Isolation of CtpA, a copper transporting P-type ATPase which has significance for virulence of *L. monocytogenes*

by

Matthew S. Francis, B.Sc. (Hons.)

Department of Microbiology and Immunology,
The University of Adelaide.

A thesis submitted for the degree of Doctor of Philosophy
in
The University of Adelaide
(Faculty of Science)

December, 1996

Abstract

Bacteria utilise changes in their environment as a means to regulate gene expression. This can be exploited by reporter gene fusion technology to identify genes regulated by these conditions. The correlation between control of virulence gene expression and environmental signals (Finlay and Falkow, 1989a; Miller *et al.*, 1989; Mekalanos, 1992; Gross, 1993), suggests that this approach may facilitate isolation of genetic loci which have significance for virulence in bacteria. In this study, a similar approach was employed to generate a library of chromosomally derived transcriptional promoter::*lacZ* fusion mutants in an environmental isolate of *L. monocytogenes* (DRDC8). Fusion mutants were constructed by transposon mutagenesis using a Tn*917* derivative containing promoterless *lacZ* (β-galactosidase) and *cat86* (chloramphenicol acetyl transferase) genes (Youngman *et al.*, 1985a). Preliminary investigation of β-galactosidase expression from promoter::*lacZ* fusion mutants identified genes regulated by iron and calcium limitation, temperature upshift (25°C → 37°C), and carbon dioxide. Similar conditions are routinely encountered by bacteria when infecting a host. Therefore, it was anticipated that several of these environmentally regulated genes may be significant for virulence of *L. monocytogenes.*

A *lacZ* transcriptional fusion mutant (*L. monocytogenes* C185) that displayed increased β-galactosidase activity in response to the calcium chelater EGTA was investigated in detail. A 4.3 kb fragment of *L. monocytogenes* chromosomal DNA flanking the *lacZ* fusion, was cloned and sequenced. A 1962 bp open reading frame was identified, designated *ctpA* (copper transport protein). Analysis of the deduced 653 aa polypeptide revealed significant similarity to the family of ATP-dependent enzymes involved in copper transport in prokaryotes and eukaryotes. DNA/RNA hybridisation studies showed that levels of *ctpA* mRNA were increased following growth in media containing low (BHI broth + 10 mM EGTA or BHI broth + 5 μM 8-hydroxyquinoline) and high (BHI broth + 4 mM $CuSO_4$) copper concentrations. Furthermore, when compared to wild type parental strains, insertion mutants in *ctpA* showed an increased sensitivity for growth in media containing low copper concentrations. This data was interpreted to mean that CtpA is responsible for maintenance of intracellular copper concentration, presumably by mediating Cu^{2+} influx in *L. monocytogenes.*

CtpA is structurally similar to other reported bacterial P-type ATPases on the basis of aligned hydropathy profiles and prediction of transmembrane topology. Using these approaches, an N-terminal truncation was observed in CtpA in a domain normally attributed to initial cation binding. This truncation has only been described for one other P-type ATPase protein involved in copper transport in *Helicobacter pylori* (Ge *et al.*, 1995). Confirmation of this finding in future studies is required using N-terminal amino acid sequence analysis of purified CtpA. Nevertheless, conserved amino acid residues critical for protein function were identified in CtpA, located in putative functional domains of this protein. These domains were predicted to lie on the cytoplasmic side of the bacterial membrane, which is consistent with other membrane topology models reported for several ATPases.

To investigate the significance of CtpA for virulence, a mutant strain was constructed by insertion of an antibiotic resistance cartridge into the *ctpA* gene. A tissue culture internalisation assay, optimised in this study using the HeLa cell line, and mouse infection studies were used to compare *ctpA* insertion mutants and parental wild type strains. Mutants in CtpA, were unaltered for intracellular growth in J774 and HeLa cell lines. However, recovery of mutants from liver of infected mice was dramatically reduced compared with the wild type, and a significant impairment in terms of *in vivo* persistence in livers and spleens of mice following mixed-infection competition experiments was observed. These results demonstrated the significance of CtpA for establishment of an *in vivo* infection by *L. monocytogenes.* Given Cu^{2+} is an essential nutrient for growth of all lifeforms, and Cu^{2+} concentration is significantly reduced in mammalian cells upon infection, this suggested CtpA may be involved in scavenging free Cu^{2+} ions from the intracellular environment of an infected host.

Furthermore, DNA homologous to *ctpA* was not detected by Southern hybridisation analysis or PCR, in non-pathogenic *Listeria* spp. or in the animal pathogen *L. ivanovii.* However, the distribution of *ctpA* in *L. monocytogenes* was restricted to a population of environmental and clinical isolates predominantly associated with RLFP groups B and B1, which contain unique *Hin*dIII restriction fragment polymorphisms within the *hly* gene (Thomas, 1995).

Statement

This work contains no material which has been accepted for the award of any other degree or diploma in any University or other tertiary institution. To the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text.

When accepted for the award of the degree I give consent for this thesis to be made available for photocopying and loan.

Matthew S. Francis
December, 1996.

Abbreviations

Ω	ohms
µF	microFarad
µg	micogram
µg	microgram
×g	relative centrifugal force
aa	amino acid
AP	alkaline phosphatase
Ap	ampicillin
APS	ammonium persulphate
ATP	adenosine 5′-triphosphate
B. megaterium	*Bacillus megaterium*
BHI	Brain Heart Infusion
bp	base pair
CFU	colony forming units
CHBA	Columbia Horse Blood agar
CIP	calf intestinal phosphatase
cm	centimetre
Cm	chloramphenicol
CsCl	caesium chloride
Ctp	copper transport protein
CTP	cytosine 5′-triphosphate
ddNTP	dideoxyribonucleotide triphosphate
DIG	digoxigenin
DMEM	Dulbeccos Modified Essential medium
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
DTT	dithiothreitol
E. coli	*Escherichia coli*
EDTA	ethylene-diamine-tetra-acetic-acid disodium salt
EGTA	Ethylene-glycol-N,N,N',N'-tetra-acetic-acid
Em	erythromycin
EMEM	Eagles Minimal Essential medium
EtBr	ethidium bromide
Gm	gentamycin
GTP	guanosine 5′-triphosphate
h	hour
HBSS	hanks balance salt solution
HCl	hydrochloric acid
IPTG	isopropyl-β-D-thio-galactopyranoside

kb	kilobase
kDa	kilodalton
Klenow	Klenow fragment of *E. coli* DNA polymerase I
Km	kanamycin
kV	kilovolt
L	litre
L. monocytogenes	*Listeria monocytogenes*
LIR	left inverted repeat region of Tn*917*
Lm	lincomycin
M	molar
mg	milligram
MIC	minimal inhibitory concentration
min	minute
ml	millilitre
mm	millimetre
mM	millimolar
MM	Minimal media
MOI	multiplicity of infection
MOPS	3-[N-Morpholino]propane-sulfonic acid
mRNA	messenger ribonucleic acid
NA	Nutrient agar
NB	Nutrient broth
NBT	4-Nitroblue tetrazolium chloride
ng	nanogram
nm	nanometre
O/N	overnight
OD	optical density
ONPG	o-Nitrophenyl-β-D-galactopyranoside
orfA	open reading frame A
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
Pc	penicillin
PCR	Polymerase Chain Reaction
PEG	polyethylene glycol
phage	bacteriophage
pmol	picomoles
POD	peroxidase
^{R}	resistant
RIR	right inverted repeat region of Tn*917*
RNA	ribonucleic acid
Rp	rifampicin
RT	room temperature

s	sensitive
SDS	sodium dodecyl sulphate
sec	second
S. flexneri	*Shigella flexneri*
SLCC	Special Listeria Culture Collection
Sm	streptomycin
SSC	standard saline citrate
Tc	tetracycline
TEMED	N,N,N',N'-Tetramethyl-ethylenediamine
TTP	thymine 5′-triphosphate
UTP	uridine 5′ triphosphates
UV	ultraviolet light
V	volt
v/v	volume per volume
vol	volume
w/v	weight per volume
X-gal	5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside
X-pho	5-Bromo-4-chloro-3-indolyl-phosphate

Conference presentations and publications

Conference presentations

1. **Francis M.S.,** and C.J. Thomas. (1993). Molecular analysis of virulence determinants of *Listeria monocytogenes.* Australian Society for Microbiology Conference, Perth, Australia. *Australian Microbiologist* **14:**A33.

2. **Francis M.S.,** and C.J. Thomas. (1994). Isolation of a calcium stress associated gene in *Listeria monocytogenes* which has significant homology to bacterial ATPases involved in cation transport. Australian Society for Microbiology Conference, Melbourne, Australia, *Australian Microbiologist.* **15:**A74.

3. **Francis M.S.,** and C.J. Thomas. (1995). Isolation of *orfC*, a gene in *Listeria monocytogenes* induced by EGTA, which has significant homology to bacterial ATPases involved in cation transport. Third Australian Conference on Molecular Analysis of Bacterial Pathogens, Marysville, Australia.

Publications

1. **Francis M.S.,** and C.J. Thomas. (1996). Analysis of multiplicity of infection on the ability of *Listeria monocytogenes* to invade HeLa and Caco-2 tissue culture cell lines. *J. Med. Microbiol.* **45:**323-330.

2. **Francis M.S.,** and C.J. Thomas. (1996). *Listeria monocytogenes ctpA* gene encodes a putative P-type ATPase involved in copper transport. *Mol. Gen. Genet.* In press.

3. **Francis M.S.,** and C.J. Thomas. (1996). Mutants in the CtpA copper transporting P-type ATPase reduce virulence of *Listeria monocytogenes. Microb. Pathog.* In press.

Acknowledgments

I would first wish to thank the Department of Microbiology and Immunology for giving me the opportunity to undertake this degree. I am also indebted to my supervisor, Dr Connor Thomas. As a result of his guidance and patience (and computer expertise), I have finally completed this project. The following people were also generous in assisting me with various aspects of this work: Dr Simon Silver (University of Illinois College of Medicine, Illinois, USA) and Christopher Clark for helpful discussions. Dr Meachery Jalajakumari for guidance with primer extension experiments. Dr Lindsay Dent for allowing me to use his tissue culture facilities and Garry Penny for assistance with animal studies. Finally, thankyou to Mark Webster, who under difficult conditions, has mastered his undoubted photographic talents. I gratefully acknowledge the support of the Australian Chicken Meat Research and Development Council having been a recipient of a Postgraduate research scholarship

To the past and present members of Lab523, a huge thankyou. We have experienced many ups and downs together, and it was the support received during these times which made the difference. I would also like to thank Dr Shaun McColl and members of Lab508, who were always supportive in my final year. The encouragement of many other departmental members at various times (often critical) throughout the course of this degree will always be remembered.

While undertaking a task such as this, it can often give you a sense of isolation. I am privileged to know a group of people who never lost sight of what will always be important, friendship. Anthony and Anna, Andrew and Leanne, Sharon, Scott, Mark, Grant, Michael and Sam, thankyou! Numerous times you guys made me feel that there was at least light at the end of the tunnel.

As the saying goes "you can choose your friends, but you can't choose your family". As God would have it, my family have been a neverending source of strength. What more can be said, except, thankyou, I love you all dearly. God bless.

To my family, the epitome of love

Be strong and courageous. Do not be terrified; do not be discouraged, for the Lord your God will be with you wherever you go.

Joshua 1:9 (NIV)

Table of Contents

Chapter 2 Materials and Methods

Errata and Corrections

The following represents a list of "Errata and Corrections" for the thesis entitled "Isolation of CtpA, a copper transporting P-type ATPase which has significance for virulence of *L. monocytogenes*" submitted for the Degree of Doctor of Philosphy by Matthew S. Francis.

General Corrections

Although photographs of agarose gel electrophorograms and Southern hybridisation analyses show Bacteriophage SPP-1 *Eco*RI digested size markers without labells, the size of the marker bands are listed in the Materials and Methods, Section 2.12.4

The legends of all photographs of Southern Blot data should indicate that the SPP-1 *Eco*RI digested size markers are digoxigenin labelled.

In the *List of Abbreviations* and elsewhere, gentamycin is spelled incorrectly as gentamicin

Specific Corrections and Errata

Page 1, Paragraph 2, Line 2
Lactobacillus, Bacillus, Streptococcus, and *Staphylococus* not *Lactobacilli, Bacilli, Streptococci* and *Staphylococci*

Page 2, Paragraph 3, Line 4
Enterococcus faecalis not *Streptococcus faecalis*

Page 5, Paragraph 3, Line 2, Page 134, Paragraph 3, Line 3, Page 150, Paragraph 1, Line 4
its not it's

Page 6, Paragraph 3, Line 3
variation not variations

Page 12, Paragraph 3, Line 5
resistant strains not resistance strains

Page 16, Paragraph 3, Line 11
were shown to be clustered on not were clustered on

Page 23, Paragraph 2, Line 5
upstream of *hly* not upstream *hly*

Page 69, Paragraph 4, Line 1
conditions not condition's

Page 85, Paragraph 2, Line 4
a short centrifugation step (see Section 2.34.4) was used not a short centrifugation step was used.

Chapter 1 Introduction

1.1 General information

Members of the genus of *Listeria* are facultatively anaerobic, non-sporeforming Gram-positive bacteria. Microscopically, they appear rod shaped with rounded ends and measure 0.4 to 0.5 μm in diameter by 0.5 to 2 μm in length. *Listeria* spp. grow in complex media, capable of growth at extreme temperatures (3°C to 42°C), wide pH range (5.5 to 9.5) and in the presence of high concentrations of sodium chloride (up to 10% to 12%) (Bille and Doyle, 1991). They are motile by few peritrichous flagella. Biochemical analysis revealed *Listeria* spp. are catalase positive and oxidase negative, hydrolyse esculin and ferment glucose without production of gas. They are methyl red and Voges-Proskauer positive, unable to produce indole or H_2S, and do not hydrolyse urea (Feresu and Jones, 1988).

1.2 Characterisation

1.2.1 Classification

The genus *Listeria* is classified into a relatively new taxonomic group including *Clostridium, Lactobacilli, Bacilli, Streptococci* and *Staphylococci* (Seeliger and Jones, 1986). Mutual features of this group are a low G+C content (<50 %), lack of mycolic acids, and presence of lipoteichoic acids. *Listeria* spp. are often confused with other genera including: *Brochothrix, Erysipelothrix, Lactobacillus, Kurthia, and Jonesia* because of morphological and biochemical similarities. The recognised species of the genus *Listeria* (displayed in Table 1.1; adapted from Lovett, 1990) are *L. monocytogenes, L. grayi, L. murrayi, L. innocua L. welshimeri, L. seeligeri* and *L. ivanovii*[1]. Three approaches (DNA-DNA hybridisation, 16S rRNA cataloguing, and G+C content of DNA), were responsible for the division of genus *Listeria* into seven species, with two genomically distinct groups. Rarely isolated *L. murrayi* and *L. grayi* belong to group one (Rocourt *et al.*, 1992). Typically, DNA from these species have a higher Mol% G+C content compared to other species (Feresu and Jones, 1988). Furthermore, a DNA-DNA hybridisation study showed that *L. murrayi* and *L. grayi* were, respectively, 1-9% and 3-29% related to reference strains of each species belonging to group two (Rocourt *et al.*, 1982b). Group two is comprised of three haemolytic species *L. monocytogenes, L. seeligeri and L. ivanovii* and two non-haemolytic species *L. innocua* and

[1] *Listeria denitrificans* was reclassified into the genus *Jonesia*, as *J. denitrificans* (Rocourt *et al.*, 1987).

Table 1.1 Recognised species of the genus *Listeria*

Species	Reference
L. monocytogenes	Murray *et al.*, (1926)
L. grayi	Larsen and Seeliger, (1986)
L. muurayi	Welshimer and Merdith, (1971)
L. innocua	Seeliger, (1981)
L. welshimeri	Rocourt and Grimont, (1983)
L. seeligeri	Rocourt and Grimont, (1983)
L. ivanovii	Seeliger *et al.*, (1984)

L. welshimeri. *L. monocytogenes* and rarely *L. ivanovii* are the only human pathogens, while *L. ivanovii* is mostly pathogenic for animals (Bille and Doyle, 1991).

1.2.2 Typing of *Listeria monocytogenes* and related species

Traditionally, the serology of *Listeria* has been routinely used to divide this genus into distinct serological types based on 'O' (somatic) heat stable antigens and 'H' (flagella) heat labile antigens. The O antigens are represented by the lipoteichoic acids which are located in the outer part of the cell wall (Kamisango *et al.*, 1983; Fiedler *et al.*, 1984). The system includes fifteen O antigens and five H antigens generating sixteen serovars (Table 1.2), which are distinguishable and associated with the five common *Listeria* spp (see Section 1.2.1). Even though *L. grayi* and *L. murrayi* are not included in this system, they share a limited number of O antigens with other *Listeria* spp., but have a unique flagella antigen (antigen E). With the exception of serovar 5 (includes only *L. ivanovii*), all serovars are not species specific (Table 1.3).

Therefore, serotyping alone cannot distinguish between pathogenic and non-pathogenic *Listeria* spp, and has limited usefulness for epidemiological analysis of listeriosis outbreaks for two reasons. First, *L. monocytogenes* has a high degree of antigenic relatedness with a number of organisms including *Staphylococcus aureus* and *Streptococcus faecalis* (Bortolussi *et al.*, 1985), and this can lead to mis-classification of bacteria. Second, three serovars (1/2a, 1/2b, and 4b) account for at least 90% of clinical cases of listeriosis (McLauchlin, 1987; Espaze *et al.*, 1989).

Thus, other systems for typing and subtyping *Listeria* spp. have been developed in an effort to overcome this limitation (reviewed by Farber and Peterkin, 1991; Schuchat *et al.*, 1991). For example, a standardised phage typing scheme for *Listeria* spp. which can reliably type up to at least 80% of isolates analysed has been developed (Rocourt *et al.*, 1985; Audurier and Martin, 1989). These systems have an advantage over serotyping for two reasons. First, they have been successfully used in the investigation of a number of outbreaks of listeriosis, and second, no cross reactivity of bacteriophage with other bacteria has been reported. Furthermore, since most *Listeria* spp. were thought to contain cryptic plasmids, plasmid profiling was investigated as a means to differentiate *L. monocytogenes* isolates (Kolstad *et al.*, 1992). Using this approach, isolates from diverse origins could be grouped together on the basis of their plasmid profile. Moreover, previously indistinguishable isolates containing a high percentage of

Table 1.2 Serovars of the genus *Listeria* and *Listeria grayi* and *Listeria murrayi*[a]

Serovar[b]	O (somatic) antigens	H (flagella) antigens
1/2a	I, II, (III)	A, B
1/2b	I, II, (III)	A, B, C
1/2c	II, (III)	B, D
3a	II, (III), IV	A, B
3b	(III), IV, (XII, XIII)	A, B, C
3c	(III), IV, (XII, XIII)	B, D
4a	(III), (V), VII, IX	A, B, C
4ab	(III), V, VI, VII, IX, X	A, B, C
4b	(III), V, VI	A, B, C
4c	(III), V, VI	A, B, C
4d	(III), V, VI, VIII	A, B, C
4e	(III), V, VI, (VIII), (IX)	A, B, C
5	(III), (V), VI, (VIII), X	A, B, C
7	(III), XII, XIII	A, B, C
6a (4f)	(III), V, (VI, VII), (IX), XV	A, B, C
6b (4g)	(III), (V, VI, VII), IX, X, XI	
L. grayi	(III), XII, XIV	E
L. murrayi	(III), XII, XIV	E

[a] reproduced from Lovett, (1990)

[b] designation of Seeliger and Donker-Voet

Roman numerals in parenthesis indicate antigens are not always present.

Table 1.3 Serovars of *Listeria* species[a]

Species	Serovar
Listeria monocytogenes	1/2a, 1/2b, 1/2c, 3a, 3b, 3c, 4a, 4ab, 4b, 4c, 4d, 4e, 7
Listeria innocua	6a, 6b, 4ab, undesignated
Listeria welshimeri	6a, 6b
Listeria seeligeri	1/2b, 4c, 4d, 6b, undesignated
Listeria ivanovii	5

[a] reproduced from Lovett, (1990)

variable sized plasmids were easily distinguished by plasmid profiles (Kolstad *et al.*, 1992). In addition, multilocus enzyme electrophoresis has successfully been applied to type *Listeria* spp. and assist in several epidemiological investigations into listeriosis outbreaks (Bibb *et al.*, 1989; Piffaretti *et al.*,, 1989; Bibb *et al.*, 1990). This technique is based on analysis of genetic variants in phenotypic characters detected by variation in the electrophoretic mobility of any of a large number of metabolic enzymes (reviewed by Selander *et al.*, 1987).

However, DNA and RNA fingerprinting appears the most promising technique for studies combining both routine typing of *Listeria* isolates and epidemiological analysis of listeriosis outbreaks. For example, genomic DNA restriction enzyme fingerprinting has enabled elucidation of the epidemiology of a number of listeriosis outbreaks proving more discriminatory then other typing methods (Nocera *et al.*, 1990; Wesley and Ashton, 1991; Lew and Desmarchelier, 1992; MacGowan *et al.*, 1993; and reference therein). Moreover, a combination of low-frequency restriction endonuclease digestion of genomic DNA and pulse field gel electrophoresis (PFGE), provides a rapid and accurate analysis of foodborne listeriosis epidemiology (Brosch *et al.*, 1991; Moore and Datta, 1994; Jacquet *et al.*, 1995; Tan *et al.*, 1995). In addition, ribotyping is routinely used to characterise *Listeria* spp. (Graves *et al.*, 1991; Jacquet *et al.*, 1992; Ryser *et al.*, 1995). Like DNA fingerprinting, this approach utilises variations in ribosomal RNA (rRNA) sequences as a means to group bacterial species. In a recent study, analysis of rRNA sequences by *Eco*R1 restriction digestion of greater than 1000 *L. monocytogenes* isolates, enabled the resolution of 50 pattern types differing in at least one polymorphic fragment (Bruce *et al.*, 1995; Hubner *et al.*, 1995), demonstrating its utility for classifying, identifying and typing *L. monocytogenes* isolates.

1.3 *Listeria* in the environment

Listeria spp. are ubiquitously distributed in the environment, but are predominantly found in soil and decaying vegetation (Welshimer and Donker-Voet, 1971; Welshimer, 1981). This suggests soil-borne organisms act as a primary source of infection for animals. Therefore, likely ecological niches could involve excretion of *Listeria* from infected animals which subsequently contaminates water, soil, vegetation and silage produced from contaminated grasses (Wehr, 1987). This would ultimately result in re-entry into animals. In addition, birds have been implicated in the initial contamination of grasses and silage by transferring organisms in sewage from one location to another (Fenlon, 1985).

1.4 Clinical significance of listeriosis in humans

A high incidence of *Listeria* infection of domestic animals is routinely reported (reviewed by Hird and Genigeorgis, 1990), which is likely to compromise the health of humans. Not suprisingly therefore, the majority of research has focused on *L. monocytogenes* infections in humans. In most cases, patients suffering with listeriosis have an underlying condition that interferes with T-cell immunity and results in a greater risk to infection. Predisposing conditions include haematological diseases, immunosuppression (resulting from disease or steroid therapy used in organ transplantation surgery), and physiological states such as extremes of age and pregnancy (reviewed by Lorber, 1990). The presence of these risk factors in both foodborne epidemics as well as in sporadic cases, indicates that an underlying risk factor is necessary for the onset of listeriosis after ingestion of *L. monocytogenes* (Lorber, 1990).

Meningitis is the most commonly recognised form of human listerial infection and demonstrates the tropism of *L. monocytogenes* for the central nervous system (Nieman and Lorber, 1980). Unlike other organisms which cause bacterial meningitis, *L. monocytogenes* infection also appears to produce clinical symptoms involving the brain. Cerebritis is one such disease state (Bach and Davis, 1987), and is analogous to Circling disease of sheep. However, the appearance of macroscopic brain abscess due to *L. monocytogenes* infection is unusually rare (Dee and Lorber, 1986). Moreover, bacteraemia and endocarditis are also common symptoms associated with human listerial infection (Nieman and Lorber, 1980; Carvajal and Frederiksen, 1988). In fact, infection of the murine central nervous system by *L. monocytogenes* is highly dependent upon the level and duration of bacteraemia (Berche, 1995).

In perinatal listeriosis, depending on the time of neonatal infection, two distinct forms of clinical syndromes are observed. First, infection of the foetus often leads to abortion or stillbirth. Moreover, early onset of septicaemic illness is usually observed shortly after birth (Lorber, 1990). Early onset septicaemia is characterised by widespread infection and high mortality. The second syndrome (late onset neonatal infection), results from a later infection at or shortly after birth which manifests into meningitis in the second to fourth week of life (Visintine *et al.*, 1977).

A relatively low incidence of clinical infection suggests that *L. monocytogenes* is an uncommon human pathogen. However, its significance as a pathogen lies in a high mortality rate, often in the order of 20-50%, with potential for producing epidemic infections. In addition, listerial infections are likely to rise with increased use of immuno-suppression therapy in organ transplantation surgery and increased incidence of immuno-compromised individuals induced by clinical infections. For these reasons, continued study of this pathogen remains an important objective in an effort to obtain a high standard of public health.

1.5 Foodborne listeriosis

1.5.1 Mode of transmission

Direct (non-foodborne) transmission from animals to humans is rarely reported. However, analysis of numerous case studies performed on large outbreaks of listeriosis throughout the world, identified ingestion of contaminated food as the major mode of transmission of *L. monocytogenes*. For example, a large outbreak of listeriosis in Canada provided the first connection between infection and ingestion of contaminated food. In this case, contaminated coleslaw was shown to be the probable vehicle of transmission (Schlech III *et al.*, 1983). The coleslaw was prepared from contaminated raw cabbage obtained from a farm known to have had cases of ovine listeriosis. Other epidemiological studies of listeriosis outbreaks have suggested pasteurised milk (Fleming *et al.*, 1985), soft cheeses (Linnan *et al.*, 1988), raw vegetables (McLauchlin, 1987), seafood (Weagant *et al.*, 1988), and pate (Watson and Ott, 1990) as probable vehicles of infection. Furthermore, *L. monocytogenes* has been isolated from other food types including; chicken, raw meat, salami and continental sausage, and prepacked salad (reviewed by Farber and Peterkin, 1991).

The increased incidence of *L. monocytogenes* in foods reflects the ability of this organism to grow at refrigeration temperatures and it's resistance to curing salts and other preservatives used in standard food preparation procedures (Galbraith, 1988; McLauchlin *et al.*, 1988). Moreover, changes in food processing resulting in the production of non perishable foods with prolonged refrigerated shelf lives may also be a contributing factor in the increased prevalence of listerial infections. Potentially, a small inoculum of *L. monocytogenes* on foods may proliferate during long term refrigeration, outgrowing competing organisms and producing sufficient numbers to cause infection and illness in a susceptible host (Galbraith, 1988). Nevertheless, in establishing risk factors involved in listeriosis infections by epidemiological

analysis, it may also be necessary to consider possible synergistic effects of coinfecting organisms and other potential cofactors (Schwartz *et al.*, 1989).

1.5.2 Assessment of infectious dose of pathogenic *L. monocytogenes* required for the onset of clinical symptoms

To date, the minimum infectious dose of pathogenic *L. monocytogenes* required to elicit a disease is unknown. In an effort to quantify the infectious dose, McLauchlin, (1995), analysed various factors associated with listeriosis patients who obtained their infection from independent sources including direct contact with infected animals, cross infection during the neonatal period, and foodborne transmission. This report concluded that widespread distribution of *L. monocytogenes* in the environment, high numbers of bacteria in food consumed by patients ($>10^3$/g), and relatively low incidence of disease, strongly indicates a requirement for high infectious doses. In agreement with this observation, feeding trials of *L. monocytogenes* using a non-human primate model, revealed that only animals which received a total of 10^9 cells, compared with 10^5 and 10^7 cells, produced symptoms of septicemia, irritability, loss of appetite and occasional diarrhoea (Farber *et al.*, 1991). Furthermore, a direct relationship between dose of *L. monocytogenes* and onset of illness has been reported for infection in mice (Berche, 1995)and normally resistant rats (Schlech III, 1990). These reports strongly indicate high initial doses of *L. monocytogenes* are required to establish an infection. The requirement for high infectious doses is most likely related to the fact that most bacteria are rapidly cleared from the host upon entry, leaving only a small proportion capable of establishing an infection (Raybourne and Bunning, 1994).

Nevertheless, analysis of listeriosis cases where the number of bacteria causing illness was approximated, revealed a distinct dependence upon a variety of factors. In particular, strain variations and host susceptibility are critical parameters in infections. Not all strains of *L. monocytogenes* are pathogenic (reviewed by Hof and Rocourt, 1992). Rough variants produce only reduced virulence, and non-haemolytic mutants are completely non-pathogenic. Moreover, several other virulence factors may be lost under natural environmental conditions. Therefore, among a population of haemolytic, pathogenic isolates there may be organisms that are non-haemolytic and/or have reduced virulence. Animal pathogenicity studies are perhaps the only way to determine the health risk of an isolate of *L. monocytogenes*. As this organism is detected in all food types, it is apparent that healthy susceptible individuals consume food which contains *L. monocytogenes* daily without falling ill. This phenomenon, may in part, be

due to most individuals harbouring T cells with reactivity to *Listeria* spp., probably as a result of sub-clinical infection with either *Listeria* or other Gram-positive bacteria, that share common antigens (Munk and Kaufmann, 1988). It is not suprising therefore, that patients with pre-disposing conditions which compromise their immunity are at most risk to *L. monocytogenes* infection (see Section 1.4). It should be remembered that most *Listeria* spp., except *L. monocytogenes*, can be regarded as harmless to man (Hof and Rocourt, 1992).

1.6 Detection of *L. monocytogenes*

Recent outbreaks of listeriosis transmitted by contaminated food has led to the necessary development of rapid procedures to isolate and identify *L. monocytogenes* in food materials (reviewed by Farber and Peterkin, 1991; Schuchat *et al.*, 1991).

1.6.1 Cultural enrichment

Cultural enrichment techniques have long been used for the direct selection of *Listeria*. These methods have involved utilising media supplements, which prevent proliferation of other contaminating organisms, and allow *L. monocytogenes* to out grow contaminating bacteria. Cold enrichment techniques have also been employed (Ralovich, 1989; Warburton *et al.*, 1991). After enrichment, *Listeria* spp. are identified on the basis of biochemical assays, direct haemolysis or serological typing (reviewed by Kerr and Lacey, 1991).

1.6.2 Rapid detection techniques

Alternative rapid detection procedures are being developed in order to overcome the time constraints associated with standard cultural methods. These techniques are based on DNA hybridisation, polymerase chain reaction, fluorescent-antibody assays, and enzyme immunoassay methodology.

1.6.2.1 Nucleic acid hybridisation

Specific DNA sequences and synthetic oligonucleotides derived from DNA encoding virulence factors produced by *L. monocytogenes*, have been successfully used as probes in colony hybridisation studies for the specific detection of *L. monocytogenes* (Datta *et al.*, 1987; Datta *et al.*, 1988; Chenevert *et al.*, 1989; Datta *et al.*, 1990; Okwumabua *et al.*, 1992; and references therein). Since *L. monocytogenes* is almost an exclusive pathogen of humans, virulence genes were considered unique to this bacterium and therefore, routinely used as markers for specific identification. However, a recent report has also identified the virulence

gene cluster of *L. monocytogenes* in the animal pathogen *L. ivanovii* and non-pathogenic *L. seeligeri* (Gouin *et al.*, 1994). Consequently, a subtracter probe hybridisation strategy, involving the concept of genomic subtraction, was developed to isolate *L. monocytogenes*-specific DNA sequences (Chen *et al.*, 1993). These unique sequences are presently being analysed for their utility in detecting *L. monocytogenes* in food.

In addition, *L. monocytogenes* specific 16S ribosomal RNA oligonucleotide sequences have been utilised in commercially available diagnostic kits for the detection of *L. monocytogenes* by RNA hybridisation (Klinger and Johnson, 1988; Klinger *et al.*, 1988; King *et al.*, 1989; Wang *et al.*, 1991). Given rRNA is present between 10^3-10^4 copies per cell, it was anticipated that this procedure would offer increased sensitivity compared to that for probes directed against single copy chromosomal genes (Datta and Benjamin, 1995).

1.6.2.2 Polymerase chain reaction

The advent of the polymerase chain reaction (PCR) has been a revolution for the detection of organisms in a suspension, capable of generating about 10^6 copies from a single DNA molecule within a few hours (Datta and Benjamin, 1995). The advantage of the PCR is that it represents a highly specific, rapid and sensitive detection technique. PCR has been routinely used to identify *L. monocytogenes* in contaminated food sources (Bessesen *et al.*, 1990; Rossen *et al.*, 1991; Thomas *et al.*, 1991; Fitter *et al.*, 1992; Wang *et al.*, 1992; Wernars *et al.*, 1992; and references therein). Furthermore, some detection protocols involve multiple pairs of primers directed against independent genes (multiplex PCR) to increase specificity by reducing the chance of false-negative results (Mingyuan *et al.*, 1995). Moreover, PCR can be coupled with restriction fragment length analysis and DNA-DNA hybridisation to provide useful tools for epidemiological investigations of *Listeria* spp (Ericsson *et al.*, 1995; Thomas, 1995). Therefore, PCR represents the most significant advancement in detection procedures.

1.6.2.3 Immunological detection assays

Methods for immunological detection of *Listeria* spp. involve monoclonal antibodies and enzyme immunoassay techniques. Monoclonal antibodies have been raised against the flagella antigens and other antigens specific to the genus *Listeria* (Farber and Speirs, 1987; Butman *et al.*, 1988). Using a direct immunofluorescent procedure, a selection of antibodies conjugated to fluorescein isothiocyanate have proven useful in the detection of *L. monocytogenes* in food samples including meats, soft cheeses and other dairy products

(Farber *et al.*, 1988; Mattingly *et al.*, 1988; McLauchlin and Pini, 1989). Furthermore, specific detection of all *L. monocytogenes* serotypes using antibodies raised against invasion-associated protein (p60)-derived synthetic peptides, has been reported (Bubert *et al.*, 1995). Detection was routinely determined by recognition of both native and denatured protein from cultured supernatants by ELISA and Western analysis. Finally, a novel approach to the detection of *L. monocytogenes* has been investigated introducing magnetic bead technology (Bruchez and Cordier, 1995). In this report, *L. monocytogenes* was reliably detected using a specific anti-*Listeria* antibody conjugated to magnetic beads.

1.7 Assays for virulent *L. monocytogenes*

Foodborne transmission of *L. monocytogenes* resulting in the onset of disease has increased the necessity to distinguish strains of *L. monocytogenes* likely to cause invasive disease from those strains that are ubiquitous in the environment and usually isolated from contaminated fresh produce and dairy products. Experimental infections in mice reliably determine the level of virulence of *Listeria* isolates either expressed as the number of colony forming units (CFU) required to kill 50% of mice in a sample population (defined as the LD_{50} value) or the degree of persistence of isolates in tissue organs of infected mice (Audurier *et al.*, 1980; Rocourt *et al.*, 1983; Hof, 1984; Gaillard *et al.*, 1986; Kathariou *et al.*, 1987a; Kathariou *et al.*, 1988; Cossart *et al.*, 1989; Michel *et al.*, 1990). Furthermore, the development of tissue culture monolayer invasion assays has provided a more convenient method for differentiating virulent and avirulent strains of *L. monocytogenes* (Gaillard *et al.*, 1987; Kuhn *et al.*, 1988; Pine *et al.*, 1991; Bhunia *et al.*, 1994). This approach can also be used to determine the degree of intracellular multiplication of internalised bacteria, an important feature of pathogenic bacteria (Jones and Portnoy, 1994). Moreover, an alternative method for measurement of lethality of *Listeria* isolates was investigated by Notermans *et al.*, (1991a). This assay involved infection of chicken embryos to determine the pathogenicity of individual strains, and was in good agreement with LD_{50} values calculated from infected mice.

In addition, phenotypic properties such as listeriolysin O (LLO) and phosphatidylinositol-specific phospholipase C (PI-PLC) activity (identified by *in vitro* culture of bacteria on suitable differentiation agar media), are useful markers in determining pathogenicity of *L. monocytogenes* isolates (Dominguez-Rodriguez *et al.*, 1986; Notermans *et al.*, 1991b). However, this approach is restricted given that individual pathogenic strains produce different levels of essential virulence determinants (Kathariou *et al.*, 1988).

1.8 Inhibitors of *L. monocytogenes* growth

Anti-microbial molecules have been isolated and characterised that are able to significantly reduce the level of *L. monocytogenes* contamination in food products and processing plants. Bacteriocins, produced by lactic acid bacteria, are biologically active proteins with anti-microbial properties that vary in spectrum of activity, mode of action, genetic determinants and biochemical characteristics (Tagg *et al.*, 1976; Klaenhammer, 1988; Stiles and Hastings, 1991). Recent research has centred on the isolation of bacteriocins that are either bactericidal or bacteriostatic towards *L. monocytogenes*. It is important that this work continues given their biological properties, as these proteins show potential for reducing the impact on microbial ecology of foods, processing plants and the environment. For example, nisin, produced by certain strains of *Lactococcus lactis*, is the best characterised anti-*Listeria* bacteriocin (Hurst, 1981). It is also non-hazardous and has been strongly recommended for use in the food industry (Delves-Broughton, 1990). Several additional bacteriocins have since been isolated which displayed specific anti-listerial activity including; leucocin A-UAL 187 (Hastings *et al.*, 1991), mesentericin Y105 (Héchard *et al.*, 1992), curvacin A (Tichaczek *et al.*, 1993), pediocin JD (Christensen and Hutkins, 1992), pediocin SJ-1 (Schved, *et al.*, 1993), and Enterocin 226NWC (Villani *et al.*, 1993).

1.9 Cellular immunity to *L. monocytogenes* infection

L. monocytogenes evade both antibody and complement mediated lysis or killing by professional macrophages by entering, multiplying and persisting within host cells (Mackaness, 1962). Therefore, immune recognition of *L. monocytogenes* infection requires a cellular response. Pamer, (1993) provides an excellent review which clearly describes the cellular response of immune recognition to foreign antigens.

Mammalian cells respond to phagocytosis of foreign particles by producing stress proteins (reviewed by Polla, 1991), in the same way that bacteria induce expression of genes in response to substantial changes in their environment upon entry into mammalian hosts. One host stress protein, Hsp60, plays a central role in protection from *L. monocytogenes* infection (Ang *et al.*, 1991). In particular, a class of T lymphocytes expressing the γδ T cell receptor, are necessary for immune recognition of self-stress proteins at an early phase of *L. monocytogenes* infection (Hiromatsu *et al.*, 1992). For example, γδ T cells that appeared

after intraperitoneal infection with *L. monocytogenes*, proliferated in the presence of Hsp60, and are essential for protection, since ablation with anti-γδ antibodies allowed the bacterium to establish an infection.

However, clearance of established *L. monocytogenes* infections also requires cytotoxic T lymphocytes expressing the αβ T cell receptor (Kaufmann *et al.*, 1986; Murray and Young, 1992). αβ T cells recognise bacterial peptides presented in association with class I Major Histocompatibility Complex (MHC) molecules on the surface of infected cells. In view of this, entry of *L. monocytogenes* into the cytosol of infected cells is critical for recognition by cytotoxic T cells (Hahn and Kaufmann, 1981; Wirsing von Koenig *et al.*, 1982; Berche *et al.*, 1987b; Brunt *et al.*, 1990). Moreover, the immunodominant target antigen for immune T cells was found to be an essential virulence determinant listeriolysin O (LLO, Berche *et al.*, 1987a). The 60 kDa secreted LLO protein is involved in lysis of the phagolysosome membrane, allowing release of internalised bacteria into the cytoplasm of host cells (see Section 1.13.1 for a description of LLO). Furthermore, an independent study has defined the specificity of cytotoxic T cells for *L. monocytogenes* to a single epitope of LLO (Pamer *et al.*, 1991). Amino acid residues 91-99 of the secreted LLO protein, are processed within the cytoplasm and presented on the surface of infected cells by class 1 MHC molecules.

The importance of LLO in cell mediated immunity has been elegantly shown. For example, specific cytotoxic T cells to *L. monocytogenes* recognise macrophages infected with *Bacillus subtilis* expressing LLO (Bouwer *et al.*, 1992). Further confirmation was obtained by Barry *et al.*, (1992), in a study which demonstrated that LLO was required to induce listerial immunity. While two avirulent LLO mutants were unable to immunise mice against a secondary challenge with virulent *L. monocytogenes*, mutant strains defective in cell-to-cell spread induced protection. Moreover, the LLO mutants were unable to escape phagolysosomes in infected J774 cells and could not transform these phagocytic cells into targets of *L. monocytogenes* specific cytotoxic T cells. Therefore, it was concluded that a cytoplasmic location of bacterial cells is necessary for the development of protective immunity.

It is proposed that cytotoxic T cells have two functions in cellular immunity: one is to lyse infected cells, and the second is to produce interferon in the region of infection, which activates resident macrophages, capable of phagocytosing and killing the released bacteria (Kaufmann, 1990). Not suprisingly, *L. monocytogenes* has been used extensively as a model for

determination of the cell mediated response to foreign antigens. In particular, this organism has enabled the study of a number of independent antigen processing and presentation pathways by virtue of the involvement of both the endosomal and cytoplasmic environments encountered during cell to cell spread (see Section 1.12 for a description of the cell biology of *L. monocytogenes*). A concise commentary of the intracellular antigen processing pathways encountered by an engulfed *Listeria* is provided by Hiltbold and Ziegler, (1994). Developments in this area are critical for the generation of successful vaccines against intracellular bacterial pathogens.

1.10 Vaccination strategies against *L. monocytogenes*

To date, antibiotics have effectively been used to treat human listeriosis (Weingärtner and Ortel, 1967; Marget and Seeliger, 1988). Several studies have analysed the therapeutic activities of a wide range of antibiotics in experimental listeriosis using an animal model (Hof, 1991b; Marget and Seeliger, 1988). In these reports, ampicillin, amoxicillin and gentamycin were considered the primary choice for treatment of human listeriosis. In addition, tissue culture infections using the mouse fibroblast cell line, L929, revealed other antibiotics such as the penicillins, rifampicin, rifapentine and erythromycin inhibited intracellular multiplication of *L. monocytogenes* (Nichterlein and Hof, 1991). However, antibiotics such as chloramphenicol and azlocillin had no effect. Moreover, a theoretical approach suggested by Hof, (1991a), may provide a suitable means of drug therapy. This technique involves the use of substances which interfere with host cellular processes essential for the establishment of infection of the pathogen, without direct anti-microbial activity.

Nevertheless, it is acknowledged that up to 30% of patients who have previously had drug therapy will still succumb to *Listeria* infection (Nieman and Lorber, 1980). A likely explanation for this result is the intracellular habitat of *L. monocytogenes* which protects the organisms from exposure to antibiotics. Other shortcomings associated with drug therapy include development of resistance strains and non-specific action of drugs against resident bacteria. Consequently, a number of researchers have sought to develop a *L. monocytogenes* vaccine that provides a high degree of protection against infection with minimal or no side effects.

The intracellular habitat utilised by *L. monocytogenes* effectively protects against antibody mediated defence mechanisms. Therefore, a cell mediated response is required for

anti-microbial protection. Protective immunity is best created by live vaccines, whereas soluble proteins and killed vaccines are generally not sufficient (Wirsing von Koenig *et al.*, 1982; Hess and Kaufmann, 1993). Presently, only two live vaccines active against intracellular pathogens are in use: *Mycobacterium bovis* BCG (Bacillus Calmette-Guérin) (Calmette *et al.*, 1927) against tuberculosis, and *Salmonella typhi* Ty21a (Germanier, 1975) providing protection from typhoid.

When developing an effective vaccine, it is necessary to consider the location of the initial immune response to the foreign organisms. This determines how the vaccine should be administered in order to achieve maximum efficacy. The initial encounter between the immune system and *L. monocytogenes* occurs at the level of the gastro-intestinal mucosa (Hess and Kaufmann, 1993). Therefore, ongoing research is directed at developing live oral vaccines capable of inducing mucosal immune responses similar in design to the oral *Salmonella* vaccines (Tagliabue, 1989). Research into the development of oral vaccines to *L. monocytogenes* and other intracellular organisms has involved the construction of genetically engineered BCG strains (Stover *et al.*, 1991) and the isolation of a transposon induced *S. typhimurium* AroA$^-$ strain (Hoiseth and Stocker, 1981). The most important feature of these attenuated strains involves their capacity to survive and grow within macrophages and other professional phagocytes (Kaufmann, 1988; Finlay and Falkow, 1989b). These attenuated bacterial strains have been used as viable foreign antigen delivery systems. For example, live recombinant BCG vaccines have been successfully used to deliver HIV antigens including the HIV-1 Nef protein (Aldovini and Young, 1991; Winter *et al.*, 1991), and oral *Salmonella* vaccines have been used to deliver malaria circumsporocoite recombinants (Sadoff *et al.*, 1988; Aggarwal *et al.*, 1990; Flynn *et al.*, 1990).

Interestingly, recombinant *L. monocytogenes* vaccine strains have also been developed as viable foreign antigen delivery systems. For example, recombinant *L. monocytogenes* constitutively expressing β-galactosidase from *E. coli* was capable of inducing a specific cellular immune response (Schafer *et al.*, 1992). Moreover, a live recombinant *L. monocytogenes* strain was used as a vaccine vehicle for the induction of protective anti viral cell-mediated immunity against lymphocytic choriomeningitis virus (LCMV) (Shen *et al.*, 1995).

For a successful recombinant vaccine affording protection towards *L. monocytogenes* infection, it is necessary to ensure the foreign antigen expressed in these strains is accessible to the cytoplasm of macrophages to allow subsequent class I MHC presentation (Hess and Kaufmann, 1993). LLO is processed and presented with class I MHC on the surface of infected cells allowing recognition by cytotoxic T cells (Pamer *et al.*, 1991). It follows that LLO is the antigen of choice for construction of a recombinant vaccine (Hess and Kaufmann, 1993). The major problem in the development of a vaccine is ensuring LLO can be secreted in an active form into the cytoplasm of an infected host cell. Naturally, the export machinery, including signal peptidase specificity, may differ between *L. monocytogenes* and the attenuated vaccine delivery vehicles.

Until recently, only the invasion associated protein (Iap) from *L. monocytogenes* (see Section 1.13.6 for a description of Iap) had been expressed in *S. typhimurium* AroA$^-$ (Gentschev *et al.*, 1992). In this study, Iap was fused with the HlyB/HlyD haemolysin export machinery of *E. coli* (Hess *et al.*, 1990), promoting secretion and presentation on the host cell surface affording partial protection against listeriosis. However, several recent studies have reported expression of LLO in recombinant strains. For example, Gentschev *et al.*, (1994), reported secretion of LLO and Iap fusion proteins in attenuated *aroA* strains using a plasmid system harbouring the secretion apparatus of *E. coli* haemolysin. Interestingly, the LLO fusion protein was cytolytically active, and when expressed in *Salmonella,* permitted these bacteria to escape into the cytoplasm of infected macrophages. In another report, synthetic class I and class II MHC-restricted T-cell epitopes of LLO, when fused to the hypervariable domain of the flagellin protein of an attenuated *S. dublin aroA* strain, were processed and presented to T cells stimulating a class specific immune response (Verma *et al.*, 1995). Finally, a 9 aa peptide from LLO (residue 91 to residue 99, see Section 1.9), expressed in a recombinant vaccinia virus and used to immunise mice, conferred partial protection against secondary *L. monocytogenes* challenge, mediated by specific cytotoxic-T-lymphocytes (An *et al.*, 1996). Together, these studies indicate that listerial antigens can be secreted in an active form by attenuated delivery vehicles, and provide the opportunity to establish a suitable *L. monocytogenes* vaccine candidate.

Several other approaches to the development of live oral vaccines have been investigated. First, virulence of *L. monocytogenes* can be attenuated through single amino acid substitutions

in LLO (Michel *et al.*, 1990). In particular, a non-haemolytic mutant persisted in the spleen of infected mice for several days post inoculation. This indicates that mutagenesis of a virulence determinant can attenuate virulence providing a novel approach to the development of a live vaccine. Upon further analysis of this attenuated non-haemolytic mutant, protection against secondary challenge in the murine model of infection was observed, and is directly dependent on the mutants ability to elicit class II MHC (Ia) expression in antigen presenting cells (Gahan and Collins, 1995). Therefore, a correlation exists between bacterial virulence, ability to induce a macrophage Ia response, and ability to protect against subsequent infections. Second, a mutant deficient in prephenate dehydratase involved in the biosynthesis of phenylalanine, has reduced virulence in mice, but affords significant protection from subsequent challenge with virulent *L. monocytogenes* (Alexander *et al.*, 1993). Finally, isolation of *L. monocytogenes* strains containing multiple antibiotic resistance markers show a significant reduction in virulence as observed in the murine model of infection (Linde *et al.*, 1991). These reports indicate a live vaccine could be developed using a strategy that attenuates virulence of the organism, while conferring a significant degree of protection against subsequent infection with a virulent strain.

1.11 Molecular genetics of *L. monocytogenes*

Understanding of the molecular mechanisms underlying virulence of *L. monocytogenes* has increased markedly over the past fifteen years as a consequence of pioneering research which established a number of genetic systems useful for elucidating virulence factors involved in *L. monocytogenes* pathogenesis. For example, Pérez-Díaz *et al.*, (1982), was first to report the presence of native plasmids in different *Listeria* species. In particular, a 38.5 Md cryptic plasmid, pRYC16, was identified in seven of thirty two strains analysed. Furthermore, evidence of conjugal transfer of the resistance plasmid, pIP501, from *Streptococcus agalactiae* BM6101 to *L. monocytogenes*, *L. grayi* and *L. murrayi* was demonstrated. Proteins encoded by plasmid pIP501 were expressed in *Listeria*, promoting its own transfer between strains of *Listeria* and from *Listeria* back to *Streptococcus*. However, stability and the effect on virulence of pIP501 in the recipient strain was not determined. Conjugative transfer of plasmids between *Listeria* spp. was also demonstrated through the acquisition and expression of antibiotic resistance determinants (Vicente *et al.*, 1988; Poyart-Salmeron *et al.*, 1990). Another Gram-positive plasmid, pAMβ1 (Clewell, 1981), and various derivatives, were investigated as possible cloning vectors for *L. monocytogenes* by Flamm *et al.*, (1984). This

plasmid was introduced into *L. monocytogenes* by conjugation from *Streptococcus faecalis.* No plasmid DNA rearrangements were detected after transfer, and the recipient strain was stable even in the absence of antibiotic selection. Moreover, plasmid pAMβ1 had no effect on *L. monocytogenes* virulence.

Using the Gram-positive based pAMβ1 replicon and *E. coli* based replicons from the cloning vectors pBR322 or pUC, a series of shuttle vectors were developed which allow conjugal transfer from *E. coli* to *L. monocytogenes* at modest efficiencies (Trieu-Cuot *et al.*, 1987; Trieu-Cuot *et al.*, 1990; Trieu-Cuot *et al.*, 1991), using a self-transmissible helper IncP plasmid in the donor cells (Simon *et al.*, 1983). Recently, the efficiency of conjugative transfer of the plasmid pAT18 (described by Trieu-Cuot *et al.*, 1991) into *L. monocytogenes* has been optimised by the addition of sub-inhibitory concentrations of penicillins in the culture medium (Trieu-Cuot *et al.*, 1993). This suggests the cell wall of Gram-positive bacteria is a physical barrier for conjugative transfer of genetic information delivered from *E. coli.* Other methods for efficient transfer of genetic material into *Listeria* spp. include protoplast transformation (Vicente *et al.*, 1987; Camilli *et al.*, 1990; Wuenscher *et al.*, 1991) and electroporation (Alexander *et al.*, 1990; Park and Stewart, 1990; Dunny *et al.*, 1991).

The establishment of DNA transfer techniques has greatly facilitated the application of recombinant DNA methodology and transposon technology for cloning and the analysis of virulence determinants in *L. monocytogenes.* Vectors carrying the transposon elements Tn*1545*, Tn*916*, Tn*917*, and their derivatives have successfully been used as vehicles for introduction of mutations in this bacterium (Gaillard *et al.*, 1986; Kathariou *et al.*, 1987a; Kuhn *et al.*, 1988; Portnoy *et al.*, 1988; Camilli *et al.*, 1989; Leimeister-Wächter *et al.*, 1989; Camilli *et al.* 1990; Sun *et al.*, 1990; Mengaud *et al.* 1991b). Transposon mediated mutagenesis techniques combined with tissue culture invasion assays and the murine model of infection have elucidated the cell biology of *L. monocytogenes* and identified a number of virulence determinants involved in pathogenesis. Using pulsed-field gel electrophoresis, virulence determinants were clustered on a single *Not* I fragment on the *L. monocytogenes* chromosome (Michel and Cossart, 1992; Sanchez-Campillo *et al.*, 1995).

1.12 Cell biology of *L. monocytogenes*

The development of tissue culture models of infection coupled with the use of electron and immunofluorescent microscopic techniques has provided detailed understanding of the cell biology of *L. monocytogenes* (Gaillard *et al.*, 1987; Tilney and Portnoy, 1989; Dabiri *et al.*, 1990; Mounier *et al.*, 1990; Sun *et al.*, 1990). Gaillard *et al.*, (1987), was first to establish an *in vitro* model of penetration and intracellular growth of *L. monocytogenes* using the human enterocyte-like cell line Caco-2. *L. monocytogenes* was shown to initiate entry into cells by inducing phagocytosis, followed by a period of bacterial intracellular multiplication. Subsequent studies have shown that *L. monocytogenes* is able to invade both professional and non-professional cell lines including macrophages, fibroblasts, epithelial cells, enterocytes and hepatocytes. Several of these cell lines were also used to establish the cell biology of *L. monocytogenes.*

The process of internalisation, multiplication, and cell spread into neighbouring uninfected cells has been recently reviewed (Tilney and Tilney, 1993; Theriot, 1995), and is diagrammatically represented in Figure 1.1. In the presence of the actin polymerisation inhibitor cytochalasin D, *L. monocytogenes* was able to initiate phagocytosis into non-professional cells. Following internalisation, *L. monocytogenes* lyse the phagolysosome membrane and enter into the cytoplasm where a period of rapid intracellular multiplication ensues. Auxotrophic mutants of *L. monocytogenes* are unaffected in intracellular multiplication within the cytoplasm. This suggests that the cytoplasm is a rich medium, supporting the growth of intracellular pathogens containing complex growth requirements (Marquis *et al.*, 1993). During intracellular multiplication, the bacteria begin to nucleate host cell actin initially around the surface of the cell. Eventually, polarisation of surface associated actin occurs at one end of the bacterial cell, thereby forming an actin tail enabling a directed movement of bacteria towards the host cell surface. At the cell surface, bacteria with actin tails directed towards the cell center, extend through the membrane forming a pseudopod-like structure which is recognised and phagocytosed by neighbouring uninfected cells. At this stage, bacteria are contained within a double membrane composed of the inner membrane from the original host cell and the second membrane from the phagosome of the new host. Lysis of the double membrane allows further intracellular multiplication, actin polymerisation and eventual movement and infection into neighbouring cells. The molecular and genetic determinants that constitute the

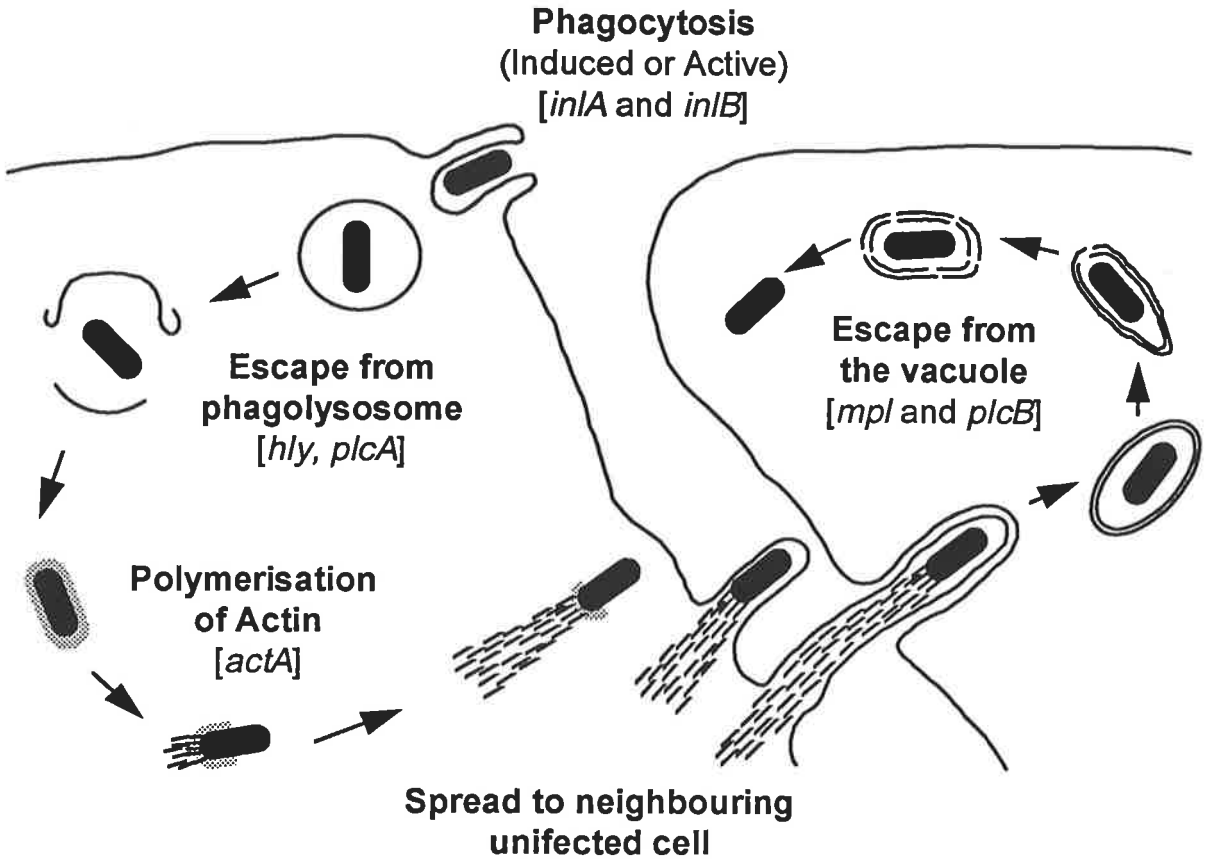


Figure 1.1: Life cycle of *L. monocytogenes* in a host cell. The genes required at each stage of the infectious process are indicated. Abbreviations: *inlAB* locus, internalin; *hly*, listeriolysin O; *plcA*, phosphatidylinositol-specific phospholipase C; *actA*, actin polymerisation; *mpl*, metalloprotease; *plcB*, phosphatidylcholine-phospholipase C (lethicinase). Adapted from Tilney and Portnoy, (1989). Shading represents initial nucleation and polymerisation of host cell actin.

L. monocytogenes infectious process have been reviewed (Portnoy *et al.*, 1992a; Sheehan *et al.*, 1994).

1.13 Determinants of pathogenesis

Transposon mutagenesis in combination with tissue culture invasion assays and a murine model of infection have identified a variety of virulence determinants responsible for *L. monocytogenes* pathogenesis. Low stringency Southern hybridisation analysis was used to determine the distribution of genes in the virulence locus within the *Listeria* genus (Gouin *et al.*, 1994). The virulence locus of *hly, plcA-prfA,* and the lecithinase operon (*mpl, actA,* and *plcB*), is only present in the three haemolytic *Listeria* spp. (*L. monocytogenes, L. ivanovii,* and *L. seeligeri*). In contrast, putative "house-keeping genes" that flank the virulence locus are present in all species. The spatial arrangement of the virulence gene cluster is represented in Figure 1.2.

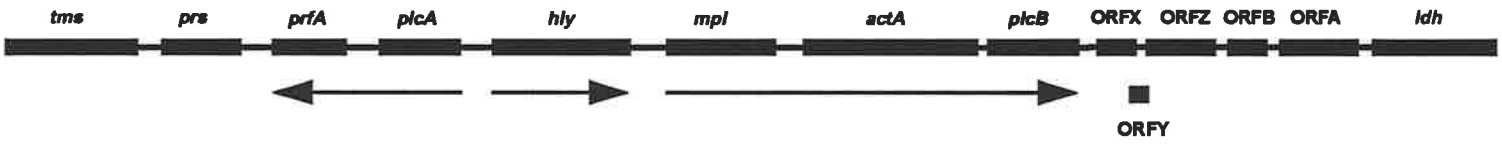
1.13.1 Listeriolysin O

1.13.1.1 History

The haemolytic characteristic of *L. monocytogenes* has attracted considerable attention for many years because of its easily detectable phenotype and its implied role in virulence. Purification of the secreted haemolysin, and subsequent analysis showed this protein to be a sulfhydryl-activated cytotoxin, antigenically related to streptolysin O and showing other properties characteristic of this group of toxins (Girard *et al.*, 1963; Njoku-Obi *et al.*, 1963; Jenkins *et al.*, 1964; Sword and Kingdon, 1971; Siddique *et al.*, 1974). Furthermore, purified haemolysin caused lysis of erythrocytes and eukaryotic membranes, and was lethal to experimental animals and damaged the reticuloendothelial system (Kingdon and Sword, 1970a; Kingdon and Sword, 1970b; Kingdon and Sword, 1970c; Watson and Lavizzo, 1973). In addition, all pathogenic strains isolated from natural infections produce haemolysin and are virulent in experimental models. Non-haemolytic strains isolated from the environment or from repeated sub-cultures, are avirulent in experimental models (Kampelmacher and Noorle-Jansen, 1972; Audurier *et al.*, 1980; Seeliger, 1981; Rocourt *et al.*, 1983; Hof, 1984). These preliminary studies provided the initial link between haemolytic activity and *L. monocytogenes* pathogenesis.

Figure 1.2: Organisation of the virulence gene cluster of *L. monocytogenes* and other *Listeria* spp. Listeriolysin O gene (*hly*) and the two adjacent operons: the *plcA-prfA* operon and the lecithinase operon (*mpl, actA* and *plcB*). *prfA* encodes a positive regulatory factor, *plcA* encodes a phosphatidylinositol-specific phospholipase C, *mpl* encodes a metalloprotease, *actA* encodes a surface protein necessary for actin assembly, and *plcB* encodes a lecithinase. Arrows indicate the direction of gene transcription. The plus (+) and minus (−) symbols indicate the representation of the virulence genes in *Listeria* spp. The virulence gene cluster is bordered by similar "housekeeping" genes in all species.

L. monocytogenes	+	+	+	+	+	+	+	+	+		+
L. ivanovii	+	+	+	+	+	+	+	-	+		+
L. seeligeri	+	+	+	+	+	+	+	-	+		+
L. innocua	+	-	-	-	-	-	-	+	+		+
L. welshimeri	+	-	-	-	-	-	-	-	+		+
L. grayi	+	-	-	-	-	-	-	-	+		+



1.13.1.2 Role of listeriolysin O in virulence

Tn*916* and Tn*1545* transposon mutagenesis of the *L. monocytogenes* genome was used to isolate haemolytic insertion mutants to enable characterisation of this putative virulence determinant. Haemolysin-negative mutants were unable to express the 58 kDa listeriolysin O (LLO) protein (Gaillard *et al.*, 1986; Kathariou *et al.*, 1987a). Haemolytic mutants are avirulent, being rapidly eliminated from the spleen and liver of infected mice, and are also deficient in intracellular growth when used to infect both professional and non-professional murine cell lines (Kuhn *et al.*, 1988; Portnoy *et al.*, 1988; Camilli *et al.*, 1989; Sun *et al.*, 1990). However, haemolytic revertants recovered through spontaneous loss of the respective transposons, subsequently regained full virulence. To ensure the mutant phenotype was not the result of polar effects on downstream genes, additional studies were performed to confirm the role of LLO in virulence of *L. monocytogenes*. First, gene complementation of a LLO mutant with the introduction of the cloned gene in *trans*, resulted in full restoration of virulence (Cossart *et al.*, 1989). Second, isogenic mutants created by single non-polar amino acid substitutions in the structural gene of LLO, attenuated virulence of these strains in the murine model of infection (Michel *et al.*, 1990).

Nevertheless, the role of LLO in infection of human cell lines remains unclear. Portnoy *et al.*, (1988), observed haemolytic mutants retained their ability to grow in human epithelial Henle 407 and fibroblast WS1 cell lines, whereas growth of mutants in the human enterocyte-like cell line Caco-2 was inhibited (Gaillard *et al.*, 1987). It appears therefore, LLO may be essential for normal growth in some, but not all human cell lines, and suggests a tropism of *L. monocytogenes* for certain host tissues. Additional factors may also be produced by this organism which complement LLO activity and enable infection of different cell types. Another interesting finding concerns the fact that levels of LLO production are not directly proportional to virulence in experimental infections in mice (Audurier *et al.*, 1981; Rocourt and Seeliger, 1987; Kathariou *et al.*, 1988; Geoffroy *et al.*, 1989). Nevertheless, since haemolytic mutants are avirulent, LLO is regarded as the most significant virulence determinant of *L. monocytogenes*.

When haemolysin mutants are used to infect cell culture monolayers, these bacteria became trapped inside a membrane limiting phagosome (Gaillard *et al.*, 1987; Kuhn *et al.*, 1988; Tilney and Portnoy, 1989). Thus, LLO was predicted to be involved in the lysis of these membranes

promoting bacterial entry into the host cell cytoplasm, although this hypothesis was originally proposed by Sword and colleagues, who reported haemolysin dependent lysis of macrophage lysosomes (Armstrong and Sword, 1966; Kingdon and Sword, 1970c). Furthermore, Geoffroy *et al.*, (1987), calculated the pH optimum of LLO to be 5.5, similar to the pH of the phagolysosome (the product of phagosome/lysosome fusion). This finding suggests that the LLO activity induced within host cell phagolysosomes, is directed towards lysis of these membranes and escape into the cytoplasm. In addition, LLO activity is intimately involved in competition between simultaneous killing and survival of *L. monocytogenes* within macrophages (De Chastellier and Berche, 1994). While most bacteria contained within a phagolysosome were killed by the oxidative burst associated with phagosome/lysosome fusion, few bacteria able to promote LLO dependent escape from the acidified phagolysosome rapidly multiplied within the cytoplasm of macrophages as a prerequisite to establishing an infection. Furthermore, this finding was in good agreement with Raybourne and Bunning, (1994), who reported survival of *L. monocytogenes* within a population of macrophages was dependent on the adaptations of a small fraction of bacteria. Presumably, LLO promoted escape from the host cell phagolysosome allowing intracellular growth in the cytoplasm.

This aspect has been conclusively demonstrated using other species of bacteria which have been engineered to express LLO. For example, *B. subtilis* strain expressing LLO under the control of an IPTG-inducible promoter was constructed (Bielecki *et al.*, 1990). When internalised by the J774 mouse macrophage-like cell line, this strain disrupted the phagolysosomal membrane when exposed to IPTG, and grew rapidly within the cytoplasm. In a follow up study, a *B. subtilis* strain expressing another member of the thiol-activated group of toxins, perfringolysin O, also promoted entry and growth of *B. subtilis* in the cytoplasm of the J774 cell line (Portnoy *et al.*, 1992b). Furthermore, a facultatively intracellular *Salmonella dublin* strain that normally remains in the phagolysosome, induced membrane lysis and bacterial release into the cytoplasm, when secreting biologically active LLO fused with the HlyB-HlyD secretion machinery of *E. coli* (Gentschev *et al.*, 1995).

1.13.1.3 Nucleotide sequence analysis and genetic characterisation of listeriolysin O

Cloning and nucleotide sequence analysis of LLO from different *L. monocytogenes* strains has been reported. Vicente *et al.*, (1985), isolated a cosmid clone from *E. coli* containing about 40 kb of *L. monocytogenes* chromosomal DNA. The haemolytic activity expressed by the *E. coli* host was LLO specific, confirmed by immunoblotting with specific anti-LLO antisera.

However, nucleotide sequence analysis of a 1.8 kb open reading frame encoding LLO was first reported by Mengaud *et al.*, (1988), and subsequently confirmed by Leimeister-Wächter and Chakraborty, (1989a). The LLO operon has been universally termed *hly*, replacing both *hlyA* and *lisA* as the accepted nomenclature (Portnoy *et al.*, 1992a).

Using immunoblotting techniques, an extracellular thiol-dependent haemolysin was detected in most strains of *L. monocytogenes* (Geoffroy *et al.*, 1989; Kreft *et al.*, 1989; Leimeister-Wächter and Chakraborty, 1989a), the animal pathogen *L. ivanovii* (Parrisius *et al.*, 1986; Barclay *et al.*, 1989; Geoffroy *et al.*, 1989; Kreft *et al.*, 1989; Leimeister-Wächter and Chakraborty, 1989a), and the haemolytic, non-pathogenic *L. seeligeri* (Geoffroy *et al.*, 1989; Leimeister-Wächter and Chakraborty, 1989a). However, Mengaud *et al.*, (1988), was unable to detect DNA homologous to *hly* in *Listeria* spp. other than *L. monocytogenes* using a *hly* specific DNA probe in DNA-DNA hybridisation studies. In contrast, when different specific *hly* DNA probes were used, DNA sequences homologous to *hly* were detected in *L. ivanovii* and *L. seeligeri* (Leimeister-Wächter and Chakraborty, 1989b; Gormley *et al.*, 1989). In addition, no homologous DNA was detected in the non-haemolytic *Listeria* spp. (Gonnley *et al.*, 1989).

Recently, the complete nucleotide sequence of the haemolysin genes, *ilo* (*L. ivanovii*) and *lso* (*L. seeligeri*) encoding ivanolysin O and seeligerolysin O respectively, have been reported (Haas *et al.*, 1992). Nucleotide identity between the three genes is at least 75%. Similar identity exists at the amino acid level after deduced amino acid sequences were compared. The high degree of identity between haemolytic sequences from *Listeria* spp., has significantly contributed to problems associated with PCR based *Listeria* detection systems. This highlights the need for careful design of synthetic oligonucleotides used in the diagnostic analysis of *Listeria* spp. by PCR.

The deduced amino acid sequence of *hly,* revealed a protein containing 504 amino acids, comparable to the determined molecular weight of purified LLO (58 kDa, Mengaud *et al.*, 1987; Mengaud *et al.*, 1988). LLO is highly homologous to the family of biologically and antigenically related membrane damaging sulfhydryl-activated toxins (Geoffroy *et al.*, 1990). The prototype toxin streptolysin O (SLO), is encoded by *Streptococcus pyogenes* (Alouf, 1980; Kehoe *et al.*, 1987). Other homologous proteins include pneumolysin from *Streptococcus pneumoniae* (Walker *et al.*, 1987; Boulnois *et al.*, 1991), alveolysin from

Bacillus alvei (Geoffroy *et al.*, 1990), perfringolysin O from *Clostridium perfringens* (Tweten, 1988), ivanolysin O from *L. ivanovii* (Vazquez-Boland *et al.*, 1989a) and seeligerolysin O (Haas *et al.*, 1992). The biochemical properties of the sulfhydryl-activated proteins have been based on analysis of SLO (Halbert, 1970; Smyth and Duncan, 1978; Alouf, 1980; Johnson *et al.*, 1980; Alouf and Geoffroy, 1984; Bernheimer and Rudy, 1986). The classical properties of bacterial sulfhydryl-activated toxins include: i) inhibition by very low amounts of cholesterol; ii) activation by reducing agents and suppression of the lytic activity by oxidation; iii) antigenic cross-reactivity with SLO, and iv) presence of a unique cysteine near the C-terminus surrounded by a conserved undecapeptide, ECTGLAWEWWR. A multiple alignment of C-terminal amino acid sequences of known sulfhydryl-activated toxins highlighting the conserved region is shown in Figure 1.3. Although the role of the unique cysteine in LLO function is unknown, site directed mutagenesis of cysteine revealed this thiol group is not essential for haemolytic activity or virulence in the mouse model (Michel *et al.*, 1990). Similar findings have also been reported with studies involving SLO (Pinkney *et al.*, 1989) and pneumolysin (Saunders *et al.*, 1989).

1.13.1.4 Mode of action

In view of the fact that LLO belongs to the family of sulfhydryl-activated toxins, LLO was purified using thiol-disulfide exchange affinity chromatography (Geoffroy *et al.*, 1987; Kreft *et al.*, 1989; Matar *et al.*, 1992). Characterisation of this purified protein revealed properties associated with sulfhydryl-activated toxins, and confirmed that LLO was a member of this family. Nevertheless, the mechanism by which LLO and other sulfhydryl-activated toxins exert their cytolytic activity on membranes is unclear. Action of these toxins is restricted to cells whose membranes contain cholesterol, which may act as the receptor for toxin binding (Bernheimer, 1974). While it has been established that LLO damages macrophage membranes, this process is inhibited by cholesterol (Yoshikawa *et al.*, 1993), and correlates with the inhibition of LLO activity on erythrocytes in the presence of low amounts of cholesterol. Furthermore, a truncated LLO protein lacking the conserved undecapeptide at the C-terminus is inactive, but still maintains the ability to bind cholesterol (Vazquez-Boland. *et al.*, 1989b), which indicates that independent protein domains are involved in cytolytic activity and cholesterol binding.

Present thinking proposes that these toxins cause cell lysis by the formation of trans-membrane pores. Immunoblotting techniques were used to confirm SLO binds to eukaryotic cell

```
alveolysin        358 PAYPISYTSVFLKDNSIAAVHNNTEYIETKTTEYSKGKIKLDHS 401
listeriolysin     381 PGVPIAYTTNFLKDNELAVIKNNSEYIETTSKAYTDGKINIDHS 424
ivanolysin        380 PGVPIAYTTNFLKDNQLAVVKNNSEYIETTSKAYSDGKINLDHS 423
perfringolysin    355 PAYPISYTSVFLKDNSVAAVHNKTDYIETTSTEYSKGKINLDHS 398
pneumolysin       393 PGLPISYTTSFLRDNVVATFQNSTDYVETKVTAYRNGDLLLDHS 436
seeligeriolysin   382 PGVPISYTTNFLKDNDLAVVKNNSEYIETTSKSYTDGKINIDHS 425
streptolysin      357 PAYPISYTSVFLKNNKIAGVNNRTEYVETTSTEYTSGKINLSHQ 400
                      *  ** **  **  *  *   *   * **    *  *     *

alveolysin        402 GAYVAQFEVYWDEFSYDADGQEIVTRKSWDGNWRDRSAHFSTEI 445
listeriolysin     425 GGYVAQFNISWDEVNYDPEGNEIVQHKNWSENNKSKLAHFTSSI 468
ivanolysin        424 GAYVARFNVTWDEVSYDANGNEVVEHKKWSENDKDKLAHFTTSI 467
perfringolysin    399 GAYVAQFEVAWDEVSYDKEGNEVLTHKTWDGNYQDKTAHYSTVI 442
pneumolysin       437 GAYVAQYYITWDELSYDHQGKEVLTPKAWDRNGQDLTAHFTTSI 480
seeligeriolysin   426 GGYVAQFNISWDEVSYDENGNEIKVHKKWGENYKSKLAHFTSSI 469
streptolysin      401 GAYVAQYEILWDEINYDDKGKEVITKRRWDNNWYSKTSPFSTVI 444
                      * ****    ***  **  * *      *             *

alveolysin        446 PLPPNAKNIRIFARECTGLAWEWWRTVVDEYNVPLASDINVSIW 489
listeriolysin     469 YLPGNARNINVYAKECTGLAWEWWRTVIDDRNLPLVKNRNISIW 512
ivanolysin        468 YLPGNARNINIHAKECTGLAWEWWRTVVDDRNLPLVKNRNVCIW 511
perfringolysin    443 PLEANARNIRIKARECTGLAWEWWRDVISEYDVPLTNNINVSIW 486
pneumolysin       481 PLKGNVRNLSVKIRECTGLAWEWWRTVYEKTDLPLVRKRTISIW 524
seeligeriolysin   470 YLPGNARNINIYARECTGLFWEWWRTVIDDRNLPLVKNRNVSIW 513
streptolysin      445 PLGANSRNIRIMARECTGLAWEWWRKVIDERDVKLSKEINVNIS 488
                        *  *  **     ***** ***** *       *       *

alveolysin        490 GTTLYPKSSITH 501
listeriolysin     513 GTTLYPKYSNKV 524
ivanolysin        512 GTTLYPAYSDTV 523
perfringolysin    487 GTTLYPGSSITY 498
pneumolysin       525 GTTLYPQVEDKV 536
seeligeriolysin   514 GTTLYPRHSNNV 525
streptolysin      489 GSTLSPYGSITY 500
                      * ** *
```

Figure 1.3: Multiple alignment of C-terminal amino acid sequences from known sulfhydryl-activated toxins. Asterisks indicate identical residues in all sequences shown. The unique cysteine (shown in bold typeface) surrounded by a conserved undecapeptide, ECTGLAWEWWR, is overlined. Sequences were aligned using CLUSTAL W (Thompson *et al.*, 1994).

membranes (Bhakdi *et al.*, 1985; Bhakdi and Tranum-Jenson, 1986). Furthermore, structural SLO pores were visualised in membrane extracts by electron microscopy and the size of these pores were estimated after measuring the ability of osmoprotectants of increasing size to migrate through the SLO induced pores (Bhakdi *et al.*, 1985). A current structural model for pneumolysin in both its monomeric and oligomeric form support this work (Morgan *et al.*, 1994). In particular, pneumolysin oligomers examined by electron microscopy revealed ring and arc shaped structures similar to features of SLO pores (Bhakdi *et al.*, 1985). This indicates that all sulfhydryl-activated toxins cause lysis of membranes by pore formation.

1.13.1.5 Regulation of *hly* expression

The isolation of non-haemolytic mutations either generated spontaneously or by transposon insertion outside the *hly* gene, have lead to the elucidation of determinants responsible for regulation of *hly* expression. Leimeister-Wächter *et al.*, (1989), isolated two haemolytic mutants, first, a spontaneous mutant which contained a 300 bp deletion in a region of DNA 1.6 kb upstream *hly*, and second, a Tn*916* insertion 200 bp upstream of *hly*. They proposed the identification of two elements within a 1.6 kb region of DNA upstream of *hly*, necessary for expression of LLO. This proposal was later confirmed by transcriptional mapping and nucleotide sequence analysis of DNA flanking *hly*. A promoter region containing a palindromic sequence considered critical for regulation was identified (Mengaud *et al.*, 1989). It is likely this sequence is the site of protein/DNA interaction (see Section 1.13.8.3). More recently, a gene required for expression of LLO, was isolated by genetic complementation of a LLO^- mutant (Leimeister-Wächter *et al.*, 1990). This gene termed *prfA* (see Section 1.13.8), is responsible for positive regulation of *hly* transcription. Nevertheless, a PrfA-independent *hly* promoter responsible for LLO expression, was also identified (Domann *et al.*, 1993). This suggests an additional level of regulatory control of *hly* expression, reflecting the importance of LLO in the intracellular survival of *L. monocytogenes*.

1.13.2 Phosphatidylinositol-specific phospholipase C

Nucleotide sequence analysis of DNA flanking *L. monocytogenes hly*, revealed an open reading frame upstream of this gene, originally designated *orfU* (Mengaud *et al.*, 1989). Using transposon mutagenesis, a variety of mutants were isolated lacking detectable phospholipase activity on egg yolk agar, and were reduced in the expression of an extracellular protein of 32 kDa, necessary for the induction of opacity on egg yolk agar (Kathariou *et al.*, 1990; Sun *et al.*, 1990). Sequence identity between the deduced amino acid sequence of OrfU

(Leimeister-Wächter *et al.*, 1991; Mengaud *et al.*, 1991b), and phosphatidylinositol-specific phospholipase C (PI-PLC) from *Bacillus cereus* (Kuppe *et al.*, 1989) and *Bacillus thuringiensis* (Henner *et al.*, 1988; Lechner *et al.*, 1989) was observed.

The gene encoding the PI-PLC-like protein was subsequently named *plcA*, replacing previous names including *pic* and *orfU*. PI-PLC activity was restricted to the pathogenic species *L. monocytogenes* and *L. ivanovii* (Mencíková, 1989; Leimeister-Wächter *et al.*, 1991; Mengaud *et al.*, 1991b; Notermans *et al.*, 1991b). The *plcA* gene is transcribed in the opposite direction to *hly*, from a non-overlapping promoter encoding a PI-PLC protein product between 32 kDa to 36 kDa (Mengaud *et al.*, 1989; Leimeister-Wächter *et al.*, 1991; Mengaud *et al.*, 1991b; Goldfine and Knob, 1992). Phenotypic evidence from *prfA* mutants, taken together with the presence of a palindromic sequence shared by the two divergent promoters specific for *plcA* and *hly*, suggests PI-PLC and LLO are coordinately regulated (Mengaud *et al.*, 1989; Kathariou *et al.*, 1990; Sun *et al.*, 1990).

PI-PLC produced by *L. monocytogenes* specifically cleaves glycosyl phosphatidylinositol (G-PI) membrane anchored proteins (Mengaud *et al.*, 1991b; Goldfine and Knob, 1992). This is a feature of PI-PLC's from other species (Cardoso de Almeida and Turner, 1983). However, *L. monocytogenes* PI-PLC exhibited low activity on G-PI anchored proteins when compared with PI-PLC from *B. thuringiensis* (Gandhi *et al.*, 1993). This may reflect a level of redundancy of PI-PLC in the pathogenicity of *L. monocytogenes*.

Preliminary analysis of the effect on virulence of *plcA* insertion mutants was inconclusive. Several independent insertion mutants produced small plaques in infected tissue culture monolayers (Kathariou *et al.*, 1990; Sun *et al.*, 1990; Camilli *et al.*, 1993), and were unable to cause progressive infection in normal mice (Camilli *et al.*, 1991; Conlon and North, 1992). However, these insertion mutants generated polar effects on other essential virulence determinants. In particular, reduced expression of *prfA* and *hly* was routinely noted (Camilli *et al.*, 1993).

This prompted the construction of an in-frame deletion within *plcA*, omitting polar effects on *prfA* (Camilli *et al.*, 1993). This mutant was significantly impaired in intracellular multiplication in infected tissue culture monolayers, reflecting an inability to mediate escape from phagolysosomes. Nevertheless, unlike LLO, PI-PLC was not shown to be an essential

determinant of pathogenicity *in vivo*. However, *plcA* clearly played a significant role in persistence of *L. monocytogenes* in certain host tissues including the liver of infected mice. Nevertheless, in a independent report, *plcA* was apparently not required for escape from primary phagolysosomes in human epithelial cells, although a LLO/PI-PLC double mutant was less efficient in comparison to the single mutants (Marquis *et al.*, 1995). Moreover, a double mutant containing in-frame deletions in both *plcA* and *plcB*, the gene encoding for phosphatidylcholine-phospholipase C (PC-PLC) (see Section 1.13.3), showed reduced virulence *in vivo* and was restricted in its ability to spread in murine cell lines compared to single mutants (Smith and Portnoy, 1994). Clearly, the mutant phenotype of *plcA* depends upon the origin and the type of cell line used, depicting a probable tropism by *L. monocytogenes* strains. It is also possible that PI-PLC functions in a synergistic capacity with LLO and PC-PLC in pathogenesis, but alone the function of PI-PLC appears redundant (D.A. Portnoy, personal communication)[2].

1.13.3 Phosphatidylcholine-phospholipase C (lecithinase)

The expression of a specific phosphatidylcholine-phospholipase C (PC-PLC) determinant by *L. monocytogenes,* has been reported for many years (Fuzi and Pillis, 1962; Leighton *et al.*, 1975). This 29 kDa protein was recently purified and found to be a zinc-dependent, phospholipase C protein independent of PI-PLC (Geoffroy *et al.*, 1991). This protein is responsible for lecithinase activity primarily directed against phosphatidylcholine substrates. Western immunoblot analysis revealed PC-PLC was specific to virulent *L. monocytogenes* strains (Geoffroy *et al.*, 1991). The lecithinase gene, *plcB*, was isolated from an *E. coli* recombinant clone containing *L. monocytogenes* DNA expressing lecithinase activity (Vazquez-Boland *et al.*, 1992). Deduced amino-acid sequence of PlcB, revealed 39% amino acid residue identity to members of the PC-PLC protein family from *B. cereus* (Johansen *et al.*, 1988; Gilmore *et al.*, 1989) and *C. perfringens* (Leslie *et al.*, 1989; Saint-Joanis *et al.*, 1989; Titball *et al.*, 1989; Tso and Siebel, 1989).

L. monocytogenes PlcB$^-$ mutants display defective intracellular multiplication. They produce small plaques on 3T3 fibroblast monolayers and electron microscopic examination showed they were defective in escape from the double membrane phagosome following entry into neighbouring cells (Vazquez-Boland *et al.*, 1992). In addition, results obtained from an

[2] D.A. Portnoy, Department of Microbiology, University of Pennsylvania, Philadelphia, Pennsylvania.

analysis of PC-PLC activity on detergent mixed micelles and biological membranes and the influence of salts, pH, and inhibitors on activity, is consistent with the proposed role for this enzyme in cell-to-cell spread of *L. monocytogenes* by lysis of the double membrane phagosome surrounding the bacterium after entry into neighbouring cells (Goldfine *et al.*, 1993). Moreover, it has also been suggested that PC-PLC and a metalloprotease (*mpl*) (see Section 1.13.4), are capable of promoting LLO-independent escape of *L. monocytogenes* from primary phagolysosome of infected Henle 407 human epithelial cells (Marquis *et al.*, 1995).

1.13.4 Zinc-dependent metalloprotease

Nucleotide sequence analysis of DNA downstream of the *hly* gene, identified an open reading frame, originally termed *orfD* (Mengaud *et al.*, 1989). This gene was independently transcribed, in the same direction as *hly*. An identical palindromic sequence was located immediately upstream of *orfD*, similar to the sequence between *plcA* and *hly*. This suggested that *hly*, *plcA* and *orfD* were coordinately regulated (see Section 1.13.8.2). The *orfD* gene product encodes a zinc dependent metalloprotease (Domann *et al.*, 1991; Mengaud *et al.*, 1991b). OrfD shows a high degree of sequence identity to metalloproteases from *Bacillus* spp. (Titani *et al.*, 1972; Vasantha *et al.*, 1984; Yang *et al.*, 1984; Takagi *et al.*, 1985; Sidler *et al.*, 1986), *Serratia marcescens*. (Nakahama *et al.*, 1986), *Legionella pneumophila* (Black *et al.*, 1990), *Pseudomonas aeruginosa* (Bever and Iglewski, 1988) and *Vibrio anguillarum* (Norqvist *et al.*, 1990). The prototype protein of this structurally related family is thermolysin from *B. thermoproteolyticus* (Titani *et al.*, 1972). Antiserum raised against thermolysin detected the OrfD peptide from *L. monocytogenes* (Domann *et al.*, 1991). This gene has since been named *mpl*, replacing the previous nomenclature of *orfD* and *prtA*.

Mutants in the *mpl* gene have reduced virulence *in vivo* and produced small plaques in tissue culture monolayers which suggested these strains are defective for intracellular multiplication (Mengaud *et al.*, 1991b; Raveneau *et al.*, 1992). Interestingly, one *mpl* mutant obtained by transposon mutagenesis was also lethicinase deficient (Raveneau *et al.*, 1992). This mutant was unable to express the 29 kDa PC-PLC protein, but an antigenically related 33 kDa protein was detected in culture supernatants. This report provided the first indication that the metalloprotease may be involved in maturation of the pre-form of PC-PLC. Metalloprotease dependent post-translational modification of PC-PLC was confirmed by gene complementation studies of a *mpl* mutant (Poyart *et al.*, 1993). Complementation of this mutant restored lecithinase activity on egg yolk agar and production of the 29 kDa PC-PLC protein in culture

supernatants. Furthermore, the virulent phenotype of the complemented strain was significantly restored.

1.13.5 Actin polymerisation

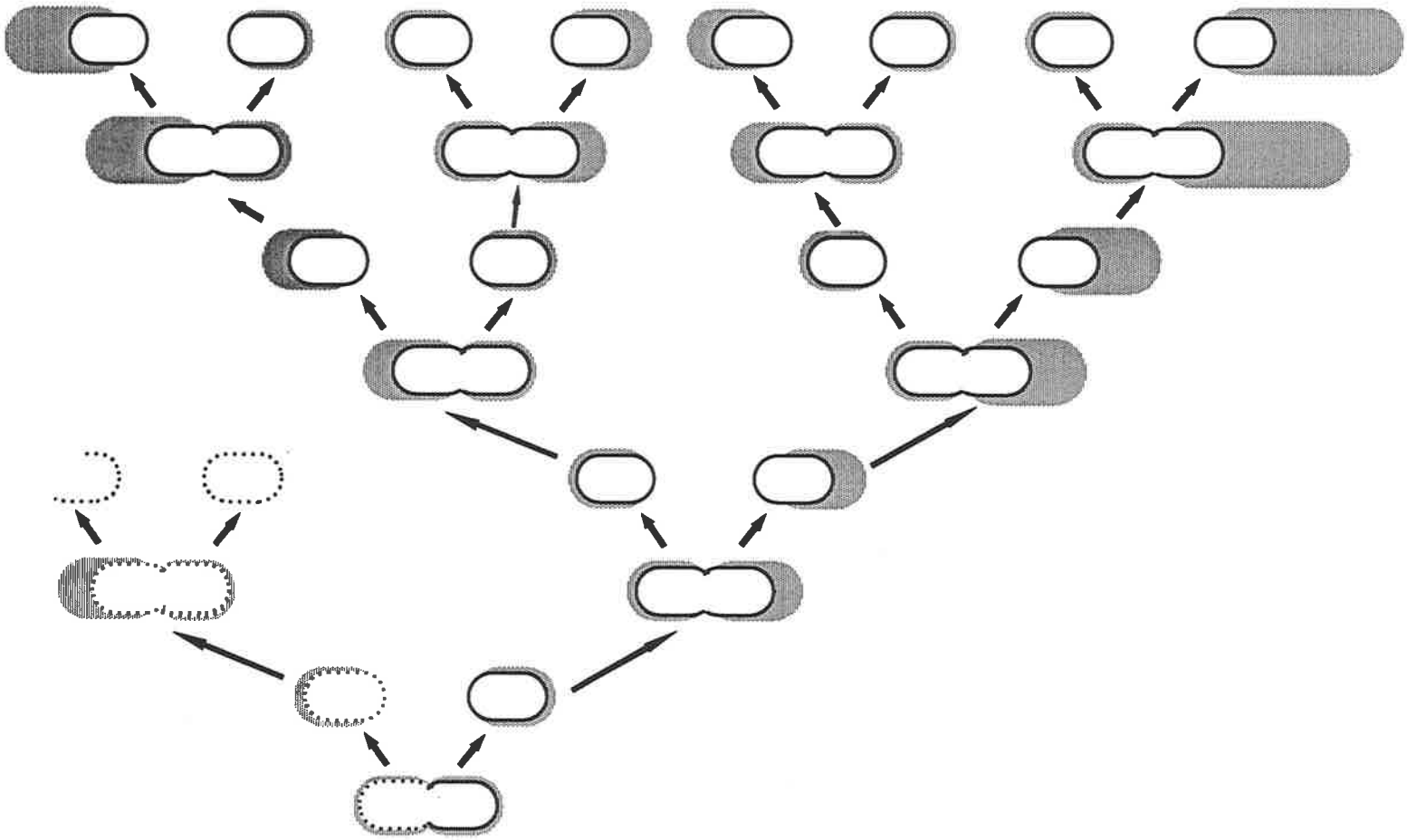
1.13.5.1 Cell to cell spread of *L. monocytogenes* involves re-organisation of host cell actin at the bacterial surface

Polymerisation of host cell F-actin filaments by *L. monocytogenes* has received intensive study, in view of the special mechanism by which this pathogen maintains itself in the host. Once inside the cytoplasm of an infected cell, *L. monocytogenes* induce polymerisation of host cell actin filaments at the bacterial surface (Tilney and Portnoy, 1989; Dabiri *et al.*, 1990). During cell division the distribution of actin filaments becomes polarised to one end of the bacterium (Tilney *et al.*, 1992b) (diagrammatically shown in Figure 1.4). Actin filaments do not form on new surfaces such as those generated during septation. Therefore, each division amplifies the polar distribution of actin filaments, and the length of the actin tail is directly proportional to the number of bacterial divisions (Sanger *et al.*, 1992; Tilney *et al.*, 1992a). The actin tail forms as a result of crosslinking of individual actin filaments presumably induced by host encoded actin bundling proteins (Tilney and Portnoy, 1989; Dabiri *et al.*, 1990; Mounier *et al.*, 1990; Tilney *et al.*, 1992b) (described in Section 1.13.5.3). The swift movement of bacteria through the cytoplasm occurs as a result of the rapid assembly of F-actin filaments at the bacterial tail (Dabiri *et al.*, 1990) and the rate of bacterial motility is apparently equal to the rate of actin polymerisation (Theriot *et al.*, 1992). Actin filaments in the tail are stationary in the cytoplasm as the bacteria projects forward, suggesting movement occurs through continuous presentation of F-actin filaments at the bacterial surface and release from the surface during migration (Sanger *et al.*, 1990; Sanger *et al.*, 1992; Theriot *et al.*, 1992; Tilney *et al.*, 1992a). Moreover, listerial actin tails may be responsible for formation of pseudopods, necessary for spreading of *L. monocytogenes* into neighbouring cells (Tilney and Tilney, 1993).

1.13.5.2 Characterisation of ActA, a bacterial protein responsible for actin polymerisation

Efforts have focused on understanding how *L. monocytogenes* interacts with F-actin. Initial observations concluded that *L. monocytogenes* secreted a protein capable of nucleating actin at the bacterial surface (Tilney *et al.*, 1990) Since then, an open reading frame (designated *actA*) has been identified, whose protein product is responsible for *L. monocytogenes* induced actin polymerisation (Domann *et al.*, 1992; Kocks *et al.*, 1992; Brundage *et al.*, 1993), and is located in the lecithinase operon (Vazquez-Boland *et al.*, 1992). Mutants in *actA* were unable

Figure 1.4: Schematic representation of the polarisation of actin filaments surrounding a *L. monocytogenes* bacterium during intracellular growth in the cytoplasm of an infected cell. Shading indicates the of extent actin tail formation during bacterial replication. After four divisions there should be 16 *Listeria,* two with a long tail, two with a shorter tail, four with tiny tails, and eight with no tails. Adapted from Tilney *et al.*, (1992a).



to accumulate actin filaments, nor spread to neighbouring cells after release into the cytoplasm, and were avirulent (Brundage *et al.*, 1993). When ActA was expressed in non-pathogenic *L. innocua,* in the absence of any additional virulence determinants, this protein induced actin assembly and propulsion of the bacteria in cytoplasmic extracts from *Xenopus laevis* eggs, as visualised by the formation of characteristic actin comet tails (Kocks *et al.*, 1995).

Clearly, ActA is not a secreted protein as originally thought, but contains key features of Gram-positive cell surface proteins including a N-terminal leader peptide sequence and a C-terminal membrane anchor region, indicative of a cell surface location for this protein. These features are schematically represented in Figure 1.5. The surface location of ActA and the exposure of the N-terminus at the extracellular face of the cell wall was confirmed by immunogold electron microscopy (Kocks *et al.*, 1993; Kocks and Cossart, 1993; Niebuhr *et al.*, 1993). Furthermore, ActA also contains an internal proline-rich repeat region (see Figure 1.5). Significantly, this region protrudes from the cell wall with potential to interact with cytoskeletal proteins (Kocks and Cossart, 1993) (see Section 1.13.5.3).

Double immunofluorescence studies with anti-ActA antibodies and phalloidin, a specific F-actin stain, confirmed that ActA was strongly expressed at one pole of the bacteria at the site of actin comet tail formation, but absent at the other pole (Kocks and Cossart, 1993). ActA was not associated with the actin tail, but remained at the surface of the bacterial cell wall. Apparently, ActA is differentially expressed along the cell wall and not expressed at the site of septation in dividing bacteria (Kocks and Cossart, 1993). In fact, asymmetric distribution of ActA is required to direct actin-based motility of intracellular *L. monocytogenes.* This was elegantly confirmed by a novel approach using an ActA-LytA fusion protein (Smith *et al.*, 1995). LytA is a major secreted autolysin of *Streptococcus pneumoniae* which specifically binds to the choline moieties within the cell wall teichoic acids of this organism. In the presence of ActA-LytA, *S. pneumoniae* were coated with this fusion product. The distribution of the fusion protein on the bacterial surface of dividing bacteria became polarised, and bacteria exhibited unidirectional actin-based movement in cell free cytoplasmic extracts, similar to the normal movement of intracellular *L. monocytogenes.* Collectively, these reports provide conclusive evidence for an essential function of ActA in actin polymerisation by *L. monocytogenes.*

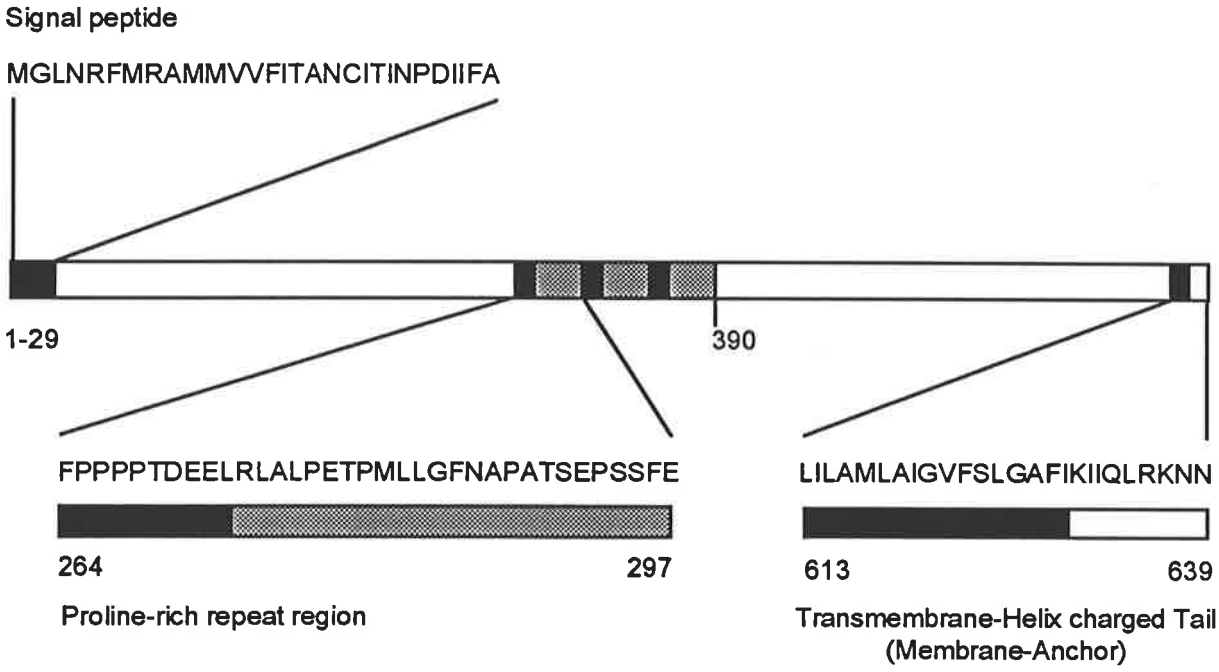


Figure 1.5: Schematic representation of the ActA 639 aa polypeptide. The position of the signal peptide, proline-rich repeat region and C-terminal membrane anchor are indicated. Adapted from Pistor *et al.*, (1994).

Two functional domains of ActA, involved in *L. monocytogenes* induced actin assembly have been proposed (Friederich *et al.*, 1995). In this study, variants of ActA, constructed with either a N-terminal deletion or the bacterial membrane anchor signal replaced by a plasma membrane localisation signal of K-ras, were expressed in mammalian cells and examined by immunofluorescence. These constructs demonstrated that the N-terminal region of ActA may promote actin filament assembly, while the proline-rich repeat region promotes or controls polymerisation. This result was confirmed in a similar study in which *L. monocytogenes* strains expressing different domains of ActA were analysed for their ability to trigger actin assembly and bacterial movement in both infected cells and cytoplasmic extracts. It was concluded that the amino terminal region of ActA is critical for F-actin assembly and movement (Lasa *et al.*, 1995).

1.13.5.3 Intracellular bacterial locomotion is induced by ActA-dependent recruitment of host cytoskeletal components

Although no extensive homology exists between the ActA polypeptide and other proteins, significant homology between a distinctive internal proline repeat region of ActA and a proline-rich region of the mammalian cytoskeletal actin associated protein vinculin was reported (Domann *et al.*, 1992; Kocks *et al.*, 1992). Sequence alignments of proline-rich regions of ActA and vinculin are shown in Figure 1.6. This suggested that *L. monocytogenes* may not directly bind actin filaments but rather, express ActA to sequester host actin binding proteins to the bacterial surface where polymerisation of actin can begin. In a normal situation, cytoskeletal actin binding proteins may represent a host cytoplasmic defence mechanism directed against intracellular pathogens by preventing the availability of free actin filaments for nucleation (Tilney and Tilney, 1993).

Several parallel studies aimed at investigating the role of ActA in binding host cytoskeletal components and the importance of the internal proline rich repeat region in these interactions have been reported. First, immunofluorescence microscopy was used to visualise a direct interaction between ActA and profilin, a protein involved in actin assembly (Theriot and Mitchinson, 1992). Profilin is an actin regulatory protein known to bind to polyproline polypeptides (Tanaka and Shibata, 1985). Additional reports have supported the involvement of profilin. For example, microinjection of mosquito oostatic factor (a naturally occurring proline-rich decapeptide) in *L. monocytogenes* infected PtK_2 epithelial cells, inhibited actin tail formation and bacterial locomotion (Southwick and Purich, 1995). Addition of profilin by

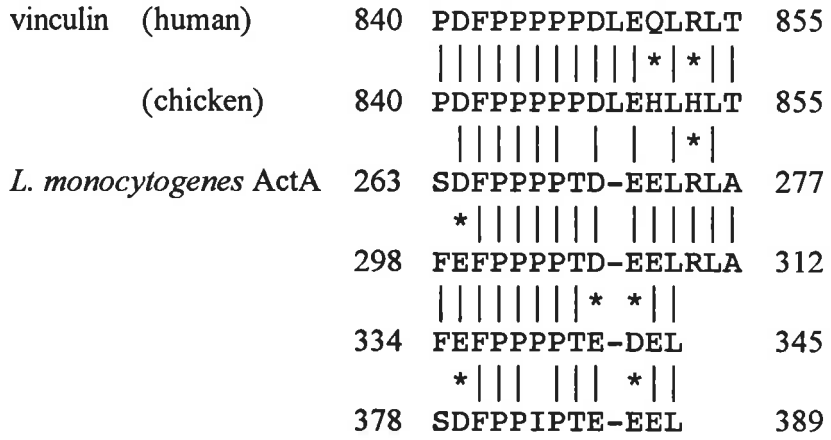


Figure 1.6: Alignment of internal repeat sequences of the *actA* gene product and homology to the proline-rich region of the mammalian cytoskeletal actin associated protein vinculin. The repeat sequences were aligned visually. Identities and conservative changes are denoted by the period and asterisk, respectively. Adapted from Domann *et al.*, (1992).

microinjection restores bacterial induced actin tail formation and locomotion. While normally the oligoproline peptide is an uncoupler of profilin action, the addition of profilin complexes with the decapeptide to neutralise its effect. This suggested profilin interacts with ActA. Furthermore, concentrated cytoplasmic extracts from the eggs of *X. laevis* support the actin-based motility of *L. monocytogenes* (Theriot *et al.*, 1994). Moreover, microinjection of a synthetic peptide, representing an internal proline-rich repeat region of ActA, into *L. monocytogenes* infected PtK_2 cells, inhibited actin-filament tail formation and intracellular bacterial motility (Southwick and Purich, 1994). Collectively, these studies clearly indicate that profilin interacts with ActA, and this complex is intimately involved in bacterial induced reorganisation of actin cytoskeleton, promoting intracellular *L. monocytogenes* locomotion.

Other host cytoskeletal components other than profilin are also known to influence *L. monocytogenes* actin-based motility. For example, expression of ActA in mammalian cells by a eukaryotic expression vector revealed a co-localisation of this polypeptide with mitochondria (Pistor *et al.*, 1994). During this time, actin and α-actinin were recruited to the surface of this organelle. Significantly, removal of the proline-rich region of ActA prevented interaction with cytoplasmic components. Furthermore, microinjection of α-actinin into cells infected with *L. monocytogenes*, induced loss of actin tails from bacteria and prevented locomotion (Dold *et al.*, 1994). These observations suggest that α-actinin plays an important role in intracellular motility, perhaps by stabilising the actin filaments in the stationary tails that are required for bacterial propulsion.

Additional host components, including vinculin and talin, are known to be associated with actin tails. These components may confer stability upon actin filaments involved in tail formation (Dold *et al.*, 1994). In contrast, Kocks and Cossart (1993), were unable to associate vinculin with *Listeria* or *Listeria*-induced actin tails, nevertheless, identified the actin bundling protein, plastin, at the site of actin assembly on the bacterial surface. Ezrin/radixin and villin were also located in the actin tail and are most likely involved in maintaining the integrity of this structure (Temm-Grove *et al.*, 1994). Recently, a novel eukaryotic factor, VASP (Vasodilator stimulated phosphoprotein), was shown to interact with ActA at a very early stage in the infection process (Ebel *et al.*, 1995). VASP is likely to interact with ActA in the internal proline-rich repeat domain (Pistor *et al.*, 1995), increasing the efficiency of actin filament elongation (Ebel *et al.*, 1995).

In summary, ActA is likely to be involved in sequestering actin binding proteins to the bacterial surface to initiate actin nucleation. However, the specific role of cellular proteins has not been elucidated and is the basis of ongoing research into the mechanism of actin polymerisation by *L. monocytogenes*. The current understanding of bacterial induced actin polymerisation is schematically shown in Figure 1.7.

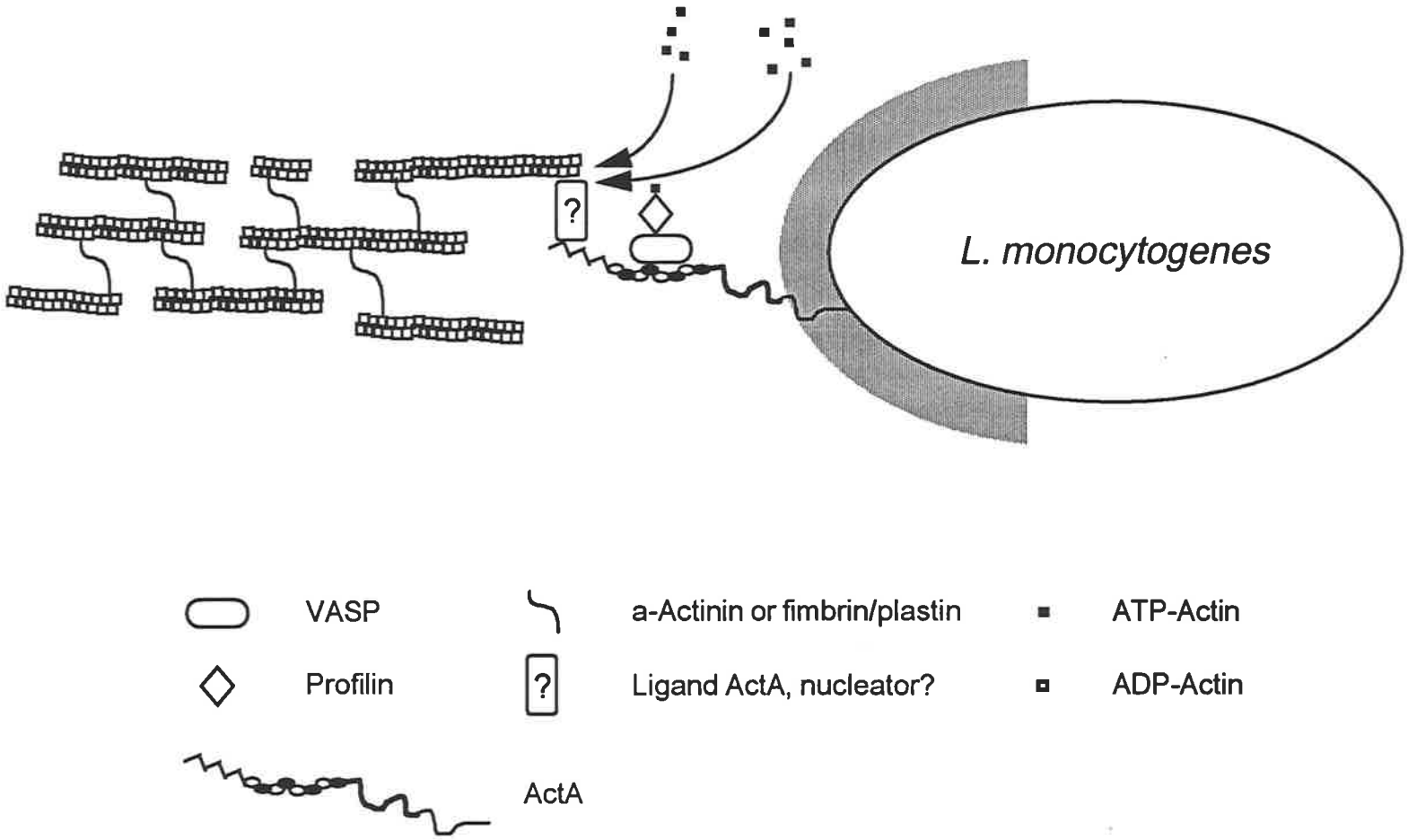
1.13.5.4 Actin polymerisation by the animal pathogen *L. ivanovii*

Interestingly, *L. ivanovii* is also capable of cell-to-cell spread by actin polymerisation, although neither DNA homologous to *actA*, nor a 90 kDa ActA protein were detected (Karunasagar *et al.*, 1993). However, the gene responsible for actin polymerisation in *L. ivanovii*, *iactA*, has recently been cloned (Gouin *et al.*, 1995; Kreft *et al.*, 1995). An N-terminal deletion mutant in *L. ivanovii iactA*, generated by allelic exchange of the wild type copy of *iactA* with an *in vitro* mutated copy, was unable to induce actin polymerisation and cell to cell spread in the Caco-2 cell line (Kreft *et al.*, 1995). Even though both IactA (*L. ivanovii*) and ActA (*L. monocytogenes*) share structural similarities including the N-terminal signal sequence, internal proline-rich repeat regions, and the C-terminal membrane anchor domain (see Figure 1.8), these proteins are only distantly related at the sequence level. Nevertheless, *iactA* restores actin tail formation and intracellular locomotion in an *L. monocytogenes actA* mutant (Gouin *et al.*, 1995).

1.13.6 Invasion associated protein

Kathariou *et al.*, (1987b), was the first to describe a series of spontaneously occurring mutants of *L. monocytogenes* distinguished by a rough phenotype. These strains were characterised by the formation of long cell chains through lack of separation of dividing cells at the site of septum formation. Apparently, R mutants have a defect in the production of a 60 kDa extracellular protein, Iap (initially termed p60), which prevents invasion of 3T3 fibroblast cells (Kuhn and Goebel, 1989). Addition of partially purified Iap caused disaggregation of the long cell chains of R mutants and restored their invasive phenotype. Therefore, Iap was initially considered necessary for adherence to 3T3 cells. Interestingly, Iap was not involved in adherence to the enterocyte-like Caco-2 cell line (Bubert *et al.*, 1992b). It appeared therefore, that R mutants can only invade certain types of mammalian cells in the presence of the Iap protein.

Figure 1.7: The role of ActA in actin assembly by *Listeria monocytogenes.* The polar distribution of ActA on the bacterial surface is represented schematically as a shaded area. Nucleation of actin would be initiated by the binding of an unknown nucleator to the N-terminal domain of ActA. The process is stimulated by the binding of vasodilator-stimulated phosphoprotein (VASP) to the proline-rich central region that, in turn, would bind profilin-ATP-actin. Host components such as α-actinin or fimbrin/plastin, considered important for the structural integrity of the actin tail are also shown. Adapted from Lasa and Cossart, (1996).


?
L. monocytogenes
VASP
a-Actinin or fimbrin/plastin
ATP-Actin
Profilin
?
Ligand ActA, nucleator?
ADP-Actin
ActA

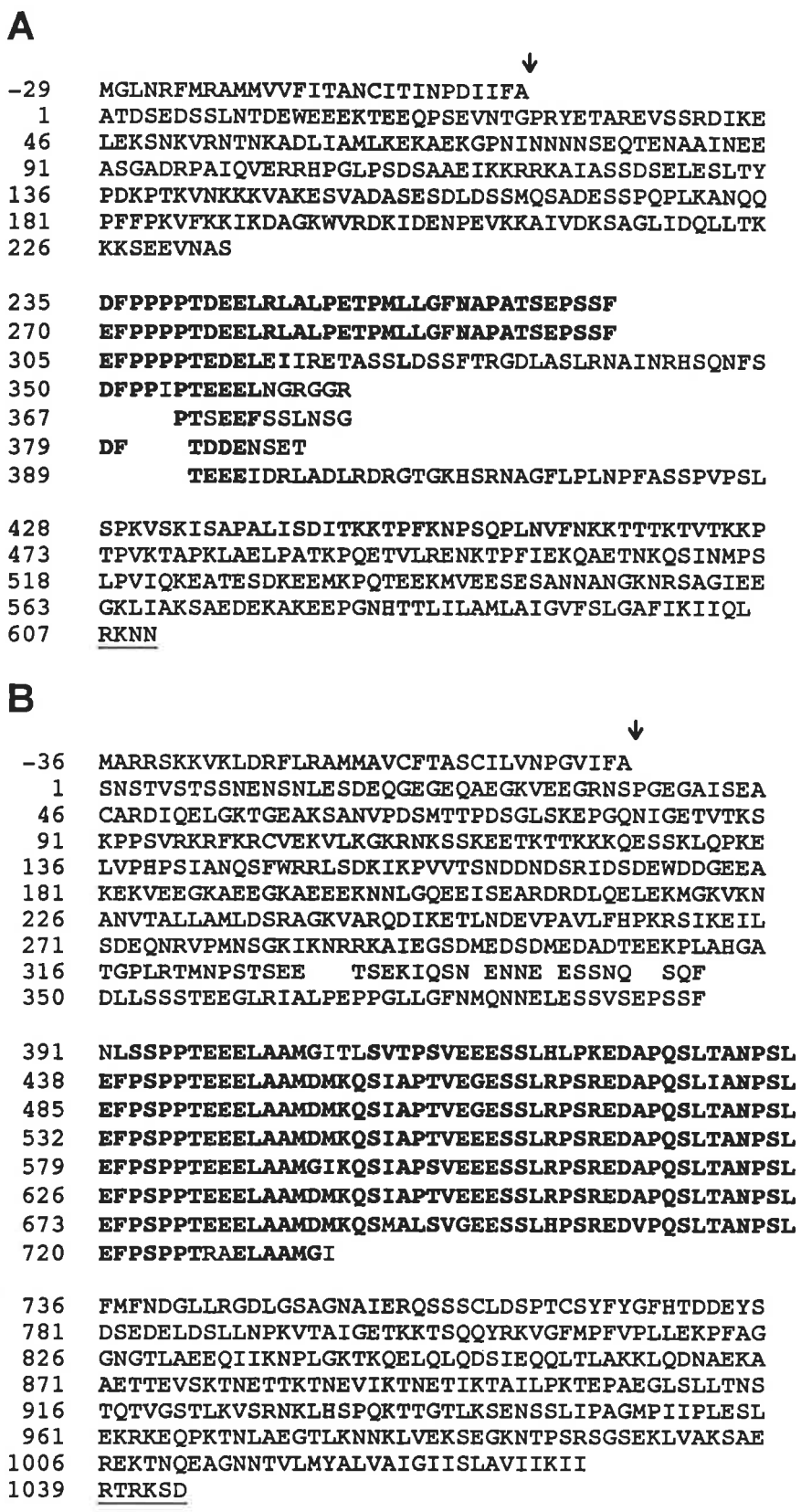


Figure 1.8: The amino acid sequence of *L. monocytogenes* ActA (Part A) and *L. ivanovii* IactA (Part B). Numbering starts at the N-terminus of the mature proteins. The arrows indicate the position of the predicted signal peptide cleavage sites. The proline-rich repeat regions and the most conserved sequences are indicated in bold type face. The C-terminal membrane anchor domain is underlined. Adapted from Gouin *et al.*, (1995).

The gene encoding Iap (*iap*, invasion associated protein), was cloned and sequenced (Köhler *et al.*, 1990). Homologues in all *Listeria* spp. except *L. grayi* were demonstrated. While a 400 bp internal fragment in *iap* was initially found to be specific for *L. monocytogenes* (Datta *et al.*, 1988), analysis of *iap*-related genes from different *Listeria* spp., revealed common and variable regions within these genes (Bubert *et al.*, 1992a). This has allowed specific identification of *Listeria* spp. by PCR using synthetic oligonucleotides to these regions. No homology with the deduced amino-acid sequence of Iap to any other known protein was detected (Köhler *et al.*, 1990), although Iap proteins from all *Listeria* spp. were cross-reactive (Bubert *et al.*, 1992b). Expression of the *iap* gene is controlled at the post-transcriptional level, and the defect in R mutants is likely to be the loss of a specific factor conferring this control, as comparable mRNA expression was observed in both wild type and R mutants (Köhler *et al.*, 1991).

The *iap* gene product is surface located (Ruhland *et al.*, 1993), and essential for cell viability (Wuenscher *et al.*, 1993). It is proposed that Iap possesses murein hydrolase activity required for the late stage in cell separation of all *Listeria* spp. This may explain the presence of *iap* in both pathogenic and non-pathogenic *Listeria* spp. With the identification of internalin, a protein essential for internalisation of *L. monocytogenes* (see Section 1.13.7), a possible role for Iap in virulence is unclear. Nevertheless, the highly basic nature of Iap suggests an involvement in promoting specific recognition between internalin and a host cell receptor, by neutralising negative charges on the host and/or bacterial surface (Kuhn and Goebel, 1989). However, in a recent study, an attenuated *S. typhimurium* strain secreting a Iap-HlyA fusion protein (utilising the *E. coli* haemolysin secretion pathway), was able to invade hepatocytes and resting macrophages and survive in spleens and livers of intravenously infected mice (Hess *et al.*, 1995). Furthermore, uptake of *L. monocytogenes* wild type and internalin deficient strains were partially blocked by anti-Iap antibodies. Together, this may imply a synergy between the two *L. monocytogenes* surface associated proteins Iap and internalin, which contributes to virulence of this organism by promoting invasion into host cells (Hess *et al.*, 1995).

1.13.7 Internalin and the *inlAB* locus

1.13.7.1 Genetic characterisation of the *inlAB* locus and functional analysis of internalin

Tn*1545* induced mutants unable to invade the human enterocyte-like Caco-2 cell line were isolated (Gaillard *et al.*, 1991), and were indistinguishable from wild type with respect to haemolytic and phospholipase activity. However, these mutants were phenotypically different from the invasion defective rough mutants isolated by Kuhn and Goebel, (1989). Nucleotide sequence analysis of DNA flanking the Tn*1545* insertions, identified an open reading frame (*inlA*) with potential to encode for a 88 kDa protein, internalin. Another open reading frame (*inlB*), was located immediately downstream of *inlA*, and has potential to encode for a 65 kDa protein. The internalin genes are organised in an operon, with two mRNA transcripts: one comprising of 5000 nucleotides and spanning *inlAB* and a smaller 2900 nucleotide transcript covering only *inlA* (Lingnau *et al.*, 1995). The promoter region of *inlA* contains a palindrome similar in sequence to promoters controlled by the positive regulatory factor, *prfA* (Dramsi *et al.*, 1993b). Nevertheless, transcriptional analysis from *inlA* is transcribed by several promoters, of which only one is PrfA-dependent (Lingnau *et al.*, 1995).

Gene complementation of *inlA*, completely restored the non-invasiveness of a Tn*1545* insertion mutant, and confirmed this phenotype was not attributed to polarity of the transposon insertion (Gaillard *et al.*, 1991). In addition, expression of *inlA* in avirulent *L. innocua*, but not *E. coli*, conferred the ability of this strain to invade Caco-2 cells, although electron microscopy confirmed that these bacteria were restricted to the host phagosome. Maximal amounts of internalin were present on the bacterial surface during exponential growth at 37°C, although internalin was also detected in significant quantities in culture supernatants (Dramsi *et al.*, 1993b). This stage of growth corresponds to maximum invasiveness of *L. monocytogenes*, reflecting the critical role surface-bound internalin plays in invasion.

1.13.7.2 Internalin possesses features consistent with Gram-positive surface proteins involved in cell recognition

Internalin is a surface protein in which two-thirds of the protein consists of two regions of internal repeat sequences called region A and region B. Region A contains 16 repeat units, highly homologous to a superfamily of leucine-rich proteins (Lingnau *et al.*, 1995), while region B is rich in glycine and proline residues (Figure 1.9, Part A). Internalin has no overall homology with any known protein including other proteins facilitating invasion. However,

A

```
1     MRKKRYVWLKSILVAILVFGSGVWINTSNGTNAQA
36    ATITQDTPINQIFTDAALAEKMKTVLGKTNVTDTVSQTDLDQVTTLQADR

86    LGIKSIDGLEYLNNLTQINFSN
108   NQLTDITPLKDLTKLVDILMNN
130   NQIADITPLANLTNLTGLTLFN
152   NQITDIDPLKNLTNLNRLELSS
174   NTISDISALSGLTNLQQLSFG
195   NQVTDLKPLANLTTLERLDISS
217   NKVSDISVLAKLTNLESLIATN
239   NQISDITPLGILTNLDELSLNG   A
261   NQLKDIGTLASLTNLTDLDLAN
283   NQISNLAPLSGLTKLTELKLGA
305   NQISNISPLAGLTALTNLELNE
327   NQLEDISPISNLKNLTYLTLYF
349   NNISDISPVSSLTKLQRLFFYN
371   NKVSDVSSLANLTNINWLSAGH
393   NQISDLTPLANLTRITQLGLND

415   QAWTNAPVNYKANVSIPNTVKNVTGALIAPATISDGGSYAEPDITWNLPSYTNEVSYTFSQPVTIGK
482   GTTTFSGTVTQPLKAIFNAKFHVDGKETTKEVEAGN

518   LLTEPAKPVKEGHTFVGWFDAQTGGTKWNFSTDKMPTNDINLYAQFSINSYTATFENDGVTTSQTVDYQG
588   LLQEPTPPTKEGYTFKGWYDAKTGGDKWDFATSKMPAKNITLYAQYSANSYTATFDVDGKSTTQAVDYQG   B
658   LLKEPKAPTKAGYTFKGWYDEKTDGKKWDFATDKMPANDITLYAQFTKN

707   PVAPPTTGGNTPPTTNNGGNTTPPSANIPGSDTSNTSTGNSASTTSTMNAYDPYNSKEASLPTTGD
773   SDNALYLLLGLLAVGTAMALTKKARASK
```

B

```
1     MKEKHNPRRKYCLISGLAIIFSLWIIIGNGAKVQA
36    ETITVSTPIKQIFPDDAFAETIKDNLKKKSVTDAVTQ

73    NELNSIDQIIANNSDIKSVQGI
95    QYLPNVTKLFLNGNKLTDIKPL
117   TNLKNLGWLFLDENKIKDLSSL
139   KDLKKLKSLSLEHNGISDINGL   A
161   VHLPQLESLYLGNNKITDITVL
183   SRLTKLDTLSLEDNQISDIVPL
205   AGLTKLQNLYLSKNHISDLRAL
227   AGLKNLDVLELFSQECLNKPIN

249   HQSNLVVPNTVKNTDGSLVTPEIISDDGDYEKPNVKWHLPE
290   FTNEVSFIFYQPVTIGKAKARFHGRVTQPLKEVYTVSYDVD
331   GTVIKTKVEAGTRITAPKPPTKQGYVFKGWYTEKNGGHEWN
372   FNTDYMSGNDFTLYAVFKAETTEKTVNLTRYVKYIRGNAGI
413   YKLPREDNSLKQGTLASHRCKALTVDREARNGGKLWYRLKN
454   IGWTKAENLSLDRYDKMEYDKGVTAYARVRNASGNSVWTKP
495   YNTAGAKHVNKLSVYQGKNMRILREAKTPITTWYQFSIGGK
536   VIGWVDTRALNTFYKQSMEKPTRLTRYVSANKAGESYYKVP
577   VADNPVKRGTLAKYKNQKLIVDCQATIEGQLWYRIRTSSTF
618   IGWTKAANLRAQK
```

Figure 1.9: The amino acid sequence of *L. monocytogenes* InlA (Part A) and InlB (Part B). The arrows indicate the position of the predicted signal peptide cleavage sites. The leucine-rich repeat regions (A), and regions rich in glycine and proline (B), are indicated in bold type face and the membrane anchor region of InlA is underlined including the double underlined conserved LPXTGX motif. Representation of InlA and InlB is adapted from Dramsi *et al.*, (1993a), and Dramsi *et al.*, (1995), respectively.

InlA contains typical cell wall sorting sequences that are distinctive features of surface proteins of Gram-positive bacteria. Appropriate location of InlA on the cell surface is achieved by an N-terminal signal peptide required for export across the cytoplasmic membrane and a 35 residue sorting signal at the C-terminus. This cell wall sorting signal consists of an LPXTGX motif, a C-terminal hydrophobic domain with limited conserved amino acid residues, and a charged tail (Schneewind *et al.*, 1993), consistent with other Gram-positive cell wall proteins including *Staphylococcus aureus* protein A (Sjödahl, 1977) and fibronectin-binding protein (Signas *et al.*, 1989) and *Streptococcus pyogenes* M protein (Hollingshead *et al.*, 1987). The C-terminal sequence similarities between these proteins are presented in Table 1.4. Interestingly, these diverse Gram-positive proteins are structurally similar consisting of numerous internal repeat domains. These proteins are all involved in cell recognition, and may reflect a strategy of gene duplication used by bacterial pathogens which has lead to the emergence of multigene families controlling specific sites of cellular infection (Dramsi *et al.*, 1993a).

1.13.7.3 InlB stimulates host cell signal transduction

Until recently, the function of the *inlB* gene product has been overlooked. Interestingly, this protein is highly homologous to the N-terminal region of internalin, containing leucine-rich repeat sequences similar to region A of internalin (Chakraborty *et al.*, 1995; Dramsi *et al.*, 1995) (see Figure 1.9, Part B). Several studies have shown that *L. monocytogenes inlB* mutants were defective in invasion of certain types of tissue culture cell lines, and virulence in the mouse model was impaired (Dramsi *et al.*, 1995; Lingnau *et al.*, 1995). These results have been confirmed by a recent study which highlights an integral role of InlB in the internalisation process. Efficient entry of bacteria into cells required stimulation of the signalling protein phosphoinositide (PI) 3-kinase p85α-p110, which is implicated in actin polymerisation and tyrosine phosphorylation (Ireton *et al.*, 1996). Stimulation of this molecule was dependent on InlB and tyrosine phosphorylation. Therefore, there is apparently a role for both internalin genes in the internalisation process.

1.13.7.4 Bacterial internalisation requires specific bacteria/host receptor interaction and stimulation of host cell signal transduction

Intracellular bacteria have developed an ability to bind to host cell surface receptors to initiate internalisation (Falkow, 1991; Isberg, 1991; Isberg and Tran Van Nhieu, 1994). The type of mammalian cell receptor involved and the nature of the interaction determines whether the

Table 1.4 Cell wall sorting signals in surface proteins of Gram-positive bacteria[a]

Bacterial species	Protein	Cell wall sorting signal[b]	Reference
L. monocytogenes	Internalin (InlA)	**LPTTGD**SDNALYLLLGLLAVGTAMALTKKARASK	Dramsi *et al.*, (1993)
L. monocytogenes	InlB	not detected	Dramsi *et al.*, (1995), Chakraborty *et al.*, (1995)
S. aureus	Protein A (Spa)	**LPETGE**ENPFIGTTVFGGLSLALGAALLAGRRREL	Uhlén *et al.*, (1984)
S. aureus	Fibronectin binding protein (FnBP)	**LPETGG**EESTNKGMLFGGLFSILGLALLRRNKKNHKA	Signäs *et al.*, (1989)
S. pyogenes	M protein (Emm6)	**LPSTGE**TANPFFTAAALTVMATAGVAAVVKRKEEN	Hollingshead *et al* (1986)

[a] reproduced from Schneewind *et al.*, 1993

[b] The conserved LPXTGX hexapeptide is shown in bold typeface, preceding a stretch of about 20 variable hydrophobic amino acids, and a short positively charged tail (underlined).

bacterium remains extracellular or enters host cells (Isberg, 1991). One class of host cell receptors that are repeatedly chosen as targets for binding by pathogens is the integrin family of cell-adhesion molecules (Hynes, 1992). *Yersinia pseudotuberculosis* and *Y. enterocolitica* bind to the β_1 chain integrins (Leong *et al.*, 1990), and *Bordetella pertussis* (Relman *et al.*, 1990) and *Legionella pneumophila* (Bellinger-Kawahara and Horwitz, 1990), bind and are internalised after interacting with β_2 chain integrins. Internalisation is the result of a series of regulated signal transduction events within the host cell. This process has been identified for the pathogens *S. typhimurium* and enteropathogenic *E. coli.* Following interaction with a host cell receptor, signal transduction events induce host cell proteins to initiate cytoskeletal rearrangement and bacterial uptake (Rosenshine *et al.*, 1992; Pace *et al.*, 1993). Signal transduction is considered a global signal initiated after pathogen-host interaction, and is required for phagocytosis. The situation is apparently no different for *L. monocytogenes.* It is likely InlB is responsible for initiating the host cell signal transduction cascade, whereas, InlA appears to interact with the cell adhesion molecule, E-cadherin (Mengaud *et al.*, 1996). This surface protein is required for entry of *L. monocytogenes* into epithelial cells.

1.13.8 Positive regulatory factor

1.13.8.1 Nucleotide sequence analysis of a positive regulatory factor of virulence gene expression in *L. monocytogenes*

The identification of 14 bp palindromic sequences situated in the −35 region of *hly, plcA* and *mpl* specific promoters presented the first indication of a common regulatory element for expression of the virulence gene cluster of *L. monocytogenes* (Mengaud *et al.*, 1989). In a subsequent study, genetic complementation of a *L. monocytogenes* LLO mutant, located a 1500 bp deletion upstream of *hly,* essential for expression of this gene (Leimeister-Wächter *et al.*, 1990). This region was subsequently sequenced and identified an open reading frame able to encode a 27 kDa protein, designated *prfA* (positive regulatory factor of *hly* transcription). The deduced amino acid sequence of PrfA shares functionally significant identity to proteins that contain N-terminal helix-turn-helix motifs involved in protein-DNA interactions (cited in Sheehan *et al.*, 1994).

1.13.8.2 PrfA-dependent expression of the virulence gene cluster from *L. monocytogenes*

Subsequent analysis of *L. monocytogenes* PrfA mutants elucidated the function of this protein. The level of mRNA transcription from *prfA, plcA, hly,* and *mpl* promoters was significantly reduced in a $PrfA^-$ mutant background compared to wild type (Mengaud *et al.*, 1991a;

Chakraborty *et al.*, 1992). Furthermore, expression of a *hly*::*lacZ* fusion in *B. subtilis* was strongly activated by the *prfA* gene product when supplied *in trans* under the control of an IPTG inducible promoter (Freitag *et al.*, 1992). Similar results were reported by Park *et al.*, (1992), using *hly*::*lux* and *plcA*::*lux* fusions. Moreover, analysis of transcription from a *inlA* specific promoter, revealed a dependence on PrfA function (Dramsi *et al.*, 1993b; Lingnau *et al.*, 1995). Collectively, results from these studies provide conclusive evidence for the PrfA-dependent expression of the virulence gene cluster from *L. monocytogenes*. In addition, further PrfA-dependent proteins in *L. monocytogenes* have been reported. For example, Sokolovic *et al.*, (1993), observed at least five secreted or surface-associated PrfA-dependent proteins that have been previously uncharacterised. These proteins are induced in stress conditions such as heat shock or stationary phase culture conditions.

PrfA is also intimately involved in autoregulation utilising a complex array of promoter regulatory elements. PrfA is expressed initially by readthrough from the *plcA* dependent promoter on a bi-cistronic 2.2 kb mRNA transcript, containing both *plcA* and *prfA* specific message (Mengaud *et al.*, 1991a). A second phase of expression from a promoter immediately 5′ to the *prfA* gene, yields a 1 kb monocistronic transcript specific for *prfA*. However, PrfA also promotes down-regulation of expression from the unique *prfA*-specific promoter region (*prfA*P1 and *prfA*P2) (Freitag *et al.*, 1993). Levels of transcripts directed from *prfA*P1 and *prfA*P2 are significantly induced in the absence of functional PrfA protein. To further investigate this control mechanism, a deletion mutant in the -35 region of *prfA*P2, was constructed. This mutant had approximately a 10 to 20 fold increase in *prfA*P1 transcripts (Freitag and Portnoy, 1994), indicating that sequences within this promoter region contributed to down-regulation of *prfA* expression. This is likely to be the palindromic sequence identified in the -35 region of the *prfA*P2 promoter which may function as the putative PrfA binding site (Freitag and Portnoy, 1994) (see Section 1.13.8.3).

Interestingly, deletion mutants in *prfA*P1 and *prfA*P2 affected the expression of PrfA-regulated genes after growth *in vitro* but not *in vivo*. Mutants maintained full virulence in both the murine model of infection and in tissue culture models of internalisation. This suggested the two *prfA* promoters are functionally redundant *in vivo* (Freitag and Portnoy, 1994). Nevertheless, the *in vitro* studies were performed in rich media and would not accurately mimic the environmental conditions usually encountered by this organism. Nevertheless, it is likely that following entry of *L. monocytogenes* into the cytoplasm, the *plcA-prfA* 2.2 kb

transcript is responsible for PrfA-dependent activation of *mpl* and *actA* promoters. However, virulence gene regulation in *L. monocytogenes* is likely to be mediated by a network of regulatory components including PrfA (Sheehan *et al.*, 1995). The current model for coordinate regulation of virulence gene expression by PrfA in *L. monocytogenes* is diagrammatically presented in Figure 1.10.

1.13.8.3 PrfA is a site-specific DNA-binding protein

Apparently, activation of PrfA-dependent genes in a heterologous *B. subtilus* host indicated a hierarchy of virulence gene expression (Sheehan *et al.*, 1995). Gene expression in this host was more efficient from the divergently transcribed *hly* and *plcA* promoters, followed by the *mpl* and *actA* promoters. The *inlA* promoter was very poorly activated, and reflects that expression of this gene is only partially dependent on PrfA. This observation has previously been reported (Lingnau *et al.*, 1995).

It is most likely that differential activation of virulence gene expression by PrfA is determined by the efficiency of binding of PrfA to promoter regulatory elements. Freitag *et al.*, (1993), provided direct evidence that PrfA is a site-specific DNA-binding protein. Gel retardation assays were used to demonstrate that the PrfA protein specifically bound to DNA fragments containing the 14 bp palindromic sequence located between the divergent *hly* and *plcA* promoter regulatory sequences. Significantly, specific PrfA-dependent expression of *hly* and *plcA* is lost upon site-directed mutagenesis within the palindromic sequence (Freitag *et al.*, 1992). These PrfA binding sites shared by *hly* and *plcA* are perfectly symmetrical (Mengaud *et al.*, 1989) (see Figure 1.11), and could be the optimum sequence for target gene activation by PrfA. On the other hand, the specific *mpl* and *actA* palindromes contain a single base substitution relative to *plcA/hly* (Mengaud *et al.*, 1989; Vazquez Boland *et al.*, 1992), whereas the *inlA* palindrome contains two base pair substitutions (Dramsi *et al.*, 1993b). This may reflect the reduced expression of *mpl, actA,* and *inlA* genes by PrfA, a conclusion supported by several independent studies (Freitag *et al.*, 1993; Bohne *et al.*, 1994; Sheehan *et al.*, 1995).

1.13.8.4 Expression of the virulence gene cluster in the animal pathogen *L. ivanovii* is dependent on a PrfA-like protein

It was initially thought that *prfA* was specific to *L. monocytogenes,* as DNA homologous to *prfA* was not detected by DNA-DNA hybridisation in any other *Listeria* spp. (Wernars *et al.*, 1992). However, a regulatory protein with a high degree of identity to *prfA* from

Figure 1.10: The coordinate regulation of virulence gene expression by the positive regulatory factor, PrfA, in *L. monocytogenes.* Abbreviations: −, negative regulation; +, positive regulation. The principle features of this model are adapted from Sheehan *et al.*, (1994).

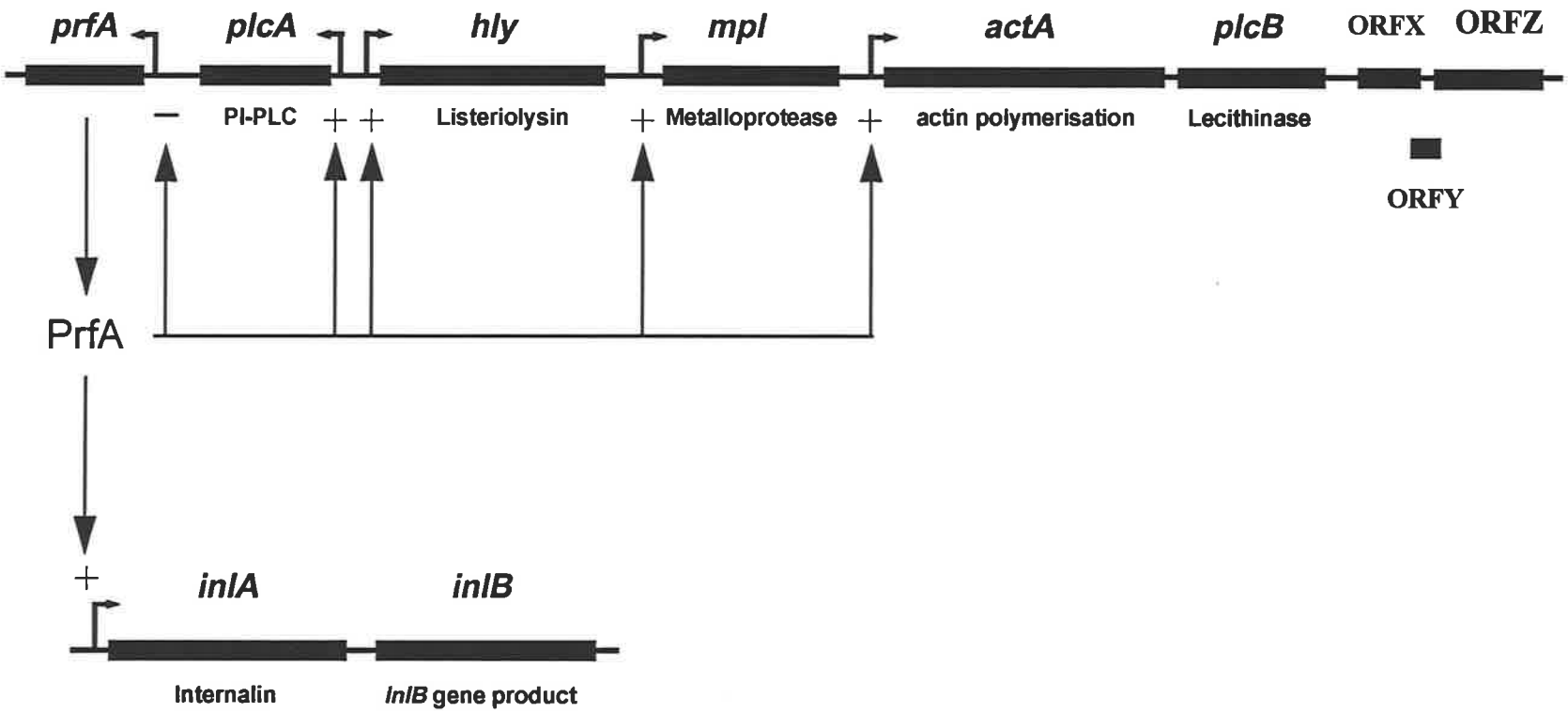
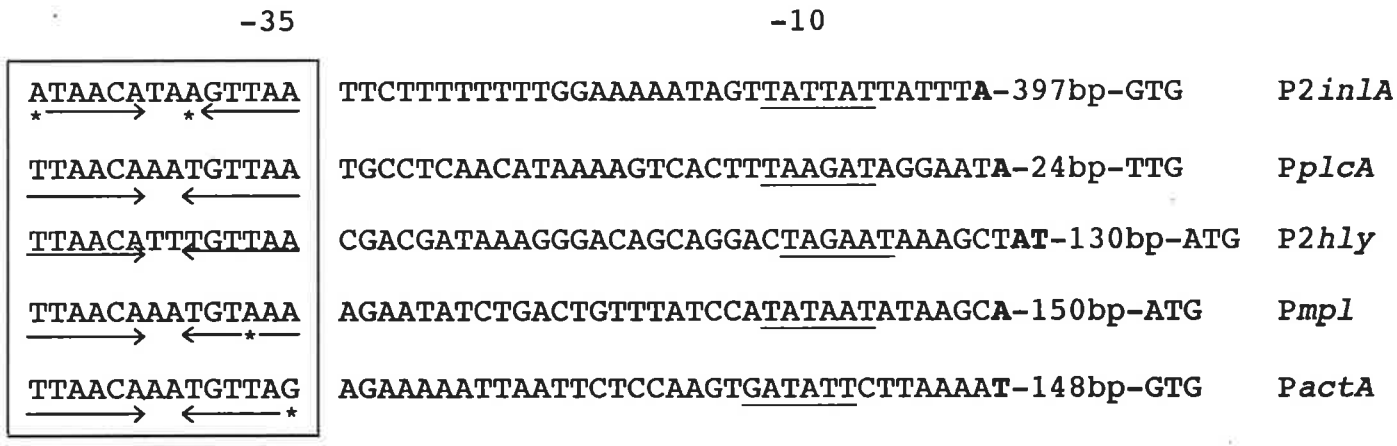

prfA
plcA
hly
mpl
actA
plcB
ORFX
ORFZ
PI-PLC
Listeriolysin
Metalloprotease
actin polymerisation
Lecithinase
ORFY
PrfA
inlA
inlB
Internalin
inlB gene product

Figure 1.11: Comparison of the 14 bp palindromes present in the –35 regions of the *prfA*-regulated virulence genes. The transcriptional start points (bold letters), and the –10 regions (underlined) of the various promoters are shown. Nucleotide substitutions with respect to the *hly* palindrome are indicated by an asterisk. Adapted from Dramsi *et al.*, (1993b).


-35
-10
ATAACATAAGTTAA
TTCTTTTTTTTTGGAAAAATAGTTATTATTATTTA-397bp-GTG
P2inlA
TTAACAAATGTTAA
TGCCTCAACATAAAAGTCACTTTAAGATAGGAATA-24bp-TTG
PplcA
TTAACATTTGTTAA
CGACGATAAAGGGACAGCAGGACTAGAATAAAGCTAT-130bp-ATG
P2hly
TTAACAAATGTAAA
AGAATATCTGACTGTTTATCCATATAATATAAGCA-150bp-ATG
Pmpl
TTAACAAATGTTAG
AGAAAAATTAATTCTCCAAGTGATATTCTTAAAAT-148bp-GTG
PactA

L. monocytogenes has been identified in *L. ivanovii* (Lampidis *et al.*, 1994). Deduced peptide sequence of both proteins show significant identity to the Crp-Fnr family of global transcriptional regulators. Transcription of the virulence gene cluster of *L. ivanovii*, identified by homology to *L. monocytogenes* (Gouin *et al.*, 1994), was dependent on the PrfA-like protein (Lampidis *et al.*, 1994).

1.13.9 Delayed type hypersensitivity

The delayed-type hypersensitivity (DTH) reaction is a major element of anti-listerial resistance (cited in Chakraborty and Goebel, 1988). A 21 kDa polypeptide was responsible for induction of a DTH response in *Listeria*-immune mice, mediated by a T-cell dependent reaction (Göhmann *et al.*, 1990). The gene encoding this protein, *lmaA* (*listeria monocytogenes* antigen), has been reported and is apparently unique to the pathogenic species *L. monocytogenes* and *L. ivanovii*. The *lmaA* gene belongs to an operon composed of four genes (*lmaDCBA*) (Portnoy *et al.*, 1992a; Schäferkordt and Chakraborty, 1995). A rho-dependent terminator was located immediately upstream of *lmaA* (Schäferkordt and Chakraborty, 1995), and expression from *lmaA* is transcriptionally thermoregulated, detectable only at temperatures below 37°C. Although the *lmaA* protein product may be an immunologically relevant antigen in listerial infections, the role of the *lma*-operon in pathogenesis is not known and remains a focus of further investigation (Schäferkordt and Chakraborty, 1995).

1.13.10 Superoxide dismutase and catalase

Facultative intracellular bacteria require mechanisms to overcome non-specific immune responses mediated by phagocytic cells. Phagocytosis of bacteria by host cells induces a significant oxidative metabolic burst releasing reactive oxygen-free radicals (O_2^-) at the phagosomal membrane (Babior *et al.*, 1973). In addition, other factors including fusion of lysosomes with phagosome, decrease in the internal pH of phagolysosomes, and activation of their contents are a collective host defence mechanism exhibiting strong anti-microbial activities (Beaman and Beaman, 1984; Hof, 1991a). This defence mechanism is activated after phagocytosis of *L. monocytogenes* (McGowan *et al.*, 1983).

Superoxide dismutase (SOD) eliminates superoxide radicals by conversion into hydrogen peroxide (H_2O_2) (McCord and Fridovich, 1969). Welch *et al.*, (1979), first demonstrated SOD

activity in *Listeria monocytogenes*. Recently, the gene encoding SOD from *L. monocytogenes* (*lmsod*) (Brehm *et al.*, 1992) and *L. ivanovii* (Haas and Goebel, 1992) have been cloned by genetic complementation of an *E. coli sodA/sodB* double mutant. Using high stringency conditions, DNA homology to *lmsod* was detected in all *Listeria* spp. (Brehm *et al.*, 1992). The by-product of SOD activity, hydrogen peroxide, is converted by catalases into H_2O and O_2 (McGowan *et al.*, 1983, Beaman and Beaman, 1984). Catalase activity in *Listeria* spp. has been detected (Welch, 1987; Dallmier and Martin, 1988; Lebond-Francillard *et al.*, 1989; Dallmier and Martin, 1990). However, only the gene from *L. seeligeri* has been identified (Haas *et al.*, 1991).

Preliminary studies were unable to correlate SOD activity to virulence of *L. monocytogenes* (Welch, 1987). Furthermore, the LD_{50} of catalase mutants for mice are not significantly different from the wild type (Welch, 1987; Lebond-Francillard *et al.*, 1989), even though catalase mutants produced elevated SOD activity. One possible explanation for the limited difference in virulence of *L. monocytogenes* SOD and catalase mutants compared to wild type, may be a legacy of limited replication of bacteria within the phagolysosome of infected cells. Only after lysis of the phagolysosome membrane and release of bacteria into the host cell cytoplasm, does replication begin. This suggests that future studies seeking to evaluate the role of SOD and catalase activity for virulence of *L. monocytogenes*, will require the murine model of infection. This will enable the degree of bacterial persistence in organ tissue of infected mice to be assessed following intravenous inoculation.

1.13.11 Iron acquisition

In attempting to colonise or invade mammalian hosts, pathogens need to overcome extreme iron-limiting conditions due to iron-protein complexes present in the host. These conditions are considered a primary defence mechanism against bacterial infection. Most of the iron is located intracellularly as haem, iron-sulphur proteins and ferritin. Consequently, iron is relatively inaccessible to bacteria (Finkelstein *et al.*, 1983; Otto *et al.*, 1992; Payne, 1993). A small concentration of iron exists extracellularly in tightly bound complexes with either transferrin or lactoferrin (Finkelstein *et al.*, 1983; Crichton and Charloteaux-Wauters, 1987; Otto *et al.*, 1992). Consequently, bacteria have developed specialised iron transport systems to overcome iron limitation. The most common means of iron transport in bacteria occurs by highly specific, small molecular weight iron binding proteins called siderophores. Siderophores act as extracellular scavenging molecules of free iron and transport this iron into the bacterial

cytosol by means of a specific receptor which recognises the iron-siderophore complex. The genetics and molecular biology of siderophore-mediated iron transport in bacteria is reviewed by Crosa, (1989). Bacteria have also developed non-siderophore mediated iron uptake systems which involve utilising transferrins and haem compounds as sources of iron by expressing specific receptors to bind these proteins at the bacterial surface (Otto *et al.*, 1992).

A direct correlation exists between virulence of *L. monocytogenes* in mice and host iron concentration (Sword 1966). Growth *in vivo* is increased during infection in iron treated mice (Hof, 1984). Several independent iron acquisition systems are employed by this organism. Ferric iron can be removed from iron-transferrin complexes by a soluble reductase, and this presumably liberates ferrous iron which inturn binds directly to the bacterial cell surface (Cowart and Foster, 1985). Binding and uptake of ferrous iron by *L. monocytogenes* was later demonstrated using radioactive iron uptake assays (Adams *et al.*, 1990). Furthermore, using a ferric reductase plate assay, *L. monocytogenes* reduced ferric iron to the ferrous form from a number of different sources including; ferric citrate, haemoglobin, ferritin and transferrin (Deneer and Boychuk, 1993). Reduction of ferric iron complexes was observed in all *Listeria* spp., and may represent a general iron scavenging system (Deneer *et al.*, 1995). Transposon mutants with reduced ferric reductase activity have been isolated. However, molecular characterisation of these mutants has not been reported.

Under iron-limiting conditions, *L. monocytogenes* can also utilise transferrin-bound iron via a 126 kDa surface located transferrin-binding protein (Hartford *et al.*, 1993). Moreover, a 32 kDa lipoprotein from *L. monocytogenes* was recently identified, and is implicated to function in iron binding and/or iron uptake in this organism (Domann *et al.*, 1995). This lipoprotein was designated ferric utilisation factor A (*fufA*), and contains significant peptide sequence identity to several binding proteins involved in iron transport from several pathogenic and non-pathogenic bacteria.

Clearly, *L. monocytogenes* can acquire iron in different forms, however, ferrous iron is essential for growth. Therefore, isolation of the ferric reductase coding region and surface located receptors for iron complexes will provide a necessary insight into the iron acquisition systems in *L. monocytogenes.*

1.13.12 Flagella

Flagella of *L. monocytogenes* were first visualised by electron microscopy and Western blot analysis. An abundance of peritrichous flagella was observed after growth at 20°C, compared with trace amounts when cells were grown at 37°C (Peel *et al.*, 1988a). Even though this corresponded to deminished motility at 37°C (Galsworthy *et al.*, 1990), chemotactic studies have shown at this temperature, directional motility by *L. monocytogenes.* This may have direct implications for penetration of the intestinal epithelium by *L. monocytogenes.*

The flagella filament of bacteria is composed of many thousands of copies of usually one or two protein subunits called flagellin. The *flaA* gene, encoding the flagellin protein of *L. monocytogenes*, has been reported (Dons *et al.*, 1992). The deduced amino acid sequence of a 30.4 kDa protein, has significant N- and C-terminal identity with other flagellin proteins including flagellin from *B. subtilis* (DeLange *et al.*, 1976) and *S. marcescens* (Harshey *et al.*, 1989). Restriction fragment polymorphisms were observed in the *flaA* gene correlating with a previous report of physical and antigenic heterogeneity in flagellin proteins of *L. monocytogenes* strains (Peel *et al.*, 1988b). In addition, nucleotide sequence analysis of a variable internal region of *flaA* from several isolates, showed that *L. monocytogenes* can be divided into three *flaA* sequence types, which correlate to the flagella antigens used in serotyping (Dons *et al.*, 1995).

Until recently, the mechanisms responsible for temperature dependent regulation of flagellin expression were unknown, despite the fact that regulation occurs at the level of transcription. The bacterial component involved in transcriptional regulation of *flaA* has been reported. This gene, *flaR*, encodes a protein of 18 kDa (FlaR) whose transcription is osmoregulated through modulation of DNA topology (Sanchez-Campillo *et al.*, 1995). Apparently, *flaR* negatively regulates its own expression. However, *flaA* expression is also thermoregulated which suggests that the effect of *flaR* on *flaA* expression is indirect. Nevertheless, regulation of flagellin gene expression by modulation of DNA topology is consistent with other organisms (Sanchez-Campillo *et al.*, 1995). Interestingly, the non-pathogenic *L. innocua*, which is otherwise indistinguishable from *L. monocytogenes*, is motile at 37°C producing substantial amounts of flagella on the bacterial surface (Kathariou *et al.*, 1995). This may reflect a different mode of regulation of flagellin gene expression between the two species.

Two genes flanking *flaA* from *L. monocytogenes* have been identified (Dons *et al.*, 1994). These genes encode polypeptides of 68.7 kDa and 13.1 kDa, homologous to the sensor protein CheA (Fuhrer and Ordal, 1991) and the response regulator CheY (Bischoff and Ordal, 1991) respectively, involved in *B. subtilis* and *E. coli* chemotaxis . DNA-DNA hybridisation results suggest that CheA- and CheY-like sequences are present in *L. innocua, L. welshimeri* and *L. ivanovii.* The high degree of conservation in the *L. monocytogenes* CheA and CheY homologues, suggests an involvement in signal transduction, although the mechanism of signal transduction controlling chemotaxis in response to environmental stimuli, awaits further characterisation.

Several unrelated studies suggest a role for flagella and chemotaxis in infection by pathogenic bacteria including *Vibrio cholerae, Campylobacter jejuni,* and *Salmonella typhimurium* (Freter *et al.*, 1981a; Freter *et al.*, 1981b; Takata *et al.*, 1992; Jones *et al.*, 1992). Similarly, the directional motility displayed by *L. monocytogenes* is considered a prerequisite for penetration of the intestinal epithelium by this organism (Galsworthy *et al.*, 1990). Moreover, consistent with expression of *flaA* at 20°C, an increase in virulence of *L. monocytogenes* in the murine model of infection was observed when bacteria were grown at similar temperatures (Czuprynski *et al.*, 1989; Stephens *et al.*, 1991). Nevertheless, as mutational analysis of the *flaA* gene has not been reported, an involvement of flagella in *L. monocytogenes* pathogenicity is yet to be established.

1.13.13 Cold tolerance

A major underlying factor promoting listeriosis outbreaks is the ability of *L. monocytogenes* to grow in food stored at refrigeration temperatures (Farber and Peterkin, 1991). However, only recently have developments been made in an attempt to understand the genetic mechanisms responsible for *L. monocytogenes* cold tolerance. Transposon-induced mutants incapable of growth at low temperature have been isolated, and localised to a 1.8 kb *Eco*R1 DNA fragment. Mutants are not interrupted in their ability to infect tissue culture monolayers, and suggests that cold tolerance is independent of previously established virulence determinants (Zheng and Kathariou, 1994). Furthermore, genetically distinct insertion mutants characterised by an increase in lag phase during growth at 4°C and 10°C have been reported (Passos *et al.*, 1995). However, these mutants have only been partially characterised, although a function is psychrotolerance is anticipated. In addition, a gene (*cspL*), homologous to the major cold shock protein gene families of *E. coli* and *Bacillus* spp. has been identified in

L. monocytogenes, using PCR methodology (Francis *et al.*, 1995). Transcriptional *cspL::luxAB* fusions have determined this gene to be significantly induced when grown at 4°C and 10°C when compared to growth at 37°C. Collectively, these preliminary studies suggest that *L. monocytogenes* has developed several independent mechanisms by which they can adapt and grow at refrigeration temperatures.

Moreover, characterisation of fatty acid and polar lipids from *L. monocytogenes* after growth at various temperatures, have revealed significant differences in these profiles (Jones *et al.*, 1995). This may have implications in the psychrotolerance of this organism. This is in agreement with a previous study that first revealed a dependence of fatty acid composition of *Listeria* spp. on growth temperature (Püttmann *et al.*, 1993). Although C_{15} and C_{17} chain length fatty acids occur in all species, enrichment with C_{15} fatty acids after growth at low temperatures, may reflect the need to increase the fluidity of the bacterial cell membrane under these conditions.

1.13.14 Stress proteins

The intracellular environment of low pH, nutritional stress and oxidising agents, encountered by invading bacteria may induce a bacterial stress response. At least 20 heat shock proteins are synthesised by *L. monocytogenes* when grown at 48°C and under oxidative stress in the presence of hydrogen peroxide (H_2O_2) (Sokolovic and Goebel, 1989; Sokolovic *et al.*, 1990). Significantly, LLO, the protein responsible for phagolysosome membrane lysis, was identified as a heat shock protein (Sokolovic and Goebel, 1989). At least 5 heat shock proteins were co-induced with LLO and were only detected in *L. monocytogenes* strains (Sokolovic *et al.*, 1990). Furthermore, heat shock protein synthesis in different *L. monocytogenes* strains and other *Listeria* spp., was induced during growth in conditions of temperature upshift, acidity and oxygen stress (Morange *et al.*, 1993). Virulent and avirulent strains of *L. monocytogenes*, exhibited differing constitutive synthesis of heat-shock proteins. This may indicate a requirement for particular heat-shock proteins in intracellular survival of pathogenic *Listeria* strains.

In a recent study, induction of stress proteins in *L. monocytogenes* including homologues of DnaK and GroEL have been observed *in vitro*, following growth in environmental conditions such as temperature upshift and H_2O_2 (Hanawa *et al.*, 1995). However, unlike the situation for

other intracellular bacteria including *Y. entercolitica, S. typhimurium,* and *L. pneumophila,* these stress proteins were not among the 32 proteins induced by *L. monocytogenes* following phagocytosis by macrophages. The stress proteins induced in this manner may be involved in the mechanism of bacterial escape from the hostile environment of host cell phagolysosome. Consequently, *L. monocytogenes* environmentally induced stress proteins including the homologues of DnaK and GroEL, may not be required for intracellular survival. Therefore, genetic analysis of proteins induced after phagocytosis could improve our understanding of bacterial survival in host cells.

1.14 Comparisons between *L. monocytogenes* and *S. flexneri* infections

The mode of infection of *L. monocytogenes* and *S. flexneri* are very similar. Both organisms induce their own internalisation into mammalian cells, and are engulfed by phagosomes. Lysis of the fused phagosome/lysosome membrane follows, and bacteria enter into the cytoplasm and undergo a rapid period of multiplication. Migration to the cell surface, pseudopod formation, and entry into neighbouring cells occurs as bacteria polymerise host cell actin. Although a common mechanism of infection is utilised, no homology exists between the genetic determinants responsible for infection.

A 220 kb plasmid is essential for *Shigella* virulence (Sansonetti *et al.*, 1981; Sansonetti *et al.*, 1982), and contains a 37 kb region which is responsible for invasiveness of *Shigella* spp. (Maurelli *et al.*, 1985). In particular, the *ipaABCD* locus (invasion plasmid antigen) is essential for the invasion phenotype (Hale *et al.*, 1985; Buysse *et al.*, 1987; Ménard *et al.*, 1996), but are unrelated to *inlA* or *inlB,* the invasion associated products of *L. monocytogenes.* However, the leucine rich repeats of InlA and InlB (region A, see Figure 1.9), that may be involved in interactions with host cytoplasmic proteins, are also observed in the IpaH virulence determinant from *S. flexneri* (Hartman *et al.*, 1990). The significance of this finding is not known. Clearly, the specificity of the bacterial-host interaction mediated by the invasion associated proteins of these organisms are different, and reflects independent internalisation pathways.

The genetic determinants for *Shigella* mediated lysis of the phagolysosome membrane are not clearly described, although the virulence plasmid is required. Independent studies have inferred that the *ipaB* gene product of *S. flexneri* may function as a contact haemolysin responsible for

lysis of the phagolysosome membrane (Sansonetti *et al.*, 1986; Mills *et al.*, 1988; High *et al.*, 1992; Ménard *et al.*, 1993). In addition, non-polar mutations in *ipaC* and *ipaD* were also defective in lysis escape from the phagolysosome (Ménard *et al.*, 1993). Nevertheless, no homology was evident between the *ipa* genes and *hly*, the gene encoding LLO from *L. monocytogenes*.

Furthermore, no significant identity at the nucleotide level between the actin nucleator *actA* from *L. monocytogenes* to any other protein was observed. However, an internal 50 aa region distal to the N-terminal proline repeats in *actA* and *L. ivanovii iactA*, was homologous to an internal region of the *icsA* gene product isolated from *S. flexneri* (Lett *et al.*, 1989). This protein is responsible for intracellular and intercellular migration of this organism, through the induction of host cell actin polymerisation (Bernardini *et al.*, 1989; d'Hauteville and Sansonetti, 1992), in a manner similar to virulent *Listeria* spp. Nevertheless, the significance of this homology has not been determined (Kreft *et al.*, 1995). Finally, *L. monocytogenes plcB* and *S. flexneri icsB* (Allaoui *et al.*, 1992), responsible for lysis of the double membrane formed after invasion of a neighbouring cell, are unrelated. Collectively, this suggests that the genetic mechanisms by which *L. monocytogenes* and *S. flexneri* establish pathogenicity have been acquired from independent sources.

1.15 Influence of environmental stimuli on *L. monocytogenes* gene expression

To overcome the complex defence mechanisms of mammalian hosts, bacteria have developed a number of strategies that enable them to survive and establish an infection within the host. Strategies used for this purpose distinguish pathogens from opportunistic bacteria that cause disease primarily in compromised hosts (Miller *et al.*, 1989). Importantly, pathogenic bacteria are predominantly found in the environment, at which time expression of virulence determinants is down regulated as a mechanism of energy conservation. A common feature of all pathogens involves their timing and regulation of virulence determinant expression (Finlay and Falkow, 1989a). It is necessary for bacteria to develop efficient mechanisms for sensing the environment and regulating expression of virulence determinants, so that individual factors are expressed only during a particular phase of infection (Gross, 1993).

Bacteria coordinately regulate expression of virulence determinants by a process termed signal transduction, enabling the bacteria to transform an environmental stimulus into a cellular signal

(Gross, 1993). This process usually involves two proteins that belong to the family of proteins known as "two component regulators". Transduction is mediated by sensor proteins on the bacterial surface that detect an environmental signal. The signal is then transported by a signal cascade to intracellular response regulator proteins involved in DNA binding that are triggered to induce gene expression. Numerous environmental signals are known to act as cues for virulence gene expression in Gram-negative and Gram-positive bacteria, examples of which are listed in Table 1.5.

The situation in *L. monocytogenes* is apparently no different. Evidence for coordinate regulation of *hly, plcA, mpl, actA, plcB* and *inlA* by the positive regulatory factor PrfA has been reported (Mengaud *et al.*, 1991; Chakraborty *et al.*, 1992; Dramsi *et al.*, 1993). To date, no cofactors have been identified to assist in the PrfA-dependent regulation of *L. monocytogenes* virulence. This suggests PrfA may be bi-functional, capable of both sensing changes in the environment and interacting with DNA. However, other traditional "two component regulators" have been identified in *L. monocytogenes.* For example, two polypeptides CheA (environmental sensor protein) and CheY (response regulator), are responsible for the signal transduction events controlling chemotaxis in this organism (Dons *et al.*, 1994). Furthermore, a PCR based strategy using degenerate oligonucleotide primers (Anderson *et al.*, 1995b), has identified several nucleotide sequences internal to genes in *L. monocytogenes* that are homologous to distinct response regulator sub-families including PhoP and KdpE (Anderson *et al.*, 1995a). Characterisation of these genes using defined mutational analysis is underway and should result in the identification of novel genes that contribute to *L. monocytogenes* pathogenicity.

The PrfA-dependent expression of *L. monocytogenes* virulence determinants is thermoregulated (Chakraborty *et al.*, 1992; Leimeister-Wächter *et al.*, 1992; Dramsi *et al.*, 1993). Similar global control of virulence genes expression by temperature has been observed for other pathogenic bacteria (Maurelli, 1989). Several phenotypic studies have also shown temperature to influence growth, haemolysin production and virulence of *L. monocytogenes* (Czuprynski *et al.*, 1989; McClure *et al.*, 1989; Stephens *et al.*, 1991; Khan *et al.*, 1993).

Nevertheless, temperature is not the sole environmental signal utilised by *L. monocytogenes* for controlling virulence gene expression. First, transcriptional *luxAB* fusions were used to demonstrated *hly* and *plcA* expression was influenced by heat stress and media composition

Table 1.5 Environmental signals controlling the expression of coordinately regulated virulence determinants in pathogenic bacteria[a]

Organism	Environmental signals	Genotype (Phenotype)	Reference
E. coli	Iron	*fur* (shiga-like toxin, siderophores)	Calderwood and Mekalanos, (1987)
	Temperature	*pil* (Pap pilus expression)	Gorgansson *et al.*, (1990)
L. monocytogenes	Heat shock	*hly* (listeriolysin-O)	Sokolovic and Goebel, (1989)
	Temperature	*prfA* (regulates expression of listeriolysin-O, phospholipase, metalloprotease and actin polymerization)	Chakraborty *et al.*, (1992); Dramsi *et al.*, (1993)
S. typhimurium	Osmolarity	*invA* (invasion)	Galan and Curtiss III, (1990)
	Starvation, Stress, pH, Growth Phase	*phoP/Q* (regulates expression of genes required for survival within macrophages)	Miller, (1991)
Shigella spp.	Temperature	*virR* (invasion)	Maurelli and Sansonetti, (1988)
V. cholerae	Osmolarity, pH, Temperature	*toxR* (regulates cholera toxin and Tcp expression)	Miller and Mekalanos, (1988)
	Iron	*fur* (regulates expression of siderophores), *irg* (iron regulated genes)	Goldberg *et al.*, (1990)
Yersinia spp.	Temperature, Calcium	*lcr* (invasion)	Barve and Straley, (1990); Rohde *et al.*, (1994)

[a] adapted from Mekalanos, (1992)

(Park *et al.*, 1992). Second, the involvement of iron in regulation of gene expression in *L. monocytogenes* has also been reported. For example, expression of LLO is inversely related to the concentration of available iron in growth media (Cowart and Foster, 1981; Cowart, 1987; Geoffroy *et al.*, 1987; Tanycz and Ziegler, 1988; Geoffroy *et al.*, 1989). In addition, as iron is an essential trace element for growth (Neilands, 1981), all bacteria utilise conditions of low iron concentration, as a means to upregulate iron scavenging systems (Payne, 1993). Third, growth conditions of heat stress, salt concentration and pH alter the level of LLO, catalase and SOD production in *L. monocytogenes* (Sokolovic and Goebel, 1989; Khan *et al.*, 1993; Myers *et al.*, 1993). Collectively, these studies implicate the involvement of several environmental factors apart from temperature, in control of virulence gene expression in this bacterium. Potentially, *L. monocytogenes* may encode other regulatory factors able to sense environmental cues other then temperature, providing an additional level of control of virulence gene expression.

1.16 Mechanisms for regulation of gene expression

Generally, the mechanism responsible for global transcriptional control of bacterial gene expression involves alterations in DNA structure or topology permitting the interaction between the RNA polymerase and gene promoter regions. Such control mechanisms have overlying implications for the regulation of virulence gene expression, and has recently been reviewed (Dorman, 1995). This control mechanism utilises the negative supercoiled nature of bacterial chromosomal DNA. In *E. coli* and *S. typhimurium*, the degree of supercoiling is controlled by two enzymes DNA gyrase and topoisomerase I (Wang, 1971; Cozzarelli, 1980). DNA gyrase introduces negative supercoils in an energy-dependent process. In contrast, topoisomerase I is a relaxing enzyme capable of removing negative supercoils independent of an energy requirement. Cellular processes such as transposition, chromosome replication, recombination and transcription are sensitive to changes in DNA supercoiling (Drlica, 1984; Wang, 1985; Drlica 1987).

Variations in DNA supercoiling have been observed in response to environmental stimulus. These observations, in part, have been made possible by the use of antibiotics which inhibit gyrase activity. Temperature, osmolarity, growth phase, nutritional status and anaerobicity all induce gene expression by altering the superhelix density of DNA (Goldstein and Drlica, 1984; Yamamoto and Droffner, 1985; Balke and Gralla, 1987; Dorman *et al.*, 1988; Higgins *et al.*, 1988; Ní Bhriain *et al.*, 1989). For example, the temperature regulated *virR* gene of *S. flexneri*

which coordinately regulates a number of plasmid encoded virulence genes, mediates its regulatory effects through changes in DNA supercoiling (Dorman *et al.*, 1990). In addition, regulation of the *Salmonella* invasion process, which is influenced by osmolarity, growth phase and anaerobiosis, is thought to involve DNA supercoiling (Galán and Curtiss, 1990).

Apparently, abundant histone-like DNA binding proteins of bacteria, such as protein H1 of *E. coli,* play a role in the regulation of a number of genes known to be sensitive to alterations in DNA topology. For example, a DNA binding protein is involved in the temperature regulation of the *E. coli* Pap pili operon (Gorgansson *et al.*, 1990). In addition, H1-defective strains of both *Shigella* and *Salmonella* show reduced virulence (Higgins *et al.*, 1990). However, given the global requirement of H1-like DNA binding proteins in regulation, this phenotype may not specifically be the result of reduced virulence gene expression, but rather a reduced growth rate due to loss of synthesis of an essential biosynthetic operon.

Nevertheless, DNA topology is apparently involved in the global regulation of virulence determinants in bacteria. However, it would be naive to suspect that regulation is the result of DNA supercoiling alone, but rather forms the foundation to which other elements become involved (Higgins *et al.*, 1990). For example, DNA binding proteins like protein H1 can affect DNA topology by initiating DNA looping or bending to bring DNA sequences in alignment to allow RNA polymerase to bind (Friedman, 1988). Furthermore, RNA polymerases are exquisitely sensitive to the relative orientation of the -35 and -10 regions, and environmental conditions can control this orientation by changing the DNA twist (Wang and Syvanen, 1992). In addition, regulatory proteins that contain both a response regulator domain to environmental stimuli and a DNA binding domain confer specificity upon the more general fluctuations of DNA topology (Dorman, 1991). A functional model of the putative DNA-protein interactions involved in regulation of gene expression is shown in Figure 1.12.

The temperature activated PrfA protein of *L. monocytogenes* is able to bind to the 14 bp palindromic sequences upstream of promoter regions of virulence determinants that presumably permits association of RNA polymerase to this region, which initiates transcription. However, it is not known if PrfA is a bi-functional protein, containing the environmental sensor domain, in addition to the DNA binding domain. It is possible other proteins are also involved in the regulation process.

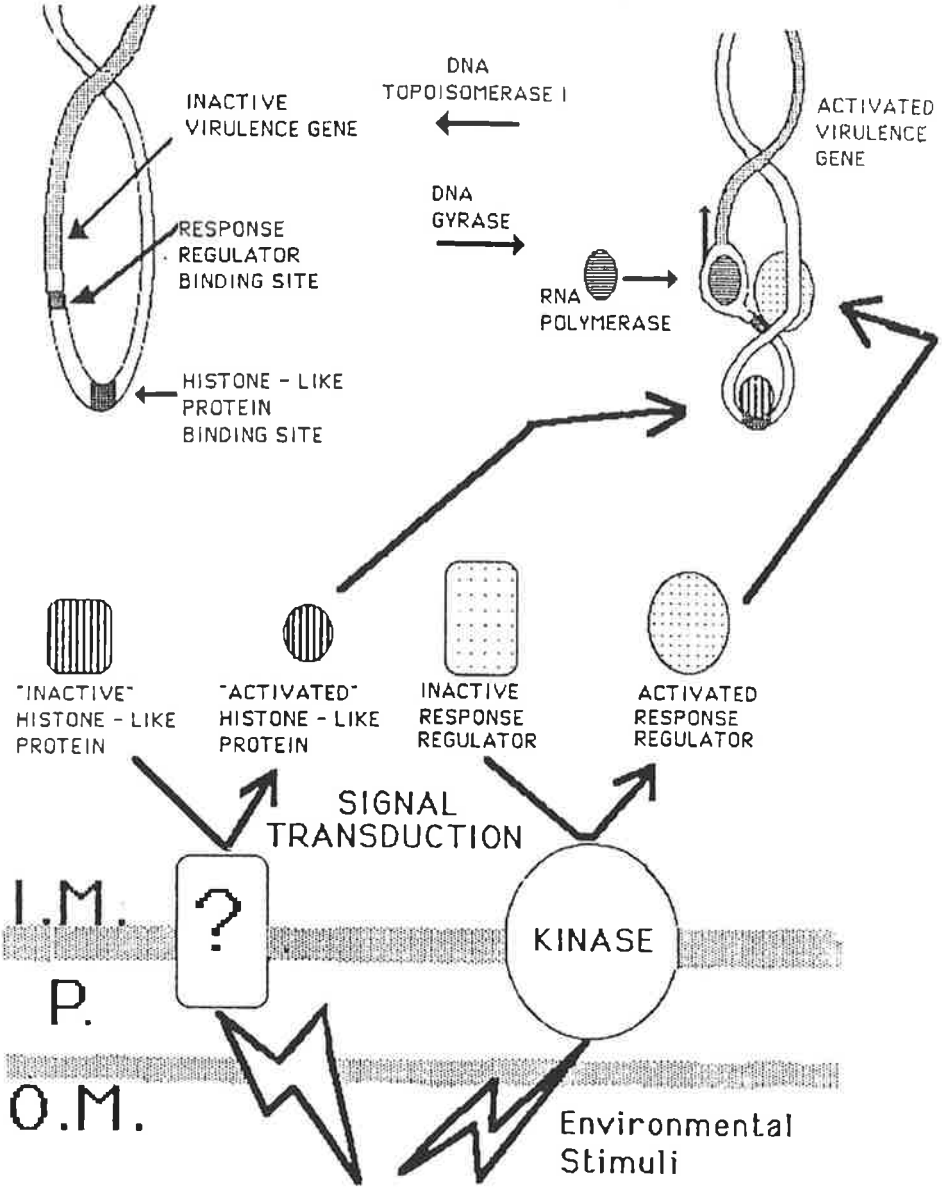


Figure 1.12: Schematic representation of the primary features of a bacterial control network for environmental regulation of gene expression (Dorman, 1991). A segment of closed circular DNA is represented by the looped ribbon, with genetic motifs represented by stipling and hatching. The molecule on the left is less supercoiled than that on the right. The question mark represents a notional signal-transducing protein involved in the covalent modification of a histone-like protein in response to environmental stimuli. A membrane-bound histidine kinase of the two-component signal transduction family is also shown. Abbreviations: I.M., Inner membrane; P., Periplasm; and O.M., Outer membrane.

1.17 Use of reporter gene fusions to isolate genes regulated by environmental conditions

Transcriptional or translational promoter fusions with reporter genes, provides a convenient method for the detection of promoter activity of genes whose products normally cannot be directly measured (Youngman, 1987). In effect, the product of the reporter gene substitutes for the natural product allowing the quantitation of changes in gene expression by measuring the enzymatic activity. This system can also be applied to the isolation of promoters whose activity is regulated by environmental cues. However, the utility of this system depends on the ability to duplicate environmental conditions in the laboratory. Nevertheless, this methodology has been routinely applied to both Gram-negative and Gram-positive bacteria. Moreover, transposon derivatives containing promoterless reporter vectors have served to enhance the ability to generate a library of gene fusions because it removes the need to first clone the gene.

However, these techniques used to identify environmentally regulated genes, rely on the ability to reproduce conditions in artificial media that control gene expression in the bacteria's natural environment. To overcome this restriction, Mahan *et al.*, (1993), have developed a rationale for the selection of novel bacterial virulence genes of *S. typhimurium*, which are specifically induced in host tissues. This approach overcomes the limitations of *in vitro* isolation techniques, allowing the detection of all genes induced *in vivo*. This study took advantage of the essential virulence gene *purA* from *Salmonella* spp. *S. typhimurium* DNA was fused with a *purA*::*lacZ* construct and introduced into a *S. typhimurium* PurA$^-$ mutant strain. The recombinant derivative when infected into mice, specifically selected surviving bacteria based on synthesis of PurA, under the control of a fused *Salmonella* promoter. All surviving bacteria were plated onto agar plates containing a colorimetric substrate (X-gal) to differentiate strains on the basis of β-galactosidase activity. Colonies that appeared white on this media were deficient in *lacZ* expression, and indicated that promoters responsible for *purA* expression were active only in the host. Genes isolated using this technique are specifically required for housekeeping function or for the establishment of an infection in a *in vivo* environment.

In addition, *L. monocytogenes* genes preferentially expressed in infected mammalian cells were identified after screening a library of Tn*917-lacZ* insertion mutants (Klarsfeld *et al.*, 1994). Genes were identified by comparing the β-galactosidase activity of insertion mutants in infected J774 macrophage-like cells, to β-galactosidase activity in rich broth medium. This

approach yielded several genes which were preferentially expressed in an intracellular environment.

These techniques are restricted to organisms that have an established gene transfer system and the availability of well defined auxotrophs. In the case of *Borrelia* spp, such genetic systems are not available, which lead Suk and collegues (Suk *et al.*, 1995), to develop an immunological strategy for the detection of *B. burgdorferi* genes selectively expressed in the infected host. An expression library of the pathogen was differentially screened using two antisera, one from animals raised against killed *in vitro* cultured bacteria, and the other from infected hosts. Those clones only reacting with the antiserum isolated from infected hosts are likely to contain genes expressed *in vivo*.

Furthermore, operon fusions have been used to characterise the phagolysosome environment of mammalian cells in which an intracellular pathogen resides. This procedure involves the construction of reporter gene fusions with well characterised virulence determinants whose expression is controlled by environmental factors. For example, a Ca^{2+}-responsive operon fusion to *lacZ* in *Y. pestis*, detected a suitable low concentration of calcium in the phagolysosome of mouse macrophages to permit gene expression (Pollack *et al.*, 1986). Similarly, *lacZ* fusions with genes regulated by Fe^{2+}, Mg^{2+}, pH, lysine or anaerobiosis in *S. typhimurium*, revealed appropriate conditions for induction of gene expression at different stages during bacterial infection of MDCK epithelial cells (Garcia-del Portillo *et al.*, 1992). In the future, this technology has the potential to characterise the intracellular environment of tissue culture cells and enable the tracking of gene expression during bacterial infection.

1.18 Project aims

Several virulence determinants of *L. monocytogenes* have been isolated and characterised. These genes tend to be grouped into a region known as the virulence gene cluster. However, additional genes from this organism have been reported and implicated to function in virulence. This suggests that there is still much to be learnt in regard to *L. monocytogenes* pathogenesis. For example, limited information is available that concerns bacterial encoded factors responsible for interaction with host cells. To date, only the products encoded by the *inlAB* and *actA* genes have been shown to interact with host encoded factors. It is likely other bacterial determinants are also involved in this critical aspect of *L. monocytogenes* pathogenicity. Another process not understood is the mode of action by the positive

regulatory factor, PrfA, intimately involved in up-regulation of the virulence gene cluster. It is possible other genes are involved in regulation, perhaps in sensing an environmental change, and mediating transmission of this signal to PrfA. Therefore, continued identification of novel virulence genes is necessary in order to increase our understanding of *L. monocytogenes* pathogenesis.

The major aim of this thesis was to isolate a library of promoter fusions in *L. monocytogenes* whose activity is up-regulated by environmental conditions prevailing within the host which have been duplicated *in vitro*. Candidate environmental conditions include temperature upshift (25°C → 37°C), calcium stress, iron stress, osmolarity, acidity or carbon dioxide. Tn*917* derivatives developed by P. Youngman and colleagues (University of Georgia, Athens, Georgia, USA) were the source of promoterless reporter genes.

Once a fusion library was established, the second aim of the thesis involved examination of each fusion mutant to assign a level of importance based on the extent of up-regulation of reporter gene activity when fusion mutants are grown in environmental stress. This approach was used to select a fusion mutant for detailed genetic characterisation.

The third aim of this thesis involved optimisation of a tissue culture internalisation assay using the human epithelial cell lines Caco-2 and HeLa. This assay provides a convenient method for assessing the level of virulence of relevant fusion mutants in comparison to wild type strains. The ability of each organism to internalise and multiply within epithelial cells was used as an indicator of virulence. This data was correlated to the level of persistence of fusion mutant and wild type strains in tissue organs of infected mice.

It was anticipated that this rationale would potentiate the isolation of uncharacterised genes of *L. monocytogenes* essential for virulence or housekeeping functions within the host.

Chapter 2 Materials and Methods

2.1 Bacterial strains and plasmids

All *L. monocytogenes* strains used in this study are listed in Table 2.1 and other *Listeria* spp. are listed in Table 2.2. *E. coli* and *B. megaterium* strains are shown in Table 2.3. Relevant characteristics of the plasmid clones and vectors used are listed in Table 2.4.

2.2 Bacterial growth media

All strains of *Listeria* spp. were routinely cultivated on Brain Heart Infusion (BHI) agar (Difco, Detroit, Michigan, USA; Oxoid Ltd., London, England; or BBL Microbiology Systems, Cockeysville, Maryland, USA) and in BHI broth. *E. coli* were cultivated on Nutrient Agar (NA) and in Nutrient Broth (NB) (Oxoid Ltd., London, England). Columbia Horse Blood Agar (CHBA) (Oxoid Ltd.) was employed to demonstrate haemolytic activity of *Listeria* spp. Minimal Medium (MM) with agar able to support growth of *L. monocytogenes*, was used as described by Premaratne *et al.*, (1991). SOC medium used in electro-transformation studies of *E. coli* consisted of 2% (w/v) Bacto tryptone (Difco), 0.5% (w/v) Bacto yeast extract (Difco), 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM glucose. Terrific broth contained 1.2% (w/v) Bacto tryptone, 2.4% (w/v) yeast extract, 0.4% (v/v) glycerol, 0.17 M KH_2PO_4, 0.72 M K_2HPO_4. Antibiotics, where indicated, were added to broth and solid media at the following final concentrations: Ap, 100 µg/ml; Cm, 25 µg/ml; Em, 10 µg/ml (*L. monocytogenes*) or 150 µg/ml (*E. coli*); Lm, 25 µg/ml; Rp, 25 µg/ml (*L. monocytogenes*) or 200 µg/ml (*E. coli*); Sm, 50 µg/ml; and Tc, 8 µg/ml.

Listeria spp. and *E. coli* were incubated at 37°C unless otherwise indicated.

2.3 Maintenance of bacterial strains

All strains were stored in Wheaton vials (Millville, New Jersey, USA) in a 1 ml solution of 32% (v/v) glycerol and 0.6% (w/v) Bacto peptone (Difco) at −70°C. Lyophilised stocks (see below) were stored *in vacuo* in sealed glass ampoules at 4°C. Fresh single colonies of *Listeria* and *E. coli* strains were prepared by streaking a loopful of glycerol stock onto the appropriate media and incubated O/N. For routine use, cultures were maintained on agar plates at 4°C.

Table 2.1 *L. monocytogenes* strains used in this study

Strain	Genotype/Phenotype	Serotype	RFLP Group[a]	Source/Reference[b]
DRDC8	environmental isolate of milk	4	B	NSW DCL
LM001	spontaneous Sm^R variant of DRDC8	4	B	This study
LM002	spontaneous Rp^R variant of DRDC8	4	B	This study
LLO17	DRDC8 containing a Tn*917-lacZ-cat86* insertion in *hly*	4	B	Stoner, (1993); Lucic, (1994)
LLO19	DRDC8 containing a Tn*917-lacZ-cat86* insertion in *prfA*	4	B	Stoner, (1993)
C044, C185	DRDC8 containing a Tn*917-lacZ-cat86* transcriptional fusion with DNA induced by EGTA, Em^R, Lm^R	4	B	This study
T390, T3619	DRDC8 containing a Tn*917-lacZ-cat86* transcriptional fusion with DNA induced by temperature, Em^R, Lm^R	4	B	This study
DSEC1	DRDC8 containing a pCT223 cointegrate in *ctpA*, Em^R, Cm^R	4	B	This study
DSE201, DSE221, DSE285, DSE294	DRDC8 containing an *erm* insertion in the internal *Pst*1 site of *ctpA*, Em^R	4	B	This study
102C, 136C	environmental isolates	1	A	IC
8T, 10T, 13T	environmental isolates	1	A	IC
37152, 37933	environmental isolates	1	A	DTS
Ing3, Ing25	environmental isolates	1	A	IC
NRN11921	environmental isolate	1	A	APL
KE1457	clinical isolate	1	A	KEH
146C, 197C	environmental isolates	1	A1	IC
1T	environmental isolate	1	A1	IC
Ing10, Ing13, Ing67	environmental isolates	1	A1	IC
DRDC1, DRDC9, DRDC10, DRDC11	environmental isolates of milk	1	B	NSW DCL
DTS22, DTS26	environmental isolates	4	B	DTS
5708	environmental isolate	4	B	IMVS
36705, 37180	environmental isolates	1	B	DTS
I40072	environmental isolates	1	B	IMVS
218C	environmental isolate	4	B	IC
KE795, KE1514	clinical isolates	1	B	KEH
KE793, KE987	clinical isolates	4	B	KEH
KE391, KE503	clinical isolates	1	B1	KEH
KE1046	clinical isolate	4	B1	KEH
5865	environmental isolate	4	C	IMVS
SLCC 2371	laboratory isolate	1/2a	A	H. Hof
SLCC 2372	laboratory isolate	1/2c	nt	H. Hof
SLCC 2373	laboratory isolate	3a	A	H. Hof
SLCC 2374	laboratory isolate	4a	nt	H. Hof
SLCC 2377	laboratory isolate	4d	nt	H. Hof
SLCC 2378	laboratory isolate	4e	nt	H. Hof
SLCC 2540	laboratory isolate	3b	nt	H. Hof
SLCC 2755	laboratory isolate	1/2b	nt	H. Hof
SLCC 5764	laboratory isolate	1/2a	nt	H. Hof
EGD (Bubert)	laboratory isolate	1/2a	nt	A. Bubert
EGD (Kaufmann)	laboratory isolate	1/2a	nt	H. Hof

[a] *Hin*dIII Restriction Fragment Length Polymorphisms (RFLP) of *prfA/plcA* and *hly* virulence operons of *L. monocytogenes* isolates (Thomas, 1995)

[b] H. Hof, University of Mannheim, Germany; A. Bubert, Biozentrum der Universität, Germany; New South Wales Dairy Corporation Laboratories (NSW DCL); Dairy Technical Services (DTS); Inghams Chickens (IC); Australian Poultry Ltd. (APL); King Edward Hospital (KEH)

nt not tested

Table 2.2 *Listeria* spp. used in this study

Strain	Genotype/Phenotype	Serotype	Source/Reference[a]
L. ivanovii	-	5	IMVS
L. seeligeri	-	nd	IMVS
L. welshimeri	-	nd	IMVS
L. grayi	-	nd	IMVS
L. innocua	-	nd	IMVS

[a] Institute of Medical and Veterinary Sciences (IMVS), Adelaide, Australia

nd not determined

Table 2.3 *Bacillus* and *E. coli* strains used in this study

Strain	Genotype/Phenotype	Source/Reference[a]
B. megaterium		
PV411/pTV53	-	P. Vary
B. subtilis		
PY1177/pLTV1	-	Camilli *et al.*, 1990
E. coli		
DH5α	ϕ80d*lacZ*ΔM15, *recA*1, *endA*1, *gyrA*96, *thi*-1, *hsdR*17(r_K^-, m_K^+), *supE*44, *relA*1, *deoR*, Δ(*lacZYA-argF*)U169	BRL
S17-1	*recA, thi, pro, hsdR*$^-M^+$, <RP4:2-Tc:Mu:Km:Tn7> Tp^R, Sm^R	Simon *et al.*, (1983)
SM10	*thi, thr, leu,* su_{III}, RP4-2-Tc::Mu, Km^R	Simon *et al.*, (1983)
CC118	*araD*, Δ(*ara leu*)7697, Δ*lacX74*, *phoA*, Δ20, *galE*, *galK*, *thi*, *rpsE*, *rpoB*, *argE*(Am), *recA1*, *sup*^{0}	Manoil and Beckwith, (1985)
HB101	*thi*-1, *hsdS*20 (r_B^-, m_B^-), *supE*44, *recA*13, *ara*-14, *leuB*6, *proA*2, *lacY*1, *rpsL*20 (Sm^R), *xyl*-5, *mtl*-1	Promega Corporation
ECC219	*his, ilv, rpsL, galKam, pgl*Δ8 (*bio-uvrB*), Δ*H1*, *htrA*63::miniTn*10*	C. Clark
MF001	ECC219 habouring pGP1-2 and pGEM-7Zf(+)	This study
MF002	ECC219 habouring pGP1-2 and pCT220	This study
MF003	ECC219 habouring pGP1-2 and pCT203	This study

[a] P. Vary, Northern Illinois University, USA; C. Clark, University of Adelaide, Australia; Bethesda Research Laboratories (BRL)

Table 2.4 Plasmids used in this study

Vector/Clone	Marker[a]	Source/Reference[b]
pTV53	Tn*917-lacZ-cat86*, Em^R. Lm^R, Tc^R	P. Vary
pLTV1	Tn*917-lacZ*, Ap^R, Cm^R, Em^R. Lm^R, Tc^R	P. Youngman
pLTV3	Tn*917-lacZ*, Cm^R, Em^R. Km^R, Lm^R, Tc^R	P. Youngman
pAT19	Em^R	P. Courvalin
pRK2013	Km^R	Figurski and Helinski, (1979)
pSUP201-1	Ap^R, Cm^R	Simon *et al.*, (1983)
pGI21	IS*10*, Ap^R, Em^R	J. Mahillon
pJIR1243	pBluescript KS containing *catP* from pJIR750, Cm^R	J. Rood
pLIS3	pUC18, harbouring 4 kb *hly* DNA fragment, Ap^R	Cossart *et al.*, (1989)
pCT006	pBluescript KS, habouring a 5.96 kb *Kpn*I/*Sac*I PCR amplified chromosomal DNA fragment from DRDC8 containing *prfA, plcA, hly,* and *mpl*, Ap^R	C. Thomas
pBR322	Ap^R, Tc^R	New England Biolabs
pGEM-T	Ap^R	Promega
pGEM-3Zf(+)	Ap^R	Promega
pGEM-7Zf(+)	Ap^R	Promega
pBluescript KS	Ap^R	Stratagene
pGP1-2	Ap^R, Em^R	Tabor and Richardson, (1985)
pUSH1	Cm^R, Km^R	BGSC
pCT200	pBR322, Ap^R	This study
pCT201	pBR322, Ap^R	This study
pCT202	pBluescript KS, Ap^R	This study
pCT203	Ap^R	This study
pCT204	Ap^R	This study
pCT205	Ap^R	This study
pCT206	Ap^R	This study
pCT207	Ap^R	This study
pCT208	Ap^R	This study
pCT209	Ap^R	This study
pCT210	Ap^R	This study
pCT211	Ap^R	This study
pCT212	Ap^R	This study
pCT214	Ap^R	This study
pCT215	Ap^R	This study
pCT217	Ap^R	This study
pCT218	Ap^R	This study
pCT220	*ctpA*, Ap^R	This study
pCT221	*ctpA*::*erm*, Ap^R, Em^R	This study
pCT222	*ctpA*::*erm, mob*, Ap^R, Em^R	This study
pCT223	*ctpA*::*erm, mob, catP*, Ap^R, Em^R, Cm^R	This study
pCT226	Ap^R	This study
pCT227	pGEM-3Zf(+), Ap^R	This study
pCT228	Ap^R	This study
pCT229	Ap^R	This study
pCT230	Ap^R	This study
pCT231	Ap^R	This study
pCT232	Ap^R	This study

[a] unless indicated, all constructs generated in this study were derived in pGEM-7Zf(+)

[b] P. Vary, Northern Illinois University, USA; P. Youngman, University of Georgia, USA; P. Courvalin, Institute Pastuer, France; J. Mahillon, Université Catholique de Louvain, Belgium; J. Rood, Monash University, Australia; C. Thomas, University of Adelaide, Australia BGSC: The Bacillus Genetic Stock Center, The Ohio State University, Columbus, USA

Lyophilisation of bacterial cultures was performed by suspending several colonies in a small volume of sterile skim milk. Approximately 0.2 ml of each bacterial suspension was dispensed into sterile 0.25 x 4 inch freeze-drying ampoules and plugged with cotton wool. The samples were then lyophilised in a Modulyo vacuum freeze drier (Edwards High Vacuum International, Crawley, West Sussex, England). After releasing the vacuum the cotton wool plugs were pushed well down the ampoule and a constriction was made just above the level of the plug. The ampoules were evacuated to a partial pressure of 30 microns, sealed at the constriction without releasing the vacuum and stored at 4°C.

2.4 Animals

Pathogen free Swiss mice were obtained from the Animal Services Branch (University of Adelaide, Adelaide, South Australia, Australia), for use in *in vivo* studies of *L. monocytogenes* pathogenesis. All mice were 6 to 8 weeks old, and handled and used in accordance with the University Ethics Committee guidelines.

2.5 Chemicals and reagents

Chemicals were Analar grade. Unless otherwise stated, all chemicals used in this study were purchased from either Ajax Chemicals (Auburn, New South Wales, Australia), BDH Laboratory Supplies (Poole, Dorset, England) or Sigma Chemical Company (St Louis, Missouri, USA). Acetic acid, HCl, CsCl, phenol, EDTA, SDS, sodium chloride, sodium acetate, copper sulphate, and sucrose were purchased from BDH Laboratory Supplies. Ethanol, methanol, propan-2-ol, iso-amyl-alcohol, Triton X-100, chloroform, formaldehyde, calcium chloride, cobalt chloride, barium chloride, cadmium sulphate, chromic chloride, magnesium chloride, magnesium sulphate, potassium chloride, potassium di-hydrogen orthophosphate, di-potassium hydrogen orthophosphate, trichloroacetic acid, potassium dichromate, acetone, and sodium hydrogen carbonate were obtained from Ajax Chemicals. Coomassie brilliant blue R250, TEMED, EGTA, ONPG, X-pho, and phalloidin rhodamine (TRITC) conjugate were obtained from Sigma Chemical Company. X-gal was purchased from Progen Industries Ltd. (Darra, Queensland, Australia). DIG DNA labelling and detection kits, DIG-11-dUTP, Tris base, IPTG, NBT, glycogen, herring sperm DNA, glycine, and Ap were purchased from Boehringer-Mannheim (Postfach, Mannheim, Germany). All other antibiotics were purchased from Sigma Chemical Company. Ficoll and dNTP's were purchased from Pharmacia (Uppsala, Sweden). Phenol (Special grade) for isolation of bacterial RNA, was

obtained from Wako Pure Chemical Industries Ltd. (Osaka, Japan). Mercuric chloride was purchased from Townson and Mercer (South Australia, Australia).

The following electrophoresis grade reagents were obtained from the sources indicated: DNA grade agarose (Progen Industries Ltd.), acrylamide and APS (Bio-Rad, Richmond, California, USA), and ultra pure N,N-methylene bis acrylamide and urea (Bethesda Research Laboratories, Grand Island, New York, USA).

Milli-Q water, an organic free reagent grade water, was used to prepare all buffers and reagents for DNA and RNA manipulation. Additional reagents and buffers were prepared with deionised water.

2.6 Enzymes and antibodies

Restriction endonucleases were routinely purchased from either Boehringer-Mannheim, New England Biolabs (Beverly, Massachusetts, USA) or Progen Industries Ltd. Lysozyme, pronase, T4 DNA ligase, terminal transferase, CIP, and Klenow were obtained from Boehringer-Mannheim. RNase A was acquired from Sigma Chemical Company, and DNase I (RNase free) was purchased from Promega (Madison, Wisconsin, USA). Stock solutions of RNase A (10 mg/ml) were heated at 95°C for 10 min prior to use, to inactivate contaminating DNases. Other DNA modifying enzymes were purchased from the following suppliers: Amplitaq DNA polymerase (Perkin-Elmer, Foster, California, USA), *Pfu* polymerase (Stratagene, La Jolla, California, USA), AMV reverse transcriptase (Boehringer-Mannheim), sequenase (Amersham International, Little Chalfont, Buckinghamshire, England), and T4 polynucleotide kinase (Bresatec Ltd., Thebarton, South Australia, Australia).

Listeria O Antiserum Poly (serotypes 1 and 4) was obtained from Difco. Goat anti-rabbit IgGAM (H+L) and fluorescein isothiocyanate (FITC) conjugate were purchased from The Binding Site Ltd., (Birmingham, England). Anti-DIG-POD (Fab fragments) and anti-DIG-AP (Fab fragments) were obtained from Boehringer-Mannheim.

2.7 Radionucleotides

γ-[^{32}P]-ATP (4000 Ci/mmole) was obtained from Bresatec Ltd, and [^{35}S]-Methionine (1270 Ci/mmole) and [^{35}S]-dATP (3000 Ci/mmole) were purchased from Amersham International.

2.8 Induction of transposition of Tn*917-lacZ-cat86* from pTV53

Two hundred independent 2 ml cultures of DRDC8/pTV53, grown O/N with agitation in sterile serology tubes containing Tc, Em and Lm at 30°C, were subcultured (0.1 vol) into 2 ml BHI broths containing Em and Lm, and incubated at 41°C O/N with agitation to induce transposition of Tn*917-lacZ-cat86*. Transcriptional promoter-*lacZ* gene fusions were isolated by plating a maximum of 20µl of O/N culture onto BHI agar containing Em, Lm, X-gal (40 µg/ml), and either 2',2'-Dipyridyl (2.5 mM) or EGTA (2.5 mM). Promoter-*lacZ* transcriptional fusions influenced by environmental signals including; temperature upshift (25°C → 37°C), iron limitation, cation limitation, osmolarity, carbon dioxide, and acidity were selected as blue pigmented colonies.

Aliquots of each independent culture were pooled, pelleted by centrifugation, and the cells resuspended in 32% (v/v) glycerol and 0.6% (w/v) bacto peptone (Difco) and stored at –70°C. Mutants containing a *lacZ* transcriptional fusion were isolated to homogeneity and stored in 32% (v/v) glycerol and 0.6% (w/v) bacto peptone (Difco) at –70°C. These insertion mutants represented a library of promoter::*lacZ* transcriptional fusions in *L. monocytogenes*.

2.9 Quantitation of β-galactosidase activity

β-galactosidase activity was assayed by ONPG hydrolysis using a modification of the procedure described by Miller, (1972). An O/N culture was subcultured (0.1 vol) and grown to mid log phase in fresh media. Cultures left on ice for 20 min were washed in chilled Z-buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, 50 mM β-mercaptoethanol, pH 7.0), resuspended in 1 ml Z-buffer containing 1 mg/ml lysozyme and incubated at 37°C for 30 min. The level of β-galactosidase activity in 0.5 ml of sample, diluted in an equal volume of Z-buffer, was determined after the addition of 0.2 ml of substrate (ONPG, 2 mg/ml). The reaction was left at RT for a maximum of 60 min, during which time yellow pigmentation developed. A 100 µl aliquot of each sample was taken and placed in a round bottom 96-well microtitre tray, and the OD at 410 nm and 570 nm was recorded on a Dynatech MR5000 Spectrophotometer (Dynatech Laboratories Ltd., Billingshurst, West Sussex, England). The β-galactosidase activity was represented by Miller units (Miller, 1972).

2.10 Preparation of Tris-HCl buffered phenol

To 500 ml phenol, an equal volume of 1 M Tris-HCl, (pH 8.0) and 500 mg of 8-hydroxyquinoline was added, mixed thoroughly and allowed to equilibrate. The upper aqueous phase was removed by aspiration and further equilibrated twice, with equal volumes of 1 M Tris-HCl (pH 8.0), followed by three additions of an equal volume of 0.1 M Tris HCl (pH 8.0). The final upper aqueous layer was left above the phenol phase.

2.11 DNA extraction procedures

2.11.1 Small scale plasmid DNA isolation

Method 1: Small scale quantities of plasmid DNA (3 to 5 µg per ml) was purified from *E. coli* by a modification of the three step alkali lysis method of Sambrook *et al.*, (1989). An O/N culture (1.5 ml), was collected in a 1.5 ml reaction tube (Sarstedt, Newton, North Carolina, USA) by centrifugation for 30 sec at 20 000×g in a Heraeus Biofuge 15 (Heraeus Sepatech GmbH, Osterode, Germany), and the pellet resuspended in 100 µl of solution 1 [50 mM glucose, 25 mM Tris-HCl (pH 8.0), 10 mM EDTA]. After the addition of 200 µl solution 2 [200 mM NaOH, 1% (w/v) SDS], the sample was incubated on ice for 5 min. A further 5 min incubation on ice was preceded by the addition of 150 µl of solution 3 [3M potassium acetate, 2 M acetic acid (pH 4.8)]. Cell debris was collected by centrifugation (1 min at 20 000×g, Heraeus Biofuge 15), and the supernatant was extracted with an equal volume of phenol:chloroform (1:1) in a fresh tube. The aqueous phase was transferred to a fresh reaction tube, plasmid DNA was precipitated in 0.7 vol of propan-2-ol, collected by centrifugation (15 min at 20 000×g, Heraeus Biofuge 15), washed with 70% (v/v) ethanol and dried *in vacuo* (Speedivac, Savant Instruments, Farmingdale, New York, USA). The DNA pellet was resuspended in 50 µl H_2O.

Method 2: Small scale quantities of plasmid DNA was extracted from *L. monocytogenes* and *B. megaterium* by the method of Kawamura *et al.*, (1985). Briefly, 1.5 ml of an O/N culture was pelleted by centrifugation (30 sec at 20 000×g, Heraeus Biofuge 15), and resuspended in 100 µl of SETL buffer [20% (v/v) sucrose, 50 mM EDTA, and 50 mM Tris-HCl (pH 7.6)]. Lysozyme was added to a final concentration of 2 mg/ml and the suspension was incubated at 37°C for 10 min. The cells were lysed by the addition of 200 µl of solution 2, followed by a 5 min incubation on ice. Following the addition of 145 µl of solution 3 and a further 5 min incubation on ice, the sample was centrifuged (1 min at

20 000×g, Heraeus Biofuge 15). The supernatant was transferred to a fresh tube and extracted with an equal volume of phenol:chloroform (1:1). Plasmid DNA was precipitated in 0.7 vol of propan-2-ol, collected by centrifugation (15 min at 20 000×g, Heraeus Biofuge 15), washed with 70% (v/v) ethanol and dried *in vacuo*. The DNA pellet was resuspended in 50 µl H_2O.

2.11.2 Large scale plasmid DNA isolation

Plasmid DNA was isolated by either of the following procedures.

Method 1: Large scale quantities of plasmid DNA (2 to 5 mg) was prepared from 300 ml O/N cultures by the 3-step alkali lysis method and CsCl gradient centrifugation (Garger *et al.*, 1983). The cell pellet, collected by centrifugation in a JA10 rotor using a Beckman J2-21M ultracentrifuge (Beckman Instruments Inc., Palo Alto, California, USA) at 11 000×g for 10 min, was resuspended in 2.4 ml of solution 1 and transferred to a SS-34 tube (Nalgene Labware, Rochester, New York, USA). The suspension was incubated at room temperature for 10 min after the addition of 0.6 ml lysozyme (20 mg/ml in solution 1). Two volumes of solution 2 was added prior to a 5 min incubation on ice. A further 15 min incubation on ice proceeded the addition of 2.8 ml of solution 3. Cell debris was removed by centrifugation at 12 000×g for 10 min at 4°C in a JA20 rotor using a Beckman J2-21M ultracentrifuge. DNA was extracted at least once with phenol:chloroform:isoamyl alcohol (25:24:1) in a 20 ml McCartney bottle, and precipitated with 0.7 vol of propan-2-ol. DNA was recovered by centrifugation (26 500×g for 20 min, Beckman J2-21M ultracentrifuge), washed with 70% (v/v) ethanol, and air dried.

A gradient was prepared after resuspension of the DNA pellet in 2.8 ml of TE buffer [10 mM Tris-HCl, 1 mM EDTA (pH 8.0)]. To the DNA solution, 2.9 g CsCl was added, prior to the addition of 0.3 ml (10 mg/ml) of EtBr. The solution was adjusted to a refractive index of 1.386 and placed into a 3.9 ml Beckman Quick-Seal ultracentrifuge polyallomer tube (Beckman Instruments Inc.). The tube was sealed and centrifuged in a Beckman Optima™ TLX Ultracentrifuge (Beckman Instruments Inc.) using a TLN-100 rotor at 391 000×g for 4 h at 20°C. The plasmid band was recovered with a 19 gauge needle attached to a 1 ml syringe. EtBr was extracted 3 to 4 times with an equal volume of iso-amyl alcohol. CsCl was removed by dialysis in 2 L of TE buffer at 4°C with at least one change of buffer.

Method 2: Large scale quantities of plasmid DNA (2 to 5 mg) isolated from 500 ml O/N cultures was prepared by a modified three step alkali lysis procedure. The cell pellet collected

by centrifugation in a JA10 rotor using a Beckman J2-21M ultracentrifuge at 11 000×g for 10 min, was resuspended in 10 ml of solution 1 containing 1 mg/ml RNase A, and transferred to a SS-34 tube (Nalgene Labware). The suspension was incubated at room temperature for 5 min after the addition of 10 ml solution 2. A further 15 min incubation on ice proceeded the addition of 10 ml of solution 3. Cell debris was carefully removed by centrifugation at 26 500×g for 30 min at 4°C in a JA-20 rotor using a Beckman J2-21M ultracentrifuge. The supernatant was applied to a Qiagen-tip 500 column (Qiagen GmbH; Hilden, Germany) equilibrated with buffer QBT [0.75 M NaCl, 0.05 M MOPS, 15% (v/v) ethanol, 0.15% (v/v) Triton X-100 (pH 7.0)], and allowed to pass through the column by gravity flow. The column was washed twice in 30 ml QC buffer [1.0 M NaCl, 0.05 M MOPS, 15% (v/v) ethanol], and the DNA eluted with 15 ml QF buffer [1.25 M NaCl, 0.05 M Tris-HCl, 15% (v/v) ethanol (pH 8.5)]. DNA was precipitated with 0.7 vol of propan-2-ol, recovered by centrifugation (26 500×g for 20 min, Beckman J2-21M ultracentrifuge), washed with 70% (v/v) ethanol, and air dried. The DNA pellet was resuspended in an appropriate volume of sterile H_2O.

2.11.3 Preparation of *E. coli* genomic DNA

Chromosomal DNA was isolated by a modification of the method described by Manning *et al.*, (1986). Briefly, bacterial cells were incubated O/N in NB, pelleted in 20 ml McCartney bottles in an MSE Minor S centrifuge (Crawley, West Sussex, England) at 310×g for 10 min, and resuspended in 2 ml 25% (w/v) sucrose, 0.05 M Tris-HCl (pH 8.0). Lysozyme (10 mg/ml) in 1 ml of 0.25 M EDTA (pH 8.0) was added and then incubated on ice for 20 min, followed by the addition of 0.75 ml TE buffer, 0.25 ml lysis solution [5% (w/v) sarkosyl, 50 mM Tris-HCl (pH 8.0), 62.5 mM EDTA], and 1 mg solid pronase. After incubating at 56°C for 1 h, the DNA was gently extracted three times with phenol and twice with diethyl ether, and dialysed against 5 L of TE buffer O/N.

2.11.4 Preparation of *L. monocytogenes* genomic DNA

Chromosomal DNA from *Listeria* spp. was isolated as previously described (Flamm *et al.*, 1984). Essentially, a 10 ml O/N culture was pelleted in an MSE Minor S centrifuge at 310×g for 10 min and washed in 1 ml of 0.1x SSC (15 mM NaCl, 1.5 mM sodium citrate). The pellet was resuspended in 0.6 ml of lysozyme solution [0.01 M sodium phosphate buffer (pH 7.0), 20% (v/v) sucrose and 2.5 mg/ml lysozyme] and incubated for 1 h at 37°C. To the sample, 5.4 ml of pronase solution [10 mM Tris-HCl (pH 8.0), 1 mM EDTA, 1% (v/v) SDS and

0.5 mg/ml pronase] was added, and incubated for a minimum of 1 h at 37°C. The solution was gently extracted once with an equal volume of phenol, followed by phenol:chloroform (1:1), and finally with chloroform. The aqueous layer was precipitated with 0.1 vol 3 M sodium acetate (pH 7.4), and 2.5 vol ethanol. The precipitated DNA was recovered, washed in 70% (v/v) ethanol, air dried and resuspended in an appropriate volume of TE buffer.

2.12 Analysis and manipulation of DNA

2.12.1 DNA quantitation

DNA concentration was determined by either measuring the OD of the DNA solution at 260 nm using a LKB Ultraspec Plus Spectrophotometer (Pharmacia), assuming for DNA, a solution containing 50 µg/ml has an absorbance of 1.0 (Miller, 1972), or by electrophoresing on an agarose minigel (EC370 Submarine gel system, E-C Apparatus Corporation, St Petersburg, Florida, USA) and comparing the fluorescence intensity of EtBr stained DNA fragments from the test sample with the intensity of DNA fragments of known concentration.

2.12.2 Restriction endonuclease digestion of DNA

Cleavage reactions with restriction enzymes were performed using restriction buffers specified by the manufacturer. The digestion reaction varied depending on the concentration of DNA and the volume of digested sample required. Essentially, a standard digest involved 6 µl of plasmid DNA (0.1 to 0.5 mg), 2 µl 10x restriction buffer (supplied by the manufacturer), 10 µl Milli-Q H_2O and 2 µl restriction enzyme (2 units/µl) in a final volume of 20 µl. The reaction mixture was incubated at the appropriate temperature for a minimum of 2 h. Prior to loading onto an agarose gel, 0.1 vol of tracking dye (15% (w/v) Ficoll, 0.1% (w/v) bromophenol blue, 0.1 mg/ml RNase A) was added.

Restriction digests involving at least two enzymes were performed as follows. Enzymes that required similar buffer conditions were digested together. When the optimal salt concentrations for enzymatic activity differed, the enzyme that required the lowest salt concentration was incubated first for 1 h, followed by the addition of the other enzyme after supplementing the reaction mix with an appropriate volume of NaCl. Digests with enzymes requiring incompatible buffers were achieved by precipitating the DNA after the first digestion, and resuspending in the second buffer prior to proceeding with the second digestion.

2.12.3 Agarose gel electrophoresis of DNA

Electrophoresis was performed at room temperature on horizontal 0.7% to 1.5% (w/v) agarose gels. The concentration of agarose depended on the expected size of the DNA fragments to be analysed. Gels were electrophoresed in a Horizontal System for Submerged gel electrophoresis, Model H5 (Bethesda Research Laboratories, Gaithersburg, Maryland, USA) at a maximum of 120 V for 1 h to 3 h in 1x TAE buffer [40 mM Tris, 1.5 mM EDTA, 0.012% (v/v) glacial acetic acid], followed by staining in distilled H_2O containing 2 µg/ml EtBr. DNA fragments were visualised by trans-illumination using a UV transilluminator (UVP Inc., Upland, California USA) and documented with a Tractel Gel Documentation System (Vision Systems, Salisbury, South Australia, Australia) or photographed on Polaroid 665 negative or 667 positive film (Polaroid Corporation, Cambridge, Massachusetts, USA).

2.12.4 Determination of restriction fragment size

The size of restriction enzyme fragments were calculated by comparing their relative mobility to *Eco*R1 digested *B. subtilis* bacteriophage SPP1 DNA. The calculated sizes of the SPP1 *Eco*R1 standard fragments used differed from those published (Ratcliff *et al.*, 1979), and were therefore re-calculated with the program DNAFRAG (Rood and Gawthorne, 1984) using bacteriophage Lambda and plasmid pBR322 as standards (Bresatec Ltd.). The sizes (kilobases, kb) used were: 8.51; 7.35; 6.11; 4.84; 3.59; 2.81; 1.95; 1.86; 1.51; 1.39; 1.16; 0.98; 0.72; 0.49; 0.36.

2.12.5 Isolation of DNA restriction fragments from agarose gels or PAGE

DNA fragments were isolated by one of the following procedures. Where required, glycogen was added to assist in the precipitation of DNA by acting as a carrier molecule, especially if the DNA was in low abundance or linearised DNA fragments were less than 1 kb.

Method 1: Digested DNA was electrophoresed at 4°C on a 1.0% (w/v) Seakem low melting temperature agarose gel (FMC BioProducts, Rockland, Maine, USA) in 0.5x TAE buffer at a maximum of 80V. The DNA fragment was excised and incubated at 65°C for at least 30 min in 5 vol TE buffer. DNA was extracted twice with 0.5 vol phenol, and the aqueous phase precipitated with 1 µl glycogen (20 mg/ml), 0.1 vol 3 M sodium acetate (pH 7.4), and 2.5 vol ethanol. DNA was collected by centrifugation (15 min at 20 000×g,

Heraeus Biofuge 15), washed in 70% (v/v) ethanol, dried *in vacuo*, and resuspended in 20 µl H_2O.

Method 2: Restricted DNA was electrophoresed at room temperature, on a 1% agarose gel in 1x TAE buffer at 100 V. DNA was excised from the gel without exposure to EtBr or UV light and placed in 400 µl of fresh 1x TAE buffer in 25 mm diameter dialysis tubing (Union Carbide Corporation, Chicago, Illinois, USA). Dialysis tubing was prepared by boiling for 10 min in sterile H_2O. The ends of the tubing were sealed with mediclips and placed into a electrophoresis tank containing 1x TAE buffer. DNA was electrophoresed from the agarose at 150 V for 2 h at 4°C. Prior to extraction of DNA from the tubing, polarity was reversed for 2 min. DNA was precipitated with 0.1 vol 3 M sodium acetate (pH 7.4), 1 µl glycogen (20 mg/ml) and 2.5 vol ethanol at –20°C for a minimum of 1 h. DNA was collected by centrifugation in a Heraeus Biofuge 15 for 15 min at 20 000×g, washed in 70% (v/v) ethanol, dried *in vacuo*, and resuspended in 20 µl H_2O.

Method 3: Phenol extracted DNA was prepared by a modification of the method of Bewsey *et al.*, (1991). Essentially, DNA electrophoresed in the standard manner, was excised from the agarose gel and finely diced using a sterile scalpel blade. An equal volume of Tris-HCl (pH 8.0) saturated phenol was added to the agarose gel pieces collected into a 1.5 ml reaction tube. After thorough mixing, the tube was place at –70°C for 90 min. The sample was then thawed and the aqueous phase collected by centrifugation in a Heraeus Biofuge 15 for 1 min at 20 000×g. DNA was extracted with phenol:chloroform (1:1), and precipitated with 1 µl glycogen and 0.7 vol iso-propyl alcohol. After collection by micro-centrifugation at RT in a Heraeus Biofuge 15 for 15 min at 20 000×g, DNA was washed in 70% (v/v) ethanol, dried *in vacuo*, and resuspended in 20 µl H_2O.

Method 4: DNA was extracted from a gel slice using the Bresaclean DNA extraction kit (Bresatec Ltd.) according to the manufacturer's instructions.

Method 5: Radiolabelled synthetic oligonucleotides were gel purified by acrylamide gel electrophoresis. A acrylamide gel stock (30% w/v) was prepared by dissolving recrystallised acrylamide and bis-acrylamide in a 50:1 ratio in Milli-Q H_2O at RT. This was de-ionised by stirring with 50 mg/ml of mixed-bed resin (Bio-Rad) for a minimum of 30 min at RT. After removing the resin by filtration through a scintered glass funnel, the gel stock was de-oxygenated under vacuum for 3 min and stored at 4°C in the dark. Gels were poured and

used on the same day. A 20 x 40 cm gel was prepared by mixing 10 ml of 30% stock solution, 6 ml 10 x TBE [0.89 M Tris-borate and 10 mM EDTA (pH 8.3)], 385 µl of 25% (w/v) APS and 95 µl of TEMED, poured into the gel sandwich, and allowed to polymerise. Immediately after removing the comb, the wells were rinsed with Milli-Q H_2O and the gel was pre-electrophoresed at 100 V for 15 min before loading the DNA. Electrophoresis was performed at a constant 400 to 500 V. The bands of end-labelled DNA were visualised by autoradiography using Kodak-Omat 100 X-ray film (Eastman Kodak Company, Rochester, New York, USA) at –70°C. Gel slices containing the DNA fragments were excised from the gel matrix by super-imposing the autoradiograph above the gel. Excised DNA was eluted by soaking the gel slices O/N in 400 µl of gel elution buffer [500 mM ammonium acetate, 10 mM magnesium acetate, 1 mM EDTA, and 0.1% (v/v) SDS (pH 7.6)] and the supernatant was ethanol precipitated. No carrier was used when precipitating the DNA fragments eluted out of polyacrylamide gel slices.

2.12.6 Dephosphorylation of DNA using alkaline phosphatase

To a 100 µl volume of digested DNA, 2-5 units of alkaline phosphatase (calf intestinal) and 0.1 vol of 10x phosphatase buffer (supplied by the manufacturer) were added. The reaction was incubated for 15 min at 37°C, followed by a 15 min incubation at 56°C. To the contents of this reaction, 0.1 vol of tracking dye was added prior to electrophoresis on a 1% (w/v) agarose gel. The linearised dephosphorylated DNA fragment was then gel purified (see Section 2.12.5).

2.12.7 End-filling enzyme restricted DNA fragments

Restricted DNA fragments with 5′ or 3′ overhanging ends were filled in by either of the following procedures.

Method 1: In a final volume of 20 µl, 1 mg of digested DNA or purified DNA fragments was mixed with 3 µl 10x Klenow polymerase buffer [20 mM Tris-HCl (pH 8.0), 100 mM $MgCl_2$], 3 µl 10 mM DTT, 1 µl of each 5 mM dNTP, and 1 µl Klenow (1 unit/µl). After incubating for 15 min at 37°C, samples were inactivated by heating at 70°C for 10 min, extracted with an equal volume of phenol:chloroform (1:1) and DNA from the aqueous phase precipitated (see Section 2.11.1).

Method 2: Blunt ending with *Pfu* polymerase was performed using a modification of the method of Costa and Weiner, (1994). Briefly, in a final volume of 50 µl, digested DNA or purified DNA fragments was mixed with 5 µl 10x *Pfu* polymerase buffer (supplied by the manufacturer), 100 µM of each dNTP, and 2.5 unit of *Pfu* polymerase. After incubating for 30 min at 72°C, samples were gel purified (see Section 2.12.5).

2.12.8 *In vitro* cloning

Where dephosphorylated vector and insert DNA had compatible cohesive termini a standard ligation reaction was performed as follows. Vector and insert were mixed in a reaction tube at a ratio of *ca.* 1:3 in a maximum volume of 16 µl. Where necessary, Milli-Q H_2O was added to make the final volume to 16 µl. The tube was heated to 45°C for 5 min to melt cohesive termini, then cooled on ice for 1 min. To the contents of the tube, 2 µl of 10x ligation buffer (supplied by the manufacturer) and T4 DNA ligase (2 units) were added. The ligation reaction was incubated O/N at RT. For blunt end ligations, 4 units T4 DNA ligase was used in a reaction. When cloning fragments of chromosomal DNA the ratio of vector to insert DNA was altered to 1:1. Before electroporation into *E. coli*, the reaction was ethanol precipitated and resuspended in 10 µl.

2.13 Construction of unidirectional deletions of cloned DNA

2.13.1 Preparation of construct DNA

Unidirectional deletions of pCT203 (see Section 5.2.3.1, Figure 5.5), were constructed using the Erase-a-Base kit (Promega). Plasmid DNA was initially purified by column extraction (Qiagen) to remove RNA and protein. Approximately, 5 µg of plasmid DNA was digested with two restriction enzymes in a final volume of 50 µl (see Section 2.12.2). Initially, pCT203 was digested with *Sph*I which generated a 4 nucleotide - 3' protrusion, resistant to exonuclease III digestion. Linearity of the sample was confirmed by electrophoresis on a 1% (w/v) agarose gel. The digest was extracted with 1 vol Tris-saturated phenol/chloroform (1:1) and the upper aqueous phase was recovered and precipitated with 0.1 vol 2 M NaCl and 2 vol absolute ethanol. DNA was pelleted by centrifugation, washed in 70% (v/v) ethanol, air dried and resuspended in an appropriate volume of TE buffer. The plasmid DNA was further digested with *Bam*HI, producing a 5' protrusion adjacent to the cloned insert, from which deletions could proceed (nesting site). Plasmid DNA was recovered after the reaction by ethanol

precipitation and resuspended in 60 µl of Exonuclease III buffer [66 mM Tris-HCl (pH 8.0), 0.66 mM $MgCl_2$].

2.13.2 Exonuclease III digestion, ligation and transformation of pCT203 derivatives

Volumes of 7.5 µl of S1 nuclease mix (0.86 vol Milli-Q H_2O, 0.135 vol 7.4x S1 buffer and 2.4 units S1 nuclease) were aliquoted into a series of microcentrifuge tubes. In addition, the DNA prepared in Section 2.13.1, was pre-warmed to the digestion temperature prior to the addition of 300-500 units of Exonuclease III. At 30 sec intervals, 2.5 µl of digested sample was removed and placed into consecutive tubes on ice, containing the aliquoted S1 nuclease mix. Following the completion of sampling, tubes were incubated at RT for 30 min, followed by an incubation at 70°C for 10 min after the addition of 1 µl S1 stop buffer (0.3 M Tris, 50 mM EDTA), to inactivate the S1 nuclease. Aliquots were removed to determine extent of digestion by electrophoresis on a 1% (w/v) agarose gel. The remaining sample from each time point was transferred to 37°C and 1 µl of Klenow mix [1 vol Klenow buffer (20 mM Tris-HCl {pH 8.0}, 100 mM $MgCl_2$), 0.15 units Klenow] was added, incubated initially for 3 min, followed by a further 5 min incubation at 37°C after the addition of 1 µl of dNTP mix (0.125 mM each of dATP, dCTP, dGTP and dTTP). Samples were then incubated O/N at RT with 40 µl of ligase mix [0.79 vol Milli-Q H_2O, 0.1 vol 10x ligase buffer (500 mM Tris-HCl {pH 7.6}, 100 mM $MgCl_2$, 10 mM ATP), 0.1 vol 50% (v/v) polyethylene glycol, 0.01 vol 100 mM DDT, 0.2 units T4 DNA ligase]. Aliquots of each reaction were transformed into chemically competent *E. coli* DH5α (see Section 2.14).

2.13.3 Rapid screening of deletion subclones

Using sterile toothpicks, individual colonies were smeared at the bottom of a microcentrifuge tube and the remainder patched onto a fresh selective plate and incubated O/N at 37°C. The bacteria contained within the tube were resuspended in 50 µl EDTA (pH 8.0), prior to the addition of 50 µl cracking buffer [0.2 M NaOH, 0.5% (v/v) SDS, 20% (v/v) sucrose]. The suspension was thoroughly mixed and incubated at RT for 5 min. A further 5 min incubation on ice followed, after which 1.5 µl of 4 M KCl and 0.5 µl of 0.4% (v/v) bromophenol blue were added. Cellular debris was removed by centrifugation in a Heraeus Biofuge 15 at 13 000×g for 3 min. Twenty five microlitres of the resultant supernatants were subsequently analysed by electrophoresis on a 0.7% (w/v) agarose gel and the sizes of the deletion clones

estimated. Clones of appropriate size differentiation were selected for restriction analysis after purification of plasmid DNA.

2.14 Chemical transformation

2.14.1 Preparation of competent cells

Competent cells were prepared by a modification of the method described by Hanahan, (1983). Briefly, 0.05 vol of an O/N culture of *E. coli* DH5α was subcultured in 100 ml of φb medium [0.5% (w/v) Bacto yeast extract, 2% (w/v) Bacto tryptone, 0.5% (w/v) $MgSO_4$ (adjusted to pH 7.6 with KOH)], and grown with agitation at 37°C until the OD at 600 nm was 0.6. The cells were chilled on ice for 20 min and pelleted in a Beckman J2-21M ultracentrifuge using a JA-14 rotor (15 000×g at 4°C for 10 min). The pellet was resuspended in 0.4 vol fresh ice cold Tfb I buffer [30 mM potassium acetate, 100 mM KCl, 10 mM $CaCl_2$, 50 mM $MnCl_2$, 15% (v/v) glycerol (adjusted to pH 5.8 with 200 mM acetic acid)], and incubated on ice for 5 min. The cells were harvested by centrifugation, resuspended in 0.04 vol fresh ice cold Tfb II [10 mM MOPS, 75 mM $CaCl_2$, 10 mM KCl, 15% (v/v) glycerol (adjusted to pH 6.5 with 1 M KOH)], and incubated on ice for at least 15 min. Cells were aliquoted into 200 µl volumes, immediately frozen on a dry ice/ethanol waterbath, and stored at –70°C.

2.14.2 Transformation

Frozen competent cells were thawed at RT and left on ice for 10 min. DNA was added in a maximum volume of 20 µl and incubated on ice for 30 min. The cells were heat shocked at 42°C for 90 sec, returned to ice for 2 min, followed by the addition of 3 vol of φb medium. The cell suspension was incubated at 37°C for 45 min with gentle agitation. Transformants were recovered by plating suitable dilutions of cells on NA plates containing appropriate antibiotics. In addition, cells could be concentrated by centrifugation before plating.

2.15 Protoplast transformation

A 100 ml bacterial culture grown to mid logarithmic phase, was harvested by centrifugation in a JA-14 at 7 400×g for 10 min, washed and resuspended in H_2O to a final cell suspension of 30 mg/ml dry weight. Cells were incubated with shaking for 15 min at 37°C in a lysozyme solution [15 mM NaCl, 30 mM Tris-HCl (pH 6.7), 0.4 M sucrose, 180 mg/ml lysozyme]. The incubation was continued in the presence of 30 mM $MgCl_2$ for a further 1 h. Protoplast formation was measured by comparing the OD at 600 nm of 1 ml of protoplast suspension

resuspended in H_2O to the OD of the original bacterial suspension. Protoplasts were harvested by centrifugation at 1 100×g for 30 min in a Heraeus Biofuge 15, and gently resuspended in 0.1 vol of the starting culture with SMMP buffer [0.5 M sucrose, 0.02 M maleate, 0.02 M $MgCl_2$ (pH 6.5), 2× Penassay broth (Difco)]. Samples of 0.1 ml were incubated with DNA (1 pg to 5 µg) and 40% PEG (v/v) for 2 min. Transformed protoplasts were incubated in 5 ml SMMP buffer with gentle agitation at 30°C for 90 min, prior to culture on DM3 regeneration plates [0.8% (w/v) agar, 0.5 M sodium succinate (pH 7.3), 0.5% (v/v) Casamino acids (Difco), 0.5% (v/v) Bacto yeast extract, 0.02 M K_2HPO_4, 0.11 M KH_2PO_4, 0.5% (v/v) glucose, 0.02 M $MgCl_2$, 0.01% (v/v) BSA] containing antibiotics and incubated at 37°C for 4 days.

2.16 Bacterial conjugation

From O/N bacterial cultures grown in NB or BHI broth, 0.1 vol were subcultured in fresh medium, and grown to early exponential phase with slow agitation to avoid damage to sex pili of the donor organism. Cells were washed in BHI broth and resuspended in an appropriate volume of this medium, such that approximately 1 x 10^6 bacteria/ml of the donor organism was mixed with 1 x 10^7 bacteria/ml of recipient. A total volume of 400 µl of cell suspension was evenly spread onto a 5 cm diameter, 0.45 micron nitrocellulose membrane filter (type HA, Millipore Corporation, Marlborough, Massachusetts, USA), placed on the surface of a BHI agar plate, and incubated for 6-16 h at 37°C. Bacteria were then resuspended in 3 ml BHI broth, and samples plated onto selective agar and incubated O/N at 37°C.

2.17 High efficiency electrotransformation of *E. coli*

A 100 ml NB was inoculated with 0.1 vol of an O/N culture and grown to mid to late log phase with shaking at 37°C. The culture was chilled for 20 min in an ice/water slurry, and centrifuged at 7 400×g in a JA14 rotor using a Beckman J2-21M ultracentrifuge at 4°C for 10 min. The pellet was consecutively washed in 100 ml and 20 ml ice cold H_2O, followed by a 20 ml ice cold 10% (v/v) glycerol wash. The bacteria were resuspended in 2 ml ice cold 10% (v/v) glycerol and aliquots of 100 µl were frozen in a dry ice/ethanol waterbath and stored at –70°C. Electrocompetent *E. coli* were thawed on ice prior to the addition of plasmid DNA contained in a maximum volume of 10 µl H_2O. The *E. coli* Pulser™ transformation apparatus (Bio-Rad), set to 2000 V, and 0.2 cm electrode gap cuvettes were used to pulse bacteria. Bacteria were immediately recovered in 1 ml of SOC medium, and incubated at 37°C for 1 h prior to plating appropriate dilutions onto selective media.

2.18 Electrotransformation of *L. monocytogenes*

Electrotransformation of *L. monocytogenes* was performed using the method of Alexander *et al.*, (1990). A 100 ml culture was grown to mid logarithmic phase (OD_{600} of 0.6 to 0.8), and chilled on ice for 20 min. Cells were harvested by centrifugation in a JA14 rotor at 4°C for 10 min (7 400×g, Beckman J2-21M ultracentrifuge), and washed twice in ice cold SMEM buffer (272 mM sucrose, 1 mM $MgCl_2$). The cell pellet was resuspended in 1 ml of SMEM buffer and 100 µl aliquots were stored at –70°C until required. A Gene-Pulser™ electroporation apparatus (Bio-Rad), set to a voltage of 2.0 kV and a capacitance of 25 µF, and a pulse controller set to a resistance of 200Ω, were used in combination with 0.2 cm electrode gap cuvettes to electroporate bacteria. Bacteria were immediately recovered in 1 ml of BHI broth and incubated at 37°C for 1 h prior to plating appropriate dilutions onto selective media.

2.19 Non-radioactive probe construction

2.19.1 Labelling of double stranded DNA

DNA was labelled by random primed incorporation of DIG-11-dUTP using the components of the DIG DNA labelling kit and the method of Feinberg and Vogelstein, (1983). In a final volume of 17 µl, purified linear DNA template (10 ng to 3 µg), and 2 µl 10x hexanucleotide mix (random primers), were denatured at 95°C for 10 min, and chilled immediately on ice. To this sample, 2 µl 10x dNTP labelling mix and 1 unit Klenow were added and incubated O/N at 37°C. The reaction was terminated by the addition of 0.1 vol 0.25 M EDTA, and unincorporated DIG-11-dUTP was removed by ethanol precipitation (see Section 2.11.1).

2.19.2 Preparation of end labelled oligonucleotide probes

To 200 ng of oligonucleotide, 1 µl 400 mM $CoCl_2$, 2.5 µl tailing buffer [1.4 mM potassium cacodylate, 300 mM Tris-HCl (pH 7.2), 1 mM DTT], 2.5 µl DIG-11-dUTP and 1 µl terminal transferase were added, and made up to a reaction volume of 25 µl with H_2O. The reaction was incubated for 1 h at 37°C and stored at –20°C until required.

2.20 Southern hybridisation analysis

2.20.1 Southern transfer

Unidirectional transfers of DNA from 1.0% agarose gels to nylon (Hybond N+, Amersham International) were performed as described by Southern, (1975) and modified by Reed, (1990), using capillary transfer. DNA was irreversibly bound to the filter after UV-crosslinking (254 nm, 2 min).

2.20.2 Hybridisation

Method 1: For double stranded DNA probes, nylon filters were incubated in a prehybridisation solution [50% (v/v) formamide, 5x SSPE (0.75 M NaCl, 0.44 M sodium phosphate buffer, 5 mM EDTA, pH 7.4), 1% (w/v) skim milk, 7% (w/v) SDS, and 250 µg/ml single stranded herring sperm DNA (Sigma)] for a minimum of 1 h at 42°C. The denatured double stranded DNA probes were combined with fresh prehybridisation solution and incubated with filters for 10 h to 16 h at 42°C in a Extron HI 2001 hybridisation oven (Bartelt Instruments Pty. Ltd., Heildelberg West, Victoria, Australia). Filters were washed 2x 5 min in 2x SSC (60 mM NaCl, 60 mM sodium citrate, pH 7.0) and 0.1% (w/v) SDS at RT, followed by 2x 20 min washes with 0.1x SSC, 0.1% (w/v) SDS at 68°C.

Method 2: For oligonucleotide probes, nylon filters were incubated in a prehybridisation solution [1 M NaCl, 0.1 M Tris-HCl (pH 7.6), 10 mM EDTA, 5x Denhardts reagent {0.1% (w/v) Ficoll, 0.1% (w/v) polyvinylpyrrolidone, 0.1% (w/v) BSA}, 0.1 mg/ml single stranded herring sperm DNA, 0.05% (w/v) SDS] for a minimum of 1 h at 5°C to 10°C below the melting temperature of the oligonucleotide probe [T_m = 67.5 + 34(G+C/primer length) – (395/primer length)]. Filters were incubated in the presence of probe DNA for 10 to 16 h in fresh prehybridisation solution at the appropriate temperature. Stringency washes (3x 10 min) were performed at the hybridisation temperature with 5x SSC, 0.1% (w/v) SDS.

2.20.3 Detection

After a brief wash in Buffer 1 [0.1 M Tris-HCl, 0.15 M NaCl (pH 7.5)], filters were incubated in Buffer 2 [5% (w/v) skim milk in Buffer 1] for 1 h prior to a 30 min incubation with anti-DIG-AP Fab fragments, diluted in Buffer 1 (1:5000). Filters were washed for 2x 15 min in Buffer 1 and then neutralised with Buffer 3 [0.1 M Tris-HCl, 0.1 M NaCl, 50 mM $MgCl_2$

(pH 9.5)]. Colorimetric detection of target DNA was performed using 0.34 mg/ml NBT and 0.18 mg/ml X-pho in Buffer 3.

2.21 Colony blot hybridisation

Bacterial colonies grown O/N on an agar plate, were chilled for 1 h at 4°C. Colonies were transferred onto a sterile 8.2 cm Hybond N+ nylon filter (Amersham International) after overlay onto the agar surface for 1 min. Colonies were recovered by reincubating the master plate O/N at 37°C. The filter was placed colony side up on Whatman 3 mm chromatography paper (Maidston, Kent, England) saturated with denaturation solution [0.5 M NaOH, 1.5 M NaCl, 0.1% (v/v) SDS] for 15 min. Care was taken to drain the absorbent paper of all excess liquid. After transfer of the filter to chromatography paper saturated with neutralisation solution [1.0 M Tris-HCl (pH 7.5), 1.5 M NaCl] for 5 min, the filter was transferred to 2x SSC saturated blotting paper for 15 min. After the DNA was fixed onto the filter by UV-crosslinking, cellular debris was removed with fine tissue paper (Kimwipes) immersed in a pre-heated solution of 2x SSC, 0.1% (w/v) SDS. Prehybridisation, hybridisation and detection were performed as previously described (see Section 2.20).

2.22 Synthesis of oligonucleotides

Oligonucleotides were either synthesised using reagents purchased from Applied Biosystems (Foster City, California, USA) or Ajax Chemicals (acetonitrile) on an Applied Biosystems 381A DNA synthesiser in the trityl-off mode and butanol extracted prior to use, or purchased from Genemed Biotechnologies (San Francisco, California, USA). All oligonucleotides used in the amplification of specific DNA by the Polymerase Chain Reaction are listed in Table 2.5. Oligonucleotides used to generate nucleotide sequence of *ctpA* (see Section 5.2.4) are listed in Table 2.6

2.23 Polymerase chain reaction (PCR)

All PCR's performed in this study followed the general reaction condition's described. In a final volume of 50 µl, the PCR reaction contained 5 µl 10x PCR buffer [500 mM KCl, 100 mM Tris-HCl (pH 8.3), 25 mM $MgCl_2$], 3.5 µg/ml of each primer, 0.2 mM of each dNTP, 2 µl chromosomal DNA [0.5 µl plasmid DNA or 3 µl boiled lysate (see Section 2.24)], and 1 unit Amplitaq DNA polymerase or *Pfu* polymerase. The reaction mixture was overlaid with

Table 2.5 Synthetic oligonucleotide pairs used in this study for amplification of DNA by the Polymerase Chain Reaction

Oligonucleotide sequence		Amplified product	Reaction protocol
p234	5′-CATCGACGGCAACCTCGGAGA-3′	417 bp fragment from the 3′ region of the *L. monocytogenes hly* gene	Step 1: 94°C for 1 min [1 cycle] Step 2: 94°C for 30 sec; 62°C for 30 sec; 72°C for 30 sec [30 cycles] Step 3: 72°C for 1 min; hold at 4°C [1 cycle]
p319	5′-ATCAATTACCGTTCTCCACCATTC-3′		
p905	5′-CTCACCATCACGCAGAAC-3′	525 bp fragment from the 5′ region of the *L. monocytogenes ctpA* gene	Step 1: 94°C for 1 min [1 cycle] Step 2: 94°C for 30 sec; 58°C for 30 sec; 72°C for 3 min [30 cycles] Step 3: 72°C for 5 min; hold at 4°C [1 cycle]
p1036	5′-TCATAATTGGTCTTGCGGTA-3′		
p945	5′-GCAATGGCACTTAGCTCAAT-3′	1454 bp fragment from the 3′ region of the *L. monocytogenes ctpA* gene and downstream flanking DNA	Step 1: 94°C for 1 min [1 cycle] Step 2: 94°C for 30 sec; 63°C for 30 sec; 72°C for 3 min [30 cycles] Step 3: 72°C for 5 min; hold at 4°C [1 cycle]
pS32	5′-CCATTGTGGAAGGCATTG-3′		
p2022	5′-ATCAGGATCCTTGCTTATCAATAT-3′	2024 bp fragment from the *L. monocytogenes ctpA* gene containing a 38 bp truncation at the 5′ end of this gene	Step 1: 94°C for 1 min [1 cycle] Step 2: 94°C for 30 sec; 61°C for 30 sec; 72°C for 5 min [30 cycles] Step 3: 72°C for 7 min; hold at 4°C [1 cycle]
p2023	5′-TTCTGGATCCGTATGCCTAGTATT-3′		

Table 2.6 Synthetic oligonucleotides used in the generation of nucleotide sequence of *ctpA* and flanking regions

Oligonucleotide	Sequence	Nucleotide position and direction[a]
p720	5′-AGAGAGATGTCACCGTCAAG-3′	na
p904	5′-TACCTACTGGTTGCCGTG-3′	2179 (F)
p905	5′-CTCACCATCACGCAGAAC-3′	974 (R)
p944	5′-CGAAGTTCTCCATTGAAG-3′	393 (R)
p945	5′-GCAATGGCACTTAGCTCAAT-3′	2407 (F)
p1036	5′-TCATAATTGGTCTTGCGGTA-3′	459 (F)
p1037	5′-TGATCCGATGACACCTATGA-3′	584 (R)
pS31	5′-TATTTAGTGTTGGTGGAA-3′	2653 (F)
pS32	5′-CCATTGTGGAAGGCATTG-3′	3861 (R)
pS33	5′-ACTTTTGCCTTACCGAGC-3′	2991 (F)
pS34	5′-AAGTAAGGCGACGATTCT-3′	3386 (R)
pS35	5′-GCCAGAATGAGGTTCGTT-3′	4151 (F)

[a] nucleotide position relative to the nucleotide sequence of *ctpA* (see Section 5.2.4, Figure 5.14)

F 5′ → 3′direction relative to *ctpA*

R 3′ → 5′ direction relative to *ctpA*

he nonsense strand of Tn*917* left inverted repeat (LIR)

sterile paraffin oil prior to thermal cycling in a Thermal Sequencer FTS-1C (Corbett Research, North Ryde, New South Wales, Australia).

2.24 Rapid detection of *L. monocytogenes* by PCR

L. monocytogenes isolates were confirmed by PCR amplification of a portion of *hly* using the method described by Fitter *et al.*, (1992). Bacterial cultures (10 ml) grown O/N were collected by centrifugation in an MSE Minor S centrifuge at 310×g for 10 min. Cells were washed twice in 1 vol saline [0.85% (w/v) NaCl] and resuspended in 0.5 vol H_2O. The contents were transferred to a 100 ml sterile plastic bottle and boiled in a 650 W Toshiba rotary microwave oven for 2 min. Lysates were reconstituted to a final volume of 2 ml. Oligonucleotide sequences (Primer 234 and Primer 319) were based on the DNA sequence of *hly*, the gene encoding listeriolysin O (LLO) (Mengaud *et al.*, 1988). These primer sequences amplified a 417 bp fragment internal to *hly*. After thermal cycling was complete (see Table 2.5 for a description of the specific cycling conditions), 10 µl of the PCR reaction mixture was analysed by electrophoresis on a 1.2% (w/v) agarose gel (see Section 2.12.3).

2.25 Polyacrylamide denaturing (sequencing) gel

The sequencing gel stock [6% (v/v) polyacrylamide, 8 M Urea in TBE buffer] was prepared by dissolving at 37°C, 57 g acrylamide, 3 g bis-acrylamide and 480 g Urea in 400 ml of Milli-Q water. This was subsequently de-ionised by an incubation with 35 g mixed-bed resin for at least 30 min, and filtered through a scintered glass funnel. After the addition of 100 ml 10x TBE buffer, the volume was made up to 1 L with Milli-Q H_2O and degassed under vacuum for 2 h. The gel stock was stored at 4°C in the dark and used for a maximum period of 2 to 3 months. To cast a 28 cm by 40 cm by 25 mm gel, 75 ml of acrylamide stock was prepared with 480 µl freshly made 25% (w/v) APS and 120 µl TEMED. The gel was left to polymerise for 1 h prior to mounting the gel onto the electrophoresis chamber. The gel containing the comb, was pre-electrophoresed for 1 h at 500 V in TBE buffer, and the wells flushed with running buffer to remove unpolymerised acrylamide prior to loading the samples.

2.26 Sequence analysis

2.26.1 Dye-primer sequencing

Plasmid DNA for sequencing was purified on a Qiagen tip-20 column. Kits for dye-primer sequencing were purchased from Perkin-Elmer. The ddNTP termination solutions were added

to template DNA in separate Gene Amp™ 0.5 ml reaction tubes (Perkin-Elmer) as follows; 4 µl ddATP, 4 µl ddCTP, 8 µl ddGTP, and 8 µl ddTTP, and 0.25 µg DNA. Each reaction was overlayed with *ca.* 40 µl Nujol mineral oil (Perkin-Elmer). Reactions were performed in a DNA thermal cycler (Perkin-Elmer), with an initial 15 cycles of 95°C for 30 sec, 55°C for 30 sec and 70°C for 1 min, followed by 15 cycles at 95°C for 30 sec and 70°C for 1 min. The DNA from the aqueous phase of each tube was then precipitated with 100 µl of 95% (v/v) ethanol, and 2 µl of 3 M sodium acetate (pH 5.5), for 15 min at RT, prior to collecting the DNA by centrifugation in a Heraeus Biofuge 15 for 20 min at 20 000×g. The DNA pellet was washed once in 250 µl 70% (v/v) ethanol, dried and resuspended in 6 µl of loading buffer [83% (v/v) formamide, 8.3 mM EDTA, pH 8.0] prior to loading onto a 6% polyacrylamide, 8 M Urea gel. The gel was electrophoresed on a Applied Biosystems 373A automated sequencer and data analysed by Analysis™ programme 1.2.1 (Applied Biosystems).

2.26.2 Dye-terminator sequencing

Plasmid DNA for sequencing was purified on a Qiagen tip-20 column. Kits for dye-terminator sequencing were purchased from Perkin-Elmer. To 1 µg DNA template, 9.5 µl terminator premix, 3.2 pmol primer and distilled H_2O were added for a final volume of 20 µl. Reactions were overlayed with *ca.* 40 µl Nujol mineral oil, and placed in a pre-heated thermal cycler. Thermal cycling began with an initial denaturation at 96°C for 30 sec, followed by annealing at 50°C for 15 sec and extension at 60°C for 4 min, for 25 cycles. Once cycling was completed, the extension product was diluted to a final volume of 80 µl with distilled H_2O, recovered from the mineral oil, and added to an equal volume of a solution containing 70% (v/v) phenol, 20% (v/v) distilled H_2O and 10% (v/v) chloroform. Following two extractions, the aqueous layer was recovered by precipitating DNA with 0.1 vol 5.2 M sodium acetate and 3 vol absolute ethanol, incubated O/N at –20°C. The DNA was pelleted at 20 000×g for 20 min in a Heraeus Biofuge 15, and air dried. Prior to loading the sample onto a pre-electrophoresed 6% polyacrylamide-8 M urea gel, the DNA was resuspended in 4 µl of loading buffer and denature at 90°C for 2 min. The gel was electrophoresed on a Applied Biosystems 373A automated sequencer and data analysed by Analysis™ programme 1.2.1.

2.27 Computer analysis of sequence

Deduced amino acid sequence was compared with sequences contained in Genpept (Release no. 90.0), Swissprot (Release no. 31.0), and Pir (Release no. 45.0) data banks using

the FASTA alignment algorithms (Pearson and Lipman, 1988). Hydropathy profiles were calculated using the method of Kyte and Doolittle, (1982). To establish evolutionary relatedness of ATPase proteins, amino acid sequences of the bacterial P-type ATPases were first aligned using the Clustal W multiple alignment tool (Thompson *et al.* 1994). Aligned sequences were then used to obtain phylogenetic distance measures using the Phylogeny Inference Package, Phylip 3.5 (J. Felsenstein, University of Washington, USA). Distance measures were obtained using the program Protdist. The Phylip program, Kitsch, was then used to estimated phylogenies by a Fitch-Margoliash and least squares method. Phenograms representing unrooted phylogenetic trees were constructed using TreeDraw.

2.28 RNA isolation from bacteria

Method 1: Total cellular RNA from *E. coli* was isolated by the hot-phenol method of Aiba *et al.*, (1981). Logarithmic phase cultures (3 ml) were centrifuged in 20 ml McCartney bottles in an MSE Minor S centrifuge at 310×g for 10 min, resuspended in 0.5 ml lysis buffer (20 mM sodium acetate, 1 mM EDTA, 0.5% SDS, pH 5.5), and immediately transferred to an 1.5 ml microfuge tube (Eppendorf-Netheler-Hinz-GmbH, Postfach, Hamburg, Germany) containing 0.5 ml phenol [equilibrated with 20 mM sodium acetate, 1 mM EDTA (pH 5.5)] at 65°C. After vortexing to mix the phases and incubating at 65°C for 5 min in a dry block heater (Ratek Instruments, Boronia, Victoria, Australia), the samples were centrifuged (3 min at 20 000×g, Heraeus Biofuge 15), and the upper aqueous phase extracted a further 3 times with hot phenol. RNA was precipitated by the addition of 0.1 vol of 3 M sodium acetate (pH 5.5), and 2 vol of absolute ethanol and then resuspended in 50 µl of 20 mM Tris-HCl (pH 7.6), 5 mM $MgCl_2$. Contaminating DNA in the samples was removed by digestion with RNase-free DNase I at 37°C for 30 min, prior to being stored in absolute ethanol at –20°C.

Method 2: Total cellular RNA from *L. monocytogenes* was isolated by a modification of the hot-phenol methods described by Gopalakrishna *et al.*, (1981) and Melin *et al.*, (1987). Logarithmic phase cultures (3 ml) were centrifuged in 20 ml McCartney bottles in an MSE Minor S centrifuge at 310×g for 10 min, washed twice in an equal volume of TES buffer [50 mM Tris-HCl (pH 7.5), 5 mM EDTA, 50 mM NaCl), and resuspended in 0.1 vol of SMM buffer [20 mM maleate buffer (pH 6.5), 0.5 M sucrose, 20 mM $MgCl_2$]. Following the addition of 20 µl lysozyme solution (40 mg/ml in SMM buffer), samples were incubated on ice for 30 min, then lysed by the addition of an equal volume of lysis buffer [0.1 M sodium acetate

(pH 4.0), 2% (w/v) SDS] and 20 µl 0.5 M EDTA (pH 8.0) and immediately transferred to hot phenol and treated as described in method 1.

2.29 RNA analysis

2.29.1 RNA quantitation

The concentration of RNA in solution was determined by measurement of absorption at 260 nm, assuming an OD_{260} of 1.0 is equal to 40 mg RNA/ml (Miller, 1972).

2.29.2 RNA slot blot and hybridisation

A suitably sized Hybond N+ nylon membrane, pre-soaked in 10x SSC, was assembled in a slot blot apparatus (Hoefer Scientific Instruments, San Francisco, California, USA). RNA samples in a final volume of 100 µl, were denatured by the addition of 300 µl of a solution containing 6.15 M formaldehyde and 10x SSC, incubated for 10 min at 65°C and stored on ice until loading. DNA controls were denatured in an equal volume of 20x SSC at 95°C for 10 min, then immediately chilled on ice. Samples (200 µl per well) were applied to the membrane under vacuum. Wells were washed once by the addition of 100 µl of 10x SSC, and RNA was fixed onto the membrane at 80°C for 2 h in a vacuum oven (Thermoline Scientific Equipment Pty. Ltd., Wetherill Park, New South Wales, Australia) prior to O/N hybridisation (see Section 2.20.2). Target RNA was detected using anti-DIG-POD (Fab fragments) and the BM Chemiluminescence blotting substrate (Boehringer-Mannheim), prepared according to the manufacturers directions.

2.29.3 Primer extension analysis

2.29.3.1 Labelling of oligonucleotides

Oligonucleotides were kinased using a reaction mix consisting of 1 µl of primer (60 to 100 ng), 1 µl 100 mM DTT, 1 µl 10x kinase buffer [500 mM Tris-HCl (pH 7.4), 100 mM $MgCl_2$], 1 µl T4 polynucleotide kinase (2 units/µl) and 5 µl γ-[^{32}P]-dATP (4000 Ci/mmole). After incubating for 30 min at 37°C, the labelled oligonucleotide was purified by PAGE (see Section 2.12.5).

2.29.3.2 Primer extension reaction

The synthetic 18-mer oligonucleotide (p1037) (see Table 2.6) complementary to the internal 5′ region of *ctpA*, was 5′ labelled with γ-[^{32}P]-dATP. Labelled primer (1 ng) was annealed to

20 μg of total cellular RNA in 10 μl of hybridisation solution containing 10 mM Tris hydrochloride (pH 8.3) and 200 mM KCl at 42°C for 90 min. Subsequently, 24 μl of extension mixture was added, resulting in a solution containing 10 units AMV reverse transcriptase, 10 mM Tris-HCl (pH 8.3), 60 mM KCl, 4 mM $MgCl_2$, 4 mM DTT, and 0.2 mM concentrations of each of the four dNTP's. The samples were incubated at 42°C for 60 min, after which 1 μl of RNase A (10 μg/ml) was added and the incubation continued for a further 20 min. The samples were recovered by ethanol precipitation and resuspended in 5 μl H_2O and 5 μl formamide dye mixture. Aliquots of 5 μl, were then heated at 95°C for 3 min and immediately loaded onto a 6% polyacrylamide-urea sequencing gel for electrophoresis. Dideoxy sequencing reactions performed using the 70770 Sequenase™ Version 2.0 DNA Sequencing Kit (Amersham International), using primer p1037 and the DNA template pCT220 containing *ctpA* (see Section 5.2.3.3, Figure 5.12), were run in parallel to allow determination of the endpoint of the extension product (see Section 2.30).

2.30 Dideoxy sequencing with Sequenase

Dideoxy sequencing was performed using the 70770 Sequenase™ Version 2.0 DNA Sequencing Kit. All reagents were stored at –20°C, and the dATP labelling and termination mixes were used as follows:

Labelling mix:	7.5 mM dGTP, dCTP, and dTTP
ddG termination mix:	80 mM dNTP, 8 mM ddGTP, 50 mM NaCl
ddA termination mix:	80 mM dNTP, 8 mM ddATP, 50 mM NaCl
ddC termination mix:	80 mM dNTP, 8 mM ddCTP, 50 mM NaCl
ddT termination mix:	80 mM dNTP, 8 mM ddTTP, 50 mM NaCl

Qiagen prepared template DNA (2 μg to 4 μg), was initially denatured in 0.2 M NaOH and 0.2 mM EDTA at 37°C for 30 min and recovered by ethanol precipitation. To 1 μl oligonucleotide p1037 (500 nM), denatured DNA resuspended in 7 μl H_2O, and 2 μl 5x Sequenase buffer [200 mM Tris-HCl (pH 7.5), 100 mM $MgCl_2$, 250 mM NaCl], was added and incubated at 65°C for 5-15 min, slowly cooled to RT (30 to 60 min) and chilled on ice. To the annealed mixture, 1 μl 100 mM DTT, 2 μl diluted labelling mixture (1:5 with H_2O), 2 μl diluted Sequenase (1:8 with enzyme dilution buffer as supplied by the manufacturer), and 1 μl α-[^{35}S]-dATP (3000 Ci/mmole) was added. The labelling reaction was incubated at 37°C for 5 min, and terminated by aliquoting 3.5 μl into four appropriately labelled tubes that contained

2.5 µl of the corresponding termination mixture pre-warmed to 37°C, and incubated for a further 5 min at this temperature. Finally, 4 µl stop solution [95% (v/v) formamide, 20 mM EDTA, 0.05% (w/v) bromophenol blue, 0.05% (w/v) xylene cyanol] was added to each reaction. Sequence reactions were heated to 100°C for 2 min and at least 1.5 µl of each reaction was loaded onto a denaturing gel (see Section 2.25).

After completion of electrophoresis, the upper glass plate was removed. The exposed gel was immediately washed with a solution containing 10% (v/v) acetic acid and 20% (v/v) ethanol, to remove all urea from the gel, transferred to 3 mm chromatography paper and dried at 65°C for 2 h using a gel drier (Bio-Rad) before exposure to X-ray film (Kodak-Omat 100), O/N at –70°C.

2.31 Protein preparations of *L. monocytogenes*

2.31.1 Whole envelope preparations

2.31.1.1 Radioactive labelling of whole envelope proteins with [^{35}S]-methionine

Stationary phase cultures of *L. monocytogenes* were subcultured (0.1 vol) in fresh BHI broth and grown to an OD of 1.0 at 600 nm. Approximately, 1 x 10^9 cell aliquots were sampled, washed in M9 media [10% (v/v) 10 x M9 salts {480 mM $Na_2HPO_4.7H_2O$, 220 mM KH_2PO_4, 85.5 mM NaCl, 190 mM NH_4Cl}, 0.8 mM $MgSO_4$, 0.5% (v/v) glucose] and grown for 2 h in Methionine assay medium (Difco) at 37°C. Samples were pulsed with 10 µCi of [^{35}S]-methionine at 37°C for 5 min collected by centrifugation for 1 min at 4°C, and cells were resuspended in 100 µl lysis solution [50 mM Tris-HCl (pH 7.5), 30 mM NaCl, 200 µg lysozyme]. The samples were freeze-thawed (3 times) in an ethanol-dry ice bath, and centrifuged in a Heraeus Biofuge 15 (9 000×g for 20 min at 4°C), to separate the soluble and insoluble protein fractions. The insoluble pellet was resuspended in 100 µl 1x Sample buffer [0.125 mM Tris-HCl (pH 6.8), 1% (w/v) SDS, 5% (v/v) glycerol, 5% (v/v) β-mercaptoethanol, 7.5% (w/v) bromophenol blue], and the soluble fraction (supernatant) was prepared by the addition of 100 µl 2x sample buffer and stored at –20°C.

2.31.1.2 Large scale protein extraction using a French pressure cell

Logarithmic phase cultures grown in conical flasks were washed in 1 vol of 50 mM Tris-HCl (pH 7.5), and resuspended in a maximum of 40 ml to obtain a cell density of approximately 1 x 10^{11} bacteria/ml. The cells were broken by three passages through a pre-cooled French

pressure cell (Paton Industries, Stepney, South Australia, Australia), and the unbroken cells removed by centrifugation (4 200×g, 10 min, JA20 rotor, Beckman J2-21M, 4°C). The supernatant was centrifuged at 170 000×g for 45 min in a Beckman L8-80 ultracentrifuge using the 50 Ti rotor at 4°C, and the pellet (whole cell envelope) was resuspended in 2x sample buffer and stored at –20°C.

2.31.1.3 Crude whole cell protein preparation

The cell pellet from 1.5 ml of logarithmic phase cultures were recovered by centrifugation in a Heraeus Biofuge 15 (20 000×g, 30 sec), solubilised in 50 µl lysing buffer [2% (w/v) SDS, 4% (v/v) β-mercaptoethanol, 10% (v/v) glycerol, 0.1% bromophenol blue, 0.86 M Tris-HCl (pH 6.8)], and boiled for 5 min. Samples were stored at –20°C until required.

2.31.2 Trichloroacetic acid (TCA) precipitation

Supernatant proteins from logarithmic phase cultures were precipitated by the addition of 0.1 vol of 100% (w/v) TCA and incubated on ice for 30 min. Proteins were collected by centrifugation in a JA-20 rotor (26 500×g at 4°C for 10 min), and the supernatant discarded. The pellet was washed twice in a suitable volume of ice cold acetone to remove residual TCA, air dried, and resuspended in 1x sample buffer and stored at –20°C.

2.32 Isolation of unprocessed CtpA from *E. coli*

2.32.1 Construction of plasmids and *E. coli* clones for over-expression of *ctpA*

Plasmid pCT203, harbouring a 2.8 kb *Pvu*II *L. monocytogenes* chromosomal DNA fragment cloned into the *Sma*I site of pGEM-7Zf(+) (see Section 5.2.3.1), and plasmid pCT220 containing a chimera of DNA fragments that resulted in a 4.3 kb DNA insert cloned into pGEM-7Zf(+) (see Section 5.2.3.3), were used to overexpress the *ctpA* gene in *E. coli*. These plasmids contain the *ctpA* gene cloned in the correct orientation to allow expression of this gene from the T7 promoter contained on pGEM-7Zf(+). Plasmids, pCT220 and pGP1-2, containing the gene for T7 RNA polymerase (Tabor and Richardson, 1985), and plasmids pCT203 and pGP1-2 were sequentially transformed into *E. coli* ECC219 to produce strains MF002 and MF003 respectively. Strain MF001, is *E. coli* ECC219[pGEM-7Zf(+) and pGP1-2] and was used for control purposes.

2.32.2 Over-expression of *ctpA*

The over-expression of *ctpA* under the control of the temperature inducible T7 RNA polymerase promoter, was performed using a modification of the method described by Tabor and Richardson, (1985).

2.32.2.1 Large scale protein over-expression

Strains MF001, MF002, and MF003 were streaked onto NA plates containing Km (50 μg/ml) and Ap (100 μg/ml) and incubated at 30°C, O/N. A single colony was used to inoculate 100 ml of Terrific broth containing Km and Ap, and incubated O/N at 30°C with agitation. Cultures were washed in 0.15 M NaCl, resuspended in 200 ml Terrific broth containing antibiotics and incubated at 30°C with agitation for 1 h. The flasks were then shifted to 42°C with agitation for 30 min to induce expression of the T7 RNA polymerase. Host mediated protein expression was inhibited by the addition of Rp (200 μg/ml), maintained at 42°C for a further 30 min, prior to a minimum 2 h incubation at 37°C with agitation. Protein extractions were performed using the French pressure cell (see Section 2.31.1.2).

2.32.2.2 [^{35}S]-methionine incorporation in CtpA

Ten millilitre O/N cultures of strains inoculated from fresh single colonies, were grown at 30°C with shaking. Cultures were diluted (1/100) into 10 ml fresh Terrific broth containing Km and Ap, and incubated with agitation at 30°C, until an OD_{600} of 0.6 had been achieved. The cultures (0.4 ml) were washed three times in 1.5 ml M9 media (Section 2.31.1.1), resuspended in 1 ml Methionine assay medium (Difco) and grown for 2 h at 30°C with agitation. Cultures were transferred to 42°C for 20 min with agitation, prior to a further 10 min incubation at 42°C in the presence of Rp (200 μg/ml). Cell cultures were incubated for 1 h at 37°C with agitation and pulsed with 10 μCi of [^{35}S]-methionine at 37°C for 5 min. Cells were recovered by centrifugation for 1 min and whole cell lysates were extracted (see Section 2.31.1.3).

2.33 SDS polyacrylamide gel electrophoresis (SDS-PAGE)

SDS PAGE was performed by the procedure of Lugtenberg *et al.*, (1975) using either a Mighty Small II Unit (Hoefer Scientific Instruments) or a Sturdier Slab Unit (Hoefer Scientific Instruments) for minigel or maxigel vertical electrophoresis, respectively. Protein preparations in sample buffer were denatured at 95°C for 3 min before loading, and electrophoresed at 80 V

through the stacking gel and 120 V through the running gel for 4 h, or until adequate migration of the dye front. Maxigels were stained for 30 min at room temperature in Coomassie quick stain [0.275% (w/v) Coomassie brilliant blue R250, 10% (v/v) ethanol, 10% (v/v) methanol, 7.5% (v/v) acetic acid], while minigels were stained for 5 min. Gels were destained with a solution of 10% (v/v) ethanol, 10% (v/v) methanol and 7.5% (v/v) acetic acid with several changes of the destain solution.

2.34 Tissue culture

2.34.1 Preparation of glass coverslips

Coverslips (12 mm) were washed O/N in a solution containing 0.34 M potassium dichromate and 1 M sulphuric acid. The acid solution was removed by repeated washing in cold sterile H_2O. Coverslips were air dried, baked for 2 h at 80°C, and stored in methanol.

2.34.2 Tissue cultures and growth media

Tissue culture cell lines used in this study were the human colon adenocarcinoma enterocyte-like cell line Caco-2 (ATCC HTB 37, American Type Culture Collection, Rockville, Maryland, USA), the human epithelial cell line HeLa (ATCC CCL 2.1) and the murine macrophage-like cell line J774 (Rathjen and Geczy, 1986). Caco-2 cells, used between passages 20 and 45 were cultured in EMEM (GIBCO Laboratories, Grand Island, New York, USA) supplemented with 10% (v/v) foetal bovine serum (GIBCO Laboratories), 2 mM glutamine, 21.4 mM $NaHCO_3$, Pc (100 µg/ml) and Sm (10 µg/ml). HeLa and J774 cells were cultured in GIBCO's DMEM supplemented with 5% (v/v) foetal bovine serum, 2 mM glutamine, 42.9 mM $NaHCO_3$, Pc (100 µg/ml) and Sm (10 µg/ml). Cells were routinely cultured in 75 cm^3 plastic tissue culture flasks (Corning, Corning, New York, USA) in a humidity cabinet with 5% CO_2 in air at 37°C (Forma Scientific, Marietta, Oaklahoma, USA). Semi-confluent monolayers (approximately 5×10^6 cells/ml) grown O/N for internalisation assays were prepared by subculture of cell monolayers into 24 well tissue culture trays (Corning), containing acid washed 12 mm coverslips, to a final concentration of about 1×10^6 cells/ml for Caco-2 cells and 5×10^5 cells/ml for HeLa and J774 cells.

2.34.3 Tissue culture monolayer infection assay

Cell monolayers were washed twice in appropriate culture medium free of antibiotics. Cultures of logarithmic phase bacteria (10 ml) were washed once in 0.15 M NaCl and resuspended in

2 ml antibiotic free culture medium. Bacteria (100 µl) diluted in antibiotic free culture medium (5×10^8, 5×10^7, 5×10^6 bacteria/ml), were overlayed onto the monolayers to achieve a MOI of 100 bacteria to 1 cell (100:1), 10:1 and 1:1, respectively. Analysis of the CFU applied to the monolayers was confirmed by plating suitable dilutions on BHI agar. Monolayers were incubated at 37°C in 5% CO_2 for 2 h, followed by three washes in antibiotic free culture medium. A final incubation at 37°C in 5% CO_2 for 2 h in culture medium supplemented with Gm (40 µg/ml, Schering Corporation, Kenilworth, New Jersey, USA) was performed to kill any extracellular bacteria. Monolayers were washed twice in HBSS [5% (v/v) solution A {2.74 M NaCl, 0.11 M KCl, 11.5 mM $MgSO_4$, 10 mM $MgCl_2$, 33.3 mM $CaCl_2$} and 5% (v/v) solution B {12.9 mM Na_2HPO_4, 8.8 mM KH_2PO_4, 55.5 mM glucose, 0.1% (v/v) phenol red} in Milli-Q H_2O]. In addition, after the HeLa cell monolayers were infected with bacteria, the monolayers were centrifuged in a GPR centrifuge (Beckman Instruments) at 1 200×g for 15 min at RT to spin the bacteria onto the monolayers. Furthermore, J774 monolayers were inoculated with bacteria at a MOI of 1:1 and were incubated at 37°C in 5% CO_2 for 30 min, followed by a 30 min incubation in media containing Gm. Monolayers were finally washed in antibiotic-free DMEM.

Intracellular bacteria were recovered by lysis of the monolayers with 1 ml ice-cold Triton X-100 in 0.15 M NaCl. The CFU recovered from cell lysates was determined by plating appropriate dilutions on BHI agar. The degree of bacterial internalisation is represented by calculating the percentage of the original inoculum recovered after lysis of infected tissue culture monolayers.

2.34.4 Immunofluorescence microscopy

Tissue culture monolayers grown on coverslips were fixed in 3.7% (v/v) formalin in PBS [140 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, 10.5 mM Na_2HPO_4 (anhydrous)] for at least 1 h at 4°C. Monolayers were washed once in PBS and the cell membranes permeabilised by the addition of 0.1% (v/v) Triton X-100 in PBS for 1 min. Monolayers were washed six times with PBS after which, 10 µl of diluted rabbit *Listeria* O Antiserum Poly (serotypes 1 and 4) was overlayed onto the monolayers and incubated at RT for 1 h in a humid chamber to prevent drying. Antisera was diluted in 10% (v/v) foetal bovine serum in PBS. Monolayers were washed a further six times with PBS. To the monolayers, 10 µl of diluted goat anti-rabbit IgGAM (H+L) fluorescein isothiocyanate (FITC) conjugate and 10 µl of phalloidin rhodamine (TRITC) conjugate, were applied and incubated at RT for 1 h in a humid chamber, followed by

six washes in PBS. The coverslips were removed, air dried, mounted face down onto microscope slides using 3 µl of moviol mounting medium and sealed using clear nail polish. Monolayers were examined using an Olympus BH-S microscope with a BH2-RFC UV fluorescent attachment (Olympus Optical Co. Ltd., Shinjuku-ku, Tokyo, Japan) and photographs taken using Kodak Tmax 400 film.

2.34.5 Giemsa staining for light microscopy

Monolayers were fixed in cold (–20°C) methanol for at least 5 min. The coverslips were air dried then stained in filter sterilised Giemsa stain (BDH Laboratory Supplies) for at least 10 min at RT. Coverslips were washed in Giemsa buffer [3.8 mM Na_2HPO_4 (anhydrous), 3.5 mM KH_2PO_4] until desired intensity of stain was achieved. Coverslips were air dried, mounted face down onto microscope slides with D.P.X. neutral mounting medium (Koch-Light Laboratories, Colnbrook, Buckinghamshire, England), then viewed using an Olympus BH-2 light microscope and photographs taken using Kodak Tmax 100 film.

2.34.6 Statistical analysis

Two-way analysis of variance was performed to compare the effect of MOI on the potential of *Listeria* spp. to invade tissue culture cell lines.

2.35 *In vitro* characterisation of mutant strains

2.35.1 Growth competition in laboratory media

The growth rates of *ctpA* mutants were compared with those of the corresponding wild-type strains by performing *in vitro* competition experiments in BHI broth. Mixed inocula of *ca.* 1 x 10^3 of each strain were inoculated into fresh media and incubated at 37°C with shaking for 8 h to 10 h. The initial ratio of the two strains in the mixed culture was determined by measuring the viable counts of suitable dilutions of culture onto BHI and selective media. This ratio was compared with that present in the culture after 8 h to 10 h incubation at 37°C.

2.35.2 Cation sensitivity

2.35.2.1 Minimal inhibitory concentration (MIC)

The MIC was calculated by a modification of the method described by Sahm and Washington, (1991). A 5 µl sample of an O/N culture washed in 0.15 M NaCl was spotted onto BHI agar plates containing dilutions of cations. Sensitivity of strains to these cations was determined

after growth at 37°C for 48 h. The MIC was recorded as the lowest concentration of cation that inhibits visible growth of an organism.

2.35.2.2 Copper sensitivity in laboratory media

Overnight cultures of *L. monocytogenes* isolates were subcultured (0.01 vol) into 10 ml BHI broth and media containing either 4 mM $CuSO_4$, or the free Cu^{2+} ion chelators (10 mM EGTA or 5 μM 8-hydroxyquinoline). The cultures, incubated at 37°C with agitation, were sampled at regular time points over 24 h for measurement of OD at 600 nm.

2.35.3 Intracellular growth of *L. monocytogenes* in tissue culture cell monolayers

The ability of *ctpA* mutants to multiply in tissue culture cell monolayers was compared to the appropriate wild type organism. Bacterial infections of tissue culture cell monolayers were performed by a modification of the method described in Section 2.34.3. HeLa and J774 cells cultured in 24 well tissue culture trays without coverslips, were inoculated with bacteria at a MOI of 0.1 and 0.05 respectively. The degree of intracellular growth was assessed after the monolayers were lysed in 1 ml of ice cold H_2O, and viable bacteria were recovered from cell lysates by growth on BHI agar.

2.36 *In vivo* studies of *L. monocytogenes* in the mouse infection model

2.36.1 Clearance of *Listeria* spp. from organ tissue

The virulence of a given strain was assessed by determining the degree of persistence within the spleen and liver of infected mice. Logarithmic phase bacterial stock cultures (1×10^8 cells/ml) were stored at −70°C. For each experiment, a vial of frozen stock was quickly thawed and diluted in 0.15 M NaCl to approximately 1×10^4 organisms for intravenous (i.v) inoculations in the tail (0.5 ml per injection). The bacterial survival in organs was determined by killing groups of five infected mice by lethal carbon dioxide anaesthesia. The spleens and livers were aseptically removed and ground for 45 sec in 8.5 ml ice cold 0.15 M NaCl using a homogeniser (Ultraturrax). Samples (0.1 ml) of serial dilutions of whole organ homogenates were surface plated on BHI agar. Viable bacteria were counted after 36 h of incubation at 37°C, and the results were expressed as the $\log_{10}$ bacterial counts per gram of tissue.

2.36.2 Competition experiments

Competition experiments were performed by i.v. injection of mice with a mixed inoculum comprising approximately equal numbers of parent and mutant organisms; the dose of each strain equal to *ca.* 1 x 10^2. The bacterial survival in organs was determined by killing groups of five infected mice by lethal carbon dioxide anaesthesia, and spleens and livers recovered (see Section 2.36.1). To determine the ratio of viable parent and mutant organisms present at the time of sacrifice, dilutions of each organ homogenate were plated onto BHI and selective agar. From the number of colonies present on these plates the ratio of parent and mutant was calculated.

Chapter 3 Optimisation of a tissue culture monolayer infection assay for analysis of *Listeria monocytogenes* pathogenicity

3.1 Introduction

L. monocytogenes has attracted worldwide attention due to the recent association of this organism with foodborne disease. However, this facultative intracellular pathogen has also been widely used as a model organism for study of cell mediated immunity. The significance of *L. monocytogenes* as a human pathogen lies in the ability of this organism to evade both antibody and complement mediated lysis or killing by professional macrophages by entering, multiplying and persisting within host cells (Mackaness, 1962). The intracellular lifecycle of *L. monocytogenes* and the interactions between this pathogen and the host, have been investigated using *in vitro* tissue culture techniques. Gaillard *et al.*, (1987), first established an *in vitro* model of penetration and intracellular growth of *L. monocytogenes* using the human enterocyte-like cell line Caco-2. Given the route of listerial infections is via the small intestine (Rácz *et al.*, 1972; Zachar and Savage, 1979; MacDonald and Carter, 1980), Caco-2 cells were chosen for the analysis of *L. monocytogenes* virulence, because they display characteristic enterocyte differentiation under standard culture conditions (Gaillard *et al.*, 1987). For example, these cells develop a brush border which contain some enzymes typical of those located in the small intestine (Pinto *et al.*, 1983; Rousset, 1986). *L. monocytogenes* can initiate entry into cells by induction of phagocytosis and multiply within the cytoplasm of the host cell. This intracellular behaviour was used as a means to distinguish *Listeria* spp. on the basis of virulence.

Subsequent investigation showed a wide variety of cell types were able to support the growth of *L. monocytogenes*, including macrophages, fibroblasts, epithelial cells, enterocytes and hepatocytes. In fact, combined use of tissue culture monolayer models of infection and electron and immunofluorescence microscopy has provided considerable insight into the cell biology of *L. monocytogenes* and the analysis of genetic determinants responsible for pathogenesis (Gaillard *et al.*, 1987; Tilney and Portnoy, 1989; Dabiri *et al.*, 1990; Mounier *et al.*, 1990; Sun *et al.*, 1990; Tilney and Tilney, 1993; De Chastellier and Berche, 1994; Karunasagar *et al.*, 1994).

Pine and colleagues, compared the ability of *L. monocytogenes* to invade 12 different mammalian tissue culture cell lines (Pine *et al.*, 1991). While all cell lines were able to internalise *L. monocytogenes*, albeit to varying degrees, the Caco-2 cell line was at least 10 fold more efficient at mediating internalisation. This unusually high efficiency of bacterial internalisation was suggested to be the result of enterocyte-like cells being the most probable initial site of entry in foodborne listeriosis patients. Thus, Caco-2 cells became the most popular model for the analysis of *L. monocytogenes* virulence primarily because of the typical enterocyte differentiation and increased susceptibility to infection by *L. monocytogenes* (Gaillard *et al.*, 1987; Kathariou *et al.*, 1990; Pine *et al.*, 1991; Karunasagar *et al.*, 1993).

Nevertheless, all comparative studies to date have neglected to consider the affect multiplicity of infection (MOI) has on the efficiency of *L. monocytogenes* to invade various cell types. In these studies, cell monolayers were routinely exposed to bacteria at MOI's of at least 10 bacteria to 1 cell (10:1) (Gaillard *et al.*, 1987; Portnoy *et al.*, 1988; Nichterlein and Hof, 1991; Pine *et al.*, 1991; Quinn *et al.*, 1993; Karunasagar *et al.*, 1994).

Another aspect overlooked in *in vitro* studies of *L. monocytogenes* internalisation concerns the method of infection of cells by bacteria. Earlier studies described enhancement of internalisation of *Y. enterocolitica* and *E. coli* (Vesikari *et al.*, 1982), and *S. flexneri* (Oaks *et al.*, 1985; Sansonetti *et al.*, 1986) in epithelial cell monolayers by impinging bacteria onto the cell surface by centrifugation. As centrifugation brings bacteria into direct contact with the cell monolayer, more efficient uptake by the cell would be expected, which has limited the acceptance of this approach for analysis of *L. monocytogenes* infection and intracellular spread (Kuhn *et al.*, 1988; Kuhn and Goebel, 1989; Mounier *et al.*, 1990). Most infection protocols exclude this step (Portnoy *et al.*, 1988; Kathariou *et al.*, 1990; Sun *et al.*, 1990; Gaillard *et al.*, 1991; Domann *et al.*, 1992). This chapter describes the optimisation of a tissue culture model of *L. monocytogenes* internalisation. In particular, MOI and use of centrifugation were examined for their influence on bacterial internalisation of cell monolayers. Furthermore, the effectiveness of the HeLa epithelial cell line and the Caco-2 enterocyte-like cell line to promote uptake of *Listeria* spp. was compared.

3.2 Results

To optimise a tissue culture model of *L. monocytogenes* internalisation, semi-confluent cell monolayers were first infected with logarithmic phase bacterial suspensions. After incubating

for 2 h, extracellular bacteria were killed by addition of gentamycin as described in the materials and methods (see Section 2.34.3). Viable intracellular bacteria recovered by lysis of cell monolayers was determined by plating appropriate dilutions of cell lysate on BHI agar. The degree of bacterial internalisation was represented by calculating the percentage of the original inoculum recovered after lysis of infected tissue culture monolayers.

3.2.1 Internalisation of *L. monocytogenes* in HeLa cell monolayers was enhanced by centrifugation

In order to determine whether physical contact between bacteria and HeLa or Caco-2 cells promoted internalisation, centrifugation was used to locate *L. monocytogenes* DRDC8 to monolayers at a MOI of 10 bacteria to 1 cell. Other monolayers were exposed to bacteria at the same concentration in the absence of centrifugation. When a short centrifugation step was used to initiate contact between bacteria and cells, significantly more viable DRDC8 cells were recovered from lysed HeLa cell monolayers ($p<0.001$) (Figure 3.1). Conversely, internalisation by DRDC8 in the Caco-2 cell line was significantly reduced following centrifugation ($p<0.001$) (Figure 3.1). Examination of Giemsa stained infected monolayers by light microscopy, indicates that Caco-2 cells were totally disrupted by centrifugation (data not shown). This finding was in direct contrast to a report by Mounier *et al.*, (1990), which showed *L. monocytogenes* infected Caco-2 cell monolayers remained viable following centrifugation. In addition, when the non-haemolytic, non-pathogenic *L. innocua* was used to infect either cell line, consistently low recovery of bacteria was obtained. On the basis of these results, all subsequent infections of HeLa cell monolayers by *Listeria* spp. included a short centrifugation step, whereas infection of Caco-2 cells were performed in the absence of centrifugation.

3.2.2 Effect of multiplicity of infection on *L. monocytogenes* internalisation of Caco-2 and HeLa cell monolayers

To determine whether inoculum size of *L. monocytogenes* effects their ability to promote internalisation of tissue culture monolayers, bacteria were applied to Caco-2 and HeLa cells at MOI of 100:1 (100 bacteria to 1 cell), 10:1 and 1:1. After lysis of Caco-2 and HeLa cells infected with *L. monocytogenes* DRDC8 and SLCC 5764, significantly more viable bacteria were recovered from monolayers when low MOI were used (100:1 < 10:1 < 1:1) ($p<0.001$) (Figure 3.2). When the phenotypically non-haemolytic *L. monocytogenes* LLO19, and the

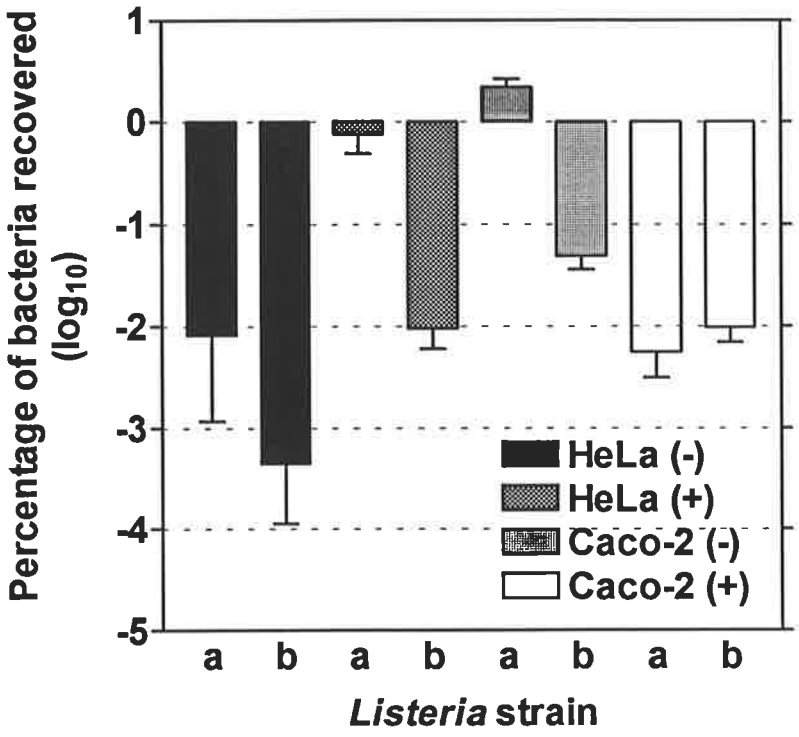


Figure 3.1: Effect of centrifugation on the percent recovery of viable *Listeria* spp. from cell lysates of HeLa and Caco-2 cell monolayers at a MOI of 10:1, 4 h post inoculation. Experiments which incorporated a short centrifugation step are indicated by (+), and those without centrifugation are indicated by (−). Bar: [a], *L. monocytogenes* DRDC8; and [b], *L. innocua.* Each column represents the mean ± SEM (log_{10}) of at least six independent experiments.

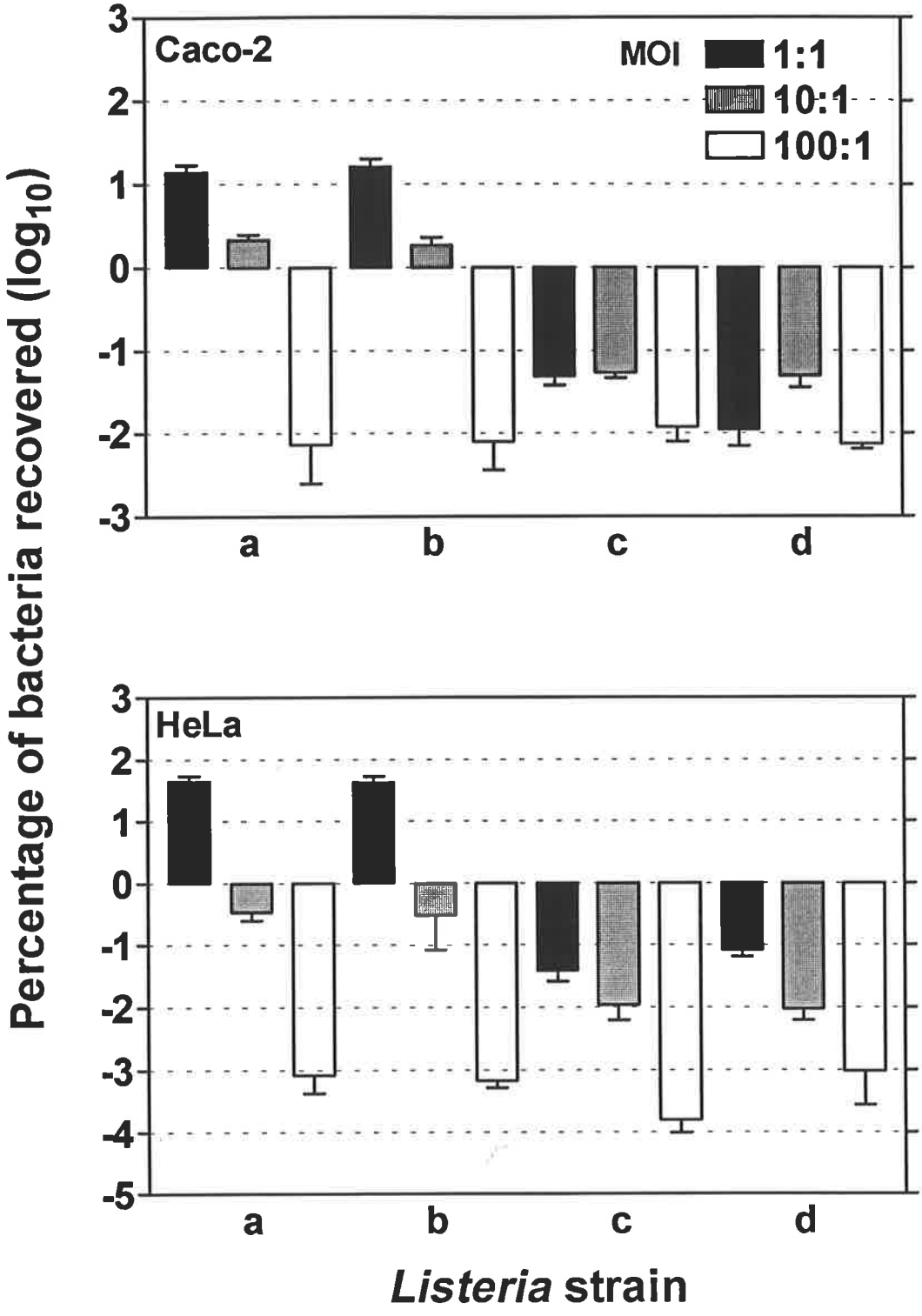


Figure 3.2: Effect of MOI on percent recovery of viable *Listeria* spp. from cell lysates of Caco-2 and HeLa cell monolayers, 4 h post inoculation. Bar: [a], *L. monocytogenes* DRDC8; [b], *L. monocytogenes* SLCC 5764; [c], *L. monocytogenes* LLO19; and [d], *L. innocua*. Each column represents the mean ± SEM (log_{10}) of at least four independent experiments.

non-haemolytic, non-pathogenic *L. innocua* were used to infect either cell line, consistently low recovery of bacteria was obtained from lysed monolayers regardless of the MOI used.

3.2.3 Microscopic analysis of Caco-2 and HeLa cell monolayers exposed to *L. monocytogenes* at various multiplicities of infection

Although studies of bacterial internalisation can provide an indication of the pathogenicity of that organism, the latter does not always follow. Consequently, light microscopy of Giemsa stained infected monolayers were used to examine the ability of internalised bacteria to induce cytopathogenic effects characteristic of virulent infections.

Giemsa stained preparations of Caco-2 and HeLa cells inoculated with *L. monocytogenes* DRDC8 at an MOI of 100:1 showed that complete disruption of the cell monolayers had occurred. Few Caco-2 cells were identified which showed extensive cytopathogenic activity when a MOI of 10:1 was used (Figure 3.3, Panel A). At an MOI of 1:1 however, intact cells which contained bacteria were detected (Figure 3.3, Panel B). The morphology of these cells was similar to that of uninfected cells or cells infected with non-invasive organisms (Figure 3.3, Panel C-G). By contrast, HeLa cell monolayers inoculated with DRDC8 at an MOI of 10:1 showed marked loss of confluence. Viable cells were heavily infected with bacteria and showed a rounded morphology with indistinct membranes (Figure 3.4, Panel A). At a MOI of 1:1, HeLa cell monolayers retained confluence with no apparent loss of viability, as observed with control cell monolayers (Figure 3.4, Panel B-G). Furthermore, pseudopod structures which facilitate bacterial cell to cell spread (Tilney and Portnoy, 1989; Dabiri *et al.*, 1990), were readily identified. Results obtained from infections of cell monolayers with *L. monocytogenes* SLCC 5764 were consistent with DRDC8 infected cell monolayers (data not shown).

3.2.4 Membrane disruption of cell monolayers by internalised *L. monocytogenes*

Overall, Section 3.2.3 revealed that pathogenic bacteria added to monolayers at high MOI resulted in an even pattern of cell monolayer disruption. One explanation for this may reflect a direct parasitism by intracellular bacteria. However, toxic substances produced during bacterial growth may also effect cell monolayer integrity and can not be overlooked. In order to determine the factors responsible for cell monolayer disruption in this tissue culture model of infection, these aspects were investigated.

Figure 3.3: Light photomicrographs (1000×) of Giemsa stained Caco-2 cell monolayers 4 h post infection with *Listeria* spp. Panel: [A], *L. monocytogenes* DRDC8 (MOI of 10:1); [B], *L. monocytogenes* DRDC8 (MOI of 1:1); [C], *L. monocytogenes* LLO19 (MOI of 10:1); [D], *L. monocytogenes* LLO19 (MOI of 1:1); [E], *L. innocua* (MOI of 10:1); [F], *L. innocua* (MOI of 1:1); [G], uninfected control. Arrows show features indicative of cytopathogenicity induced by rapid multiplication and cell to cell spread by intracellular *L. monocytogenes*. Rounded morphology and loss of cell membrane integrity defined cytopathogenicity by *L. monocytogenes*.

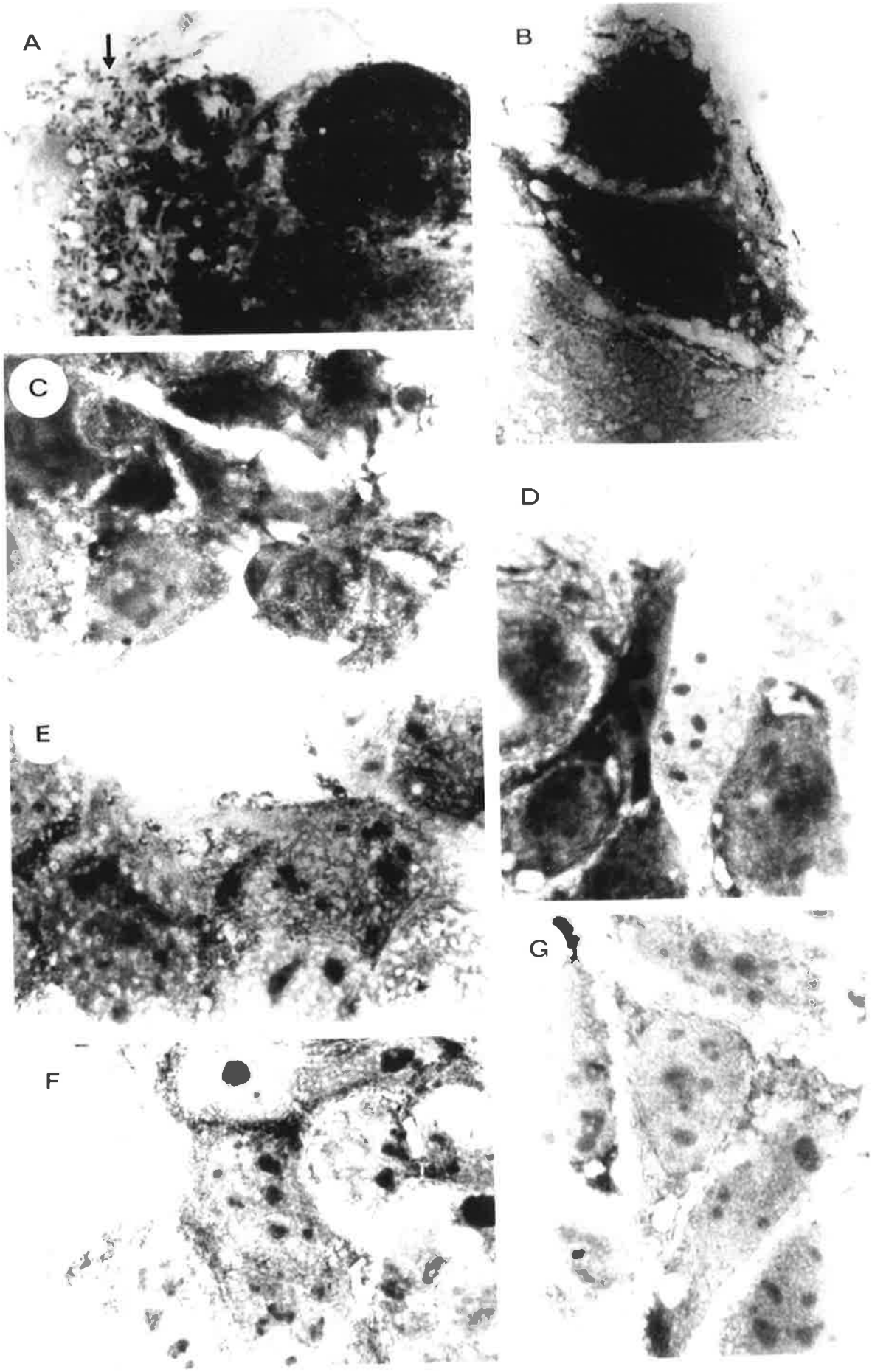
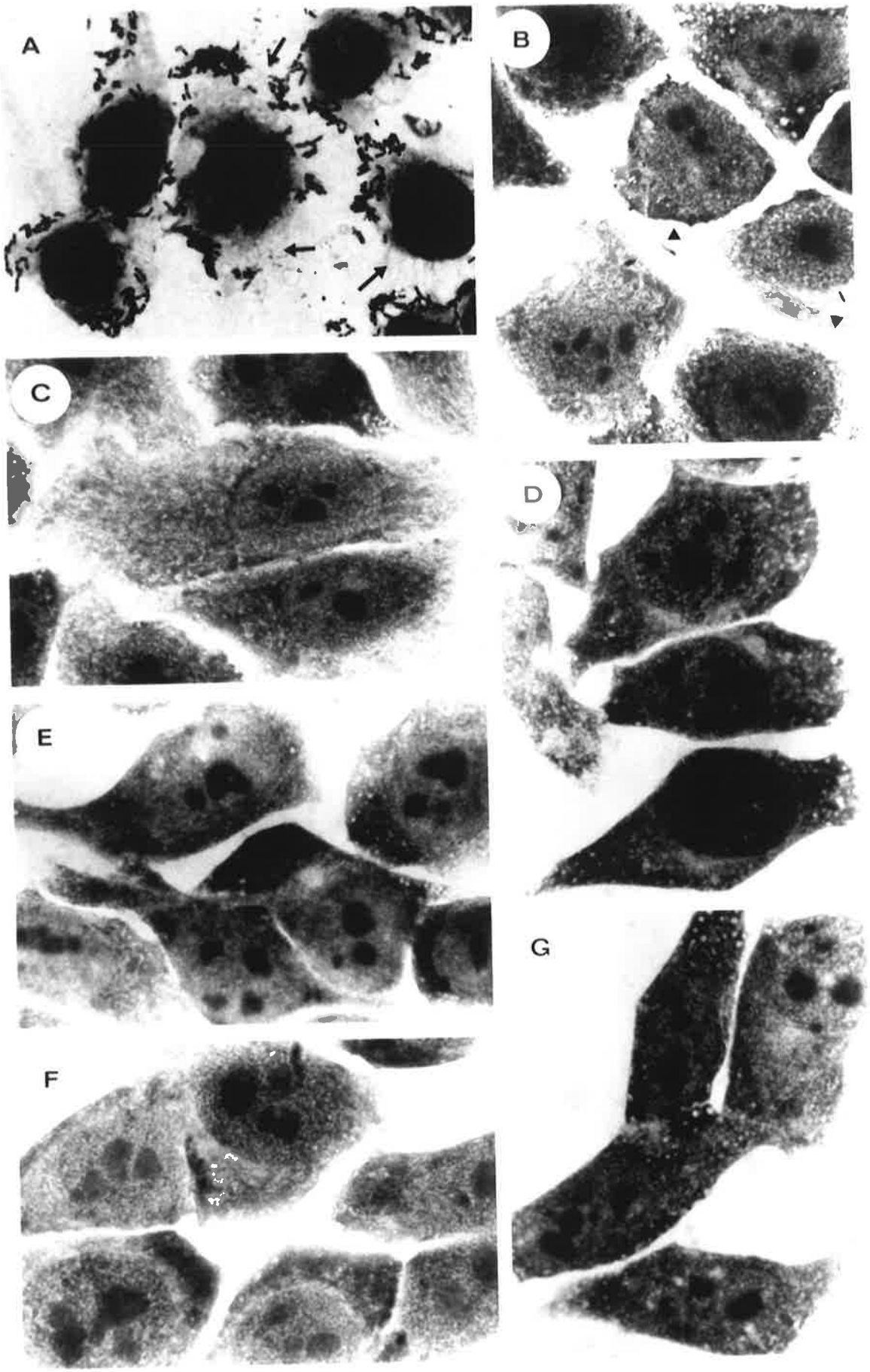

A
B
C
D
E
F
G

Figure 3.4: Light photomicrographs (1000×) of Giemsa stained HeLa cell monolayers 4 h post infection with *Listeria* spp. Panel: [A], *L. monocytogenes* DRDC8 (MOI of 10:1); [B], *L. monocytogenes* DRDC8 (MOI of 1:1); [C], *L. monocytogenes* LLO19 (MOI of 10:1); [D], *L. monocytogenes* LLO19 (MOI of 1:1); [E], *L. innocua* (MOI of 10:1); [F], *L. innocua* (MOI of 1:1); [G], uninfected control. Arrows show features indicative of cytopathogenicity induced by rapid multiplication and cell to cell spread by intracellular *L. monocytogenes*. Rounded morphology and loss of cell membrane integrity defined cytopathogenicity by *L. monocytogenes*. Arrowheads indicate pseuodopod-like structures characteristic of cell to cell spread of *L. monocytogenes*.


A
B
C
D
E
F
G

3.2.4.1 Parasitism by intracellular bacteria leads to membrane disruption

To establish a direct correlation between cell monolayer disruption and intracellular parasitism by internalised bacteria, the percentage of individual tissue culture cells that contain bacteria was determined. Bacteria added to Caco-2 and HeLa cells at a range of MOI were examined microscopically after monolayers were stained with Giemsa stain. Significantly, more Caco-2 and HeLa cells contained intracellular DRDC8 and SLCC 5764, when high MOI were used (1:1 < 10:1) ($p<0.001$) (Table 3.1). At a MOI of 10:1, all cells examined contained bacteria regardless of the cell line used. However, when a MOI of 100:1 was used, the cell monolayer was totally disrupted. Furthermore, non-haemolytic *L. monocytogenes* LLO19, and non-haemolytic, non-pathogenic *L. innocua* were rarely internalised and the cell monolayers maintained their integrity. As intracellular bacteria were evenly distributed throughout the cell monolayer, it is likely that these organisms were responsible for the widespread disruption of the monolayer observed, especially when high MOI were employed.

3.2.4.2 Bacterial culture supernatants were not cytotoxic to HeLa cell monolayers

To examine whether bacterial culture supernatants may contain toxic factors that contribute to cell membrane disruption, culture supernatants collected from HeLa cell monolayers inoculated with bacteria at a range of MOI, were applied to uninfected cells. Monolayers were subsequently examined for loss of confluency as a measure of supernatant cytotoxicity. Irrespective of the organism's pathogenic status or the MOI used, supernatants from these infections did not reduce the number of viable HeLa cells within the monolayer when compared to uninfected cell monolayer controls (Table 3.2). Furthermore, there was no evidence of cytopathogenic effects by culture supernatants when Giemsa stained cell monolayers were viewed using light microscopy (Figure 3.5, Panel B, D, F, and H). In contrast, when *L. monocytogenes* DRDC8 was added to cell monolayers at high MOI's (10:1 and 100:1) prior to collection of the culture supernatant, significant cell monolayer disruption was consistently observed (Figure 3.5, Panel A). These data are in good agreement with initial experiments that described membrane disruption after pathogenic *Listeria* spp were added to cell monolayers at high MOI (see Section 3.2.3).

This study provides evidence that cell monolayer disruption was apparently caused by intracellular bacterial parasitism, and not by cytotoxic activity of the bacterial culture supernatant. On the basis of this data, a MOI of 1:1 was routinely used in further experiments

Table 3.1 Percentage of tissue culture cells infected with *Listeria* spp.

Strain	Percent infected cells[a]					
	HeLa			Caco-2		
	1:1	10:1	100:1	1:1	10:1	100:1
DRDC8	29.27	100	nd	20.89	nd	nd
SLCC5764	19.78	100	nd	19.25	nd	nd
LLO19	0.007	0.03	nd	0.006	nd	nd
L. innocua	0.004	0.08	nd	0.018	nd	nd

[a] The presence of intracellular bacteria in a minimum of 100 cells was determined from Giemsa stained monolayers inoculated with bacteria at a MOI of 1:1, 10:1, and 100:1, from three independent experiments

nd not detected due to total monolayer disruption

Table 3.2 Cytolytic activity of bacteria and bacterial culture supernatants for HeLa cell monolayers

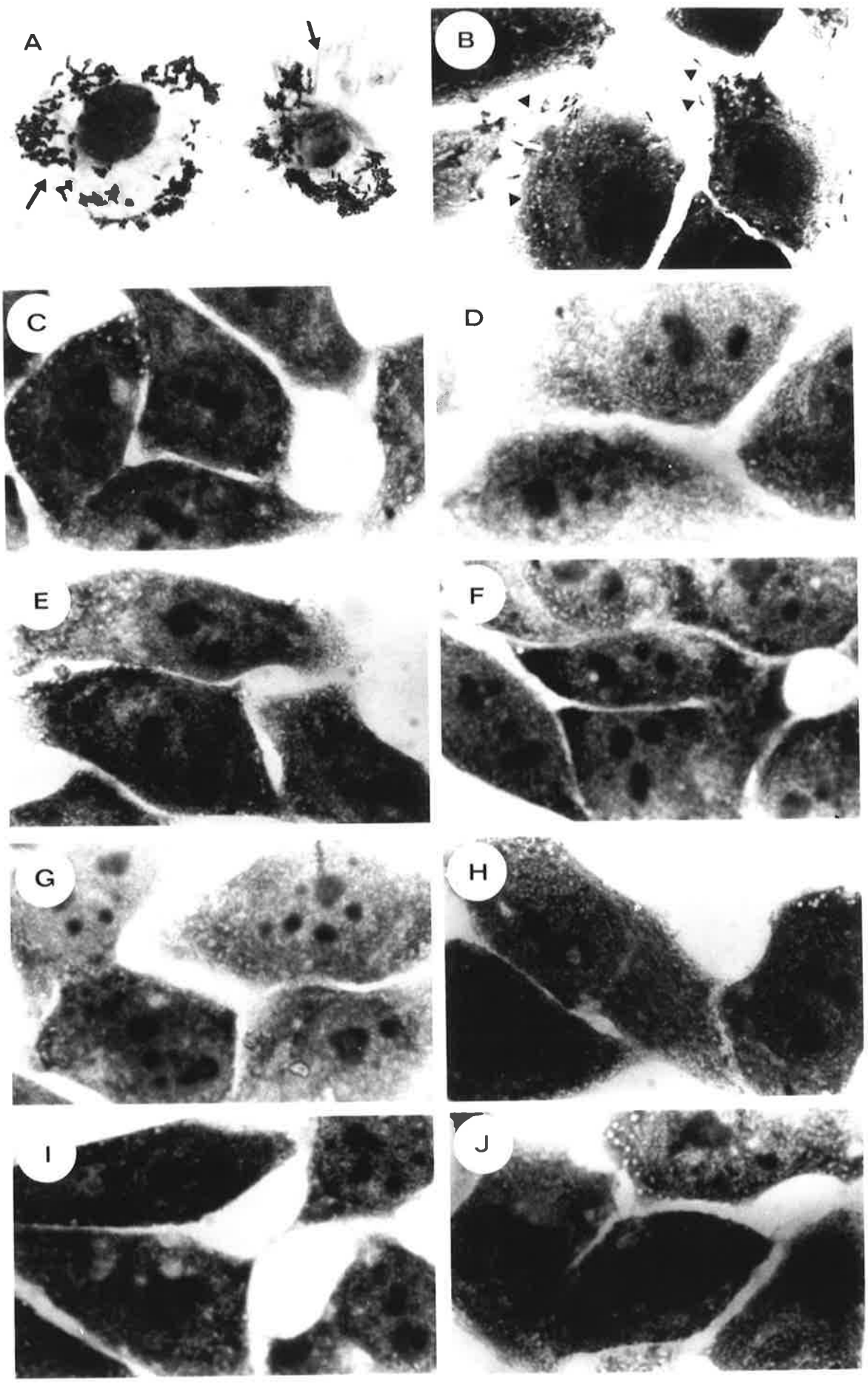
Condition	Percent monolayer disruption by *L. monocytogenes*[a]									
	DRDC8			LLO17			LLO19			Uninfected
	1:1	10:1	100:1	1:1	10:1	100:1	1:1	10:1	100:1	
Primary infection[b]	3.24	66.91	100	4.29	5.71	5.43	5.00	4.75	5.16	0
Supernatant[c]	1.05	3.09	2.59	2.86	3.31	3.76	4.05	3.57	4.13	0

[a] A minimum of 400 cells counted from at least six random locations on Giemsa stained cell monolayers inoculated with bacteria at 1:1, 10:1, and 100:1 (bacteria:cell), from two independent experiments. Percentages are calculated relative to uninfected monolayer controls. LLO17 and LLO19 are Tn*917-lacZ-cat86* insertion mutants in *hly* and *prfA* respectively.

[b] Initial bacterial inoculum was incubated with cell monolayers for 2 h to promote bacterial internalisation, followed by a 2 h incubation in media containing gentamycin.

[c] The supernatant was collected from infected cell monolayers after the initial 2 h incubation. Bacteria were removed by centrifugation, and 100 µl of supernatant was incubated with uninfected cell monolayers for 4 h prior to Giemsa staining.

Figure 3.5: Light photomicrographs (1000×) of Giemsa stained HeLa cell monolayers 4 h post inoculation with *L. monocytogenes* strains or infected cell monolayer culture supernatant. Panel: [A], DRDC8 (MOI of 10:1); [B], supernatant from DRDC8 infection (MOI of 10:1); [C], DRDC8 (MOI of 1:1); [D], supernatant from DRDC8 infection (MOI of 1:1); [E], LLO17 (MOI of 10:1); [F], supernatant from LLO17 infection (MOI of 10:1); [G], LLO19 (MOI of 10:1); [H], supernatant from LLO19 infection (MOI of 10:1); [I], uninfected control; [J], supernatant from uninfected control. Arrows show features indicative of cytopathogenicity induced by rapid multiplication and cell to cell spread by intracellular *L. monocytogenes*. Rounded morphology and loss of cell membrane integrity defined cytopathogenicity by *L. monocytogenes*. Arrowheads indicate pseuodopod-like structures characteristic of cell to cell spread of *L. monocytogenes*.


A
B
C
D
E
F
G
H
I
J

to compare ability of Caco-2 and HeLa cell lines to mediate uptake of *Listeria* spp. and to discriminate isolates on their capacity to promote internalisation.

3.2.5 Comparison of the ability of *Listeria* spp. to be internalised by Caco-2 and HeLa cell monolayers when added at a multiplicity of infection of 1:1

Recovery of viable bacteria from lysed cells, revealed comparable levels of internalisation of cell monolayers was obtained when either DRDC8 or SLCC 5764 were added at a MOI of 1:1, irrespective of the cell line used (Figure 3.6A, Bar a and b). Furthermore, similar numbers of intracellular bacteria per cell 4 hours post inoculation, were also obtained when Giemsa stained infected monolayers were viewed by light microscopy (Figure 3.6B, Bar a and b). However, neither cell line reliably discriminated the internalisation potential of DRDC8 or SLCC 5764 on the basis of bacterial recovery from infected monolayers. Nevertheless, both cell lines were able to discriminate between *Listeria* isolates able to induce their own phagocytosis vs isolates that were not (eg. *L. seeligeri, L. welshimeri, L. grayi, L. innocua and L. monocytogenes* LLO19) ($p<0.001$) (Figure 3.6).

When cell monolayers were inoculated with a range of *L. monocytogenes* serotypes at a MOI of 1:1, the ability of a number of these strains to mediate internalisation was dependent on the cell line used (Figure 3.7). *L. monocytogenes* SLCC 2371 and SLCC 2374 (Figure 3.7, Bar b and e) were efficiently internalised by the Caco-2 cell line, while SLCC 2378 and SLCC 2540 (Figure 3.7, Bar g and h), were more capable of promoting uptake in HeLa cells. These cell lines were able to clearly discriminate strains on the basis of internalisation potential, determined by percent bacterial recovery. For example, serotypes 3a (SLCC 2373), 3b (SLCC 2540), 4a (SLCC 2374), 4d (SLCC 2377), and 4e (SLCC 2378) were consistently less able to mediate internalisation of cell monolayers compared to serotypes 1/2a [SLCC 2371, SLCC 5764, EGD (Bubert), and EGD (Kaufmann)] and 1/2b (SLCC 2755). This is an interesting observation considering 90% of reported human listeriosis cases are due to serotypes 1/2a, 1/2b, and 4b (Gellin and Broome, 1989). However, definite differences in virulence among representative isolates from these serovar has been reported (Hof and Hefner, 1988; Kathariou *et al.*, 1988). Therefore, before a significant correlation between internalisation potential and serotype can be determined, or otherwise, the internalisation ability of individual isolates from a large sample population representing each serotype is required. Furthermore, the effect of extensive laboratory culturing on artificial media on attenuation of internalisation potential of bacteria should not be overlooked.

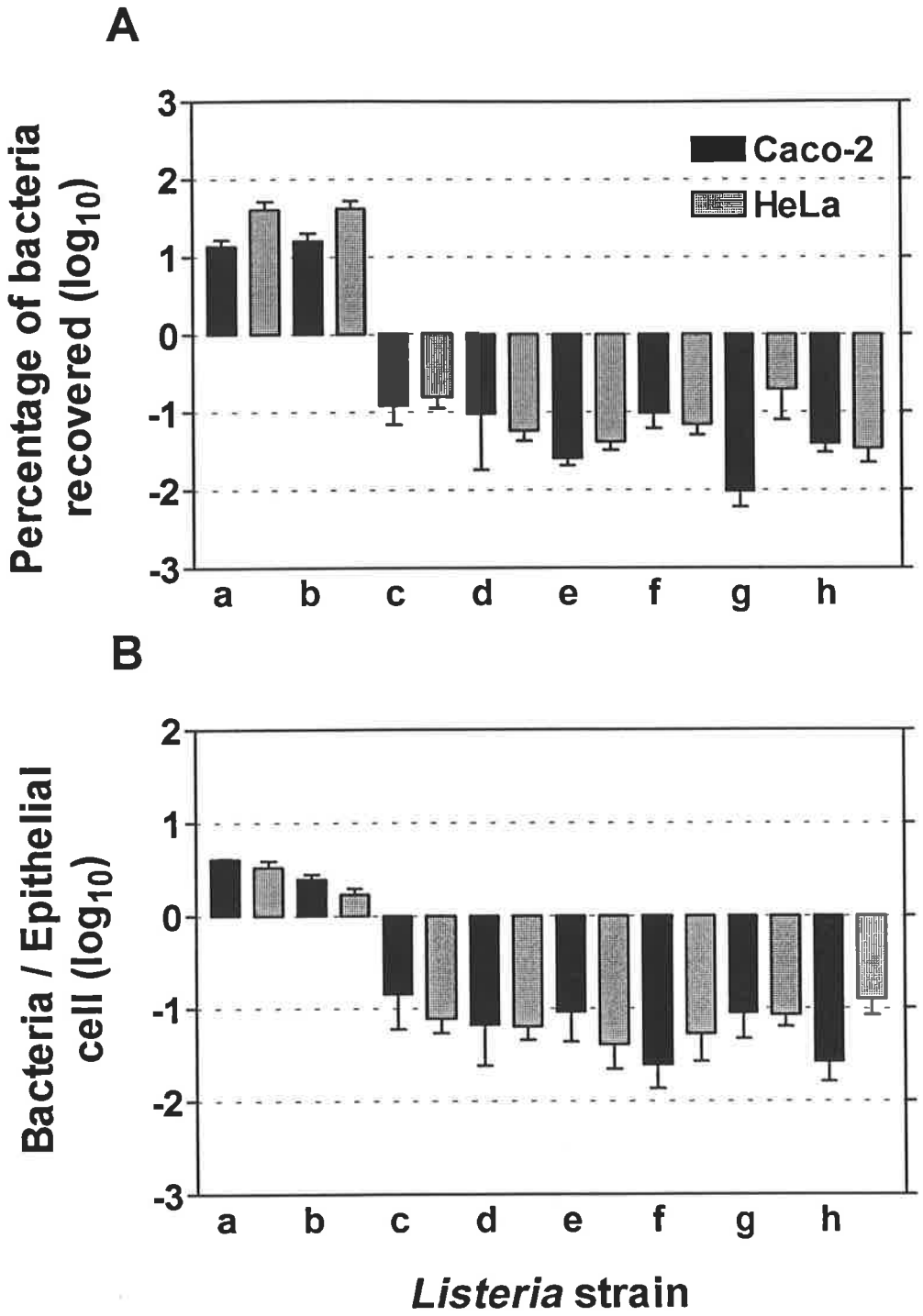


Figure 3.6: Internalisation of *Listeria* spp. by Caco-2 and HeLa cell monolayers exposed to bacteria at a MOI of 1:1. Part A: Results are expressed as percent bacterial recovery of viable bacteria from infected cell lysates 4 h post inoculation. Part B: Results are represented by the number of bacteria per epithelial cell determined by light microscopy of Giemsa stained infected cell monolayers 4 h post inoculation. Bar: [a], *L. monocytogenes* DRDC8; [b], *L. monocytogenes* SLCC 5764; [c], *L. ivanovii*; [d], *L. seeligeri*; [e], *L. welshimeri*; [f], *L. grayi*; [g], *L. innocua*; and [h], *L. monocytogenes* LLO19. Each column represents the mean ± SEM (log_{10}) of at least four independent experiments.

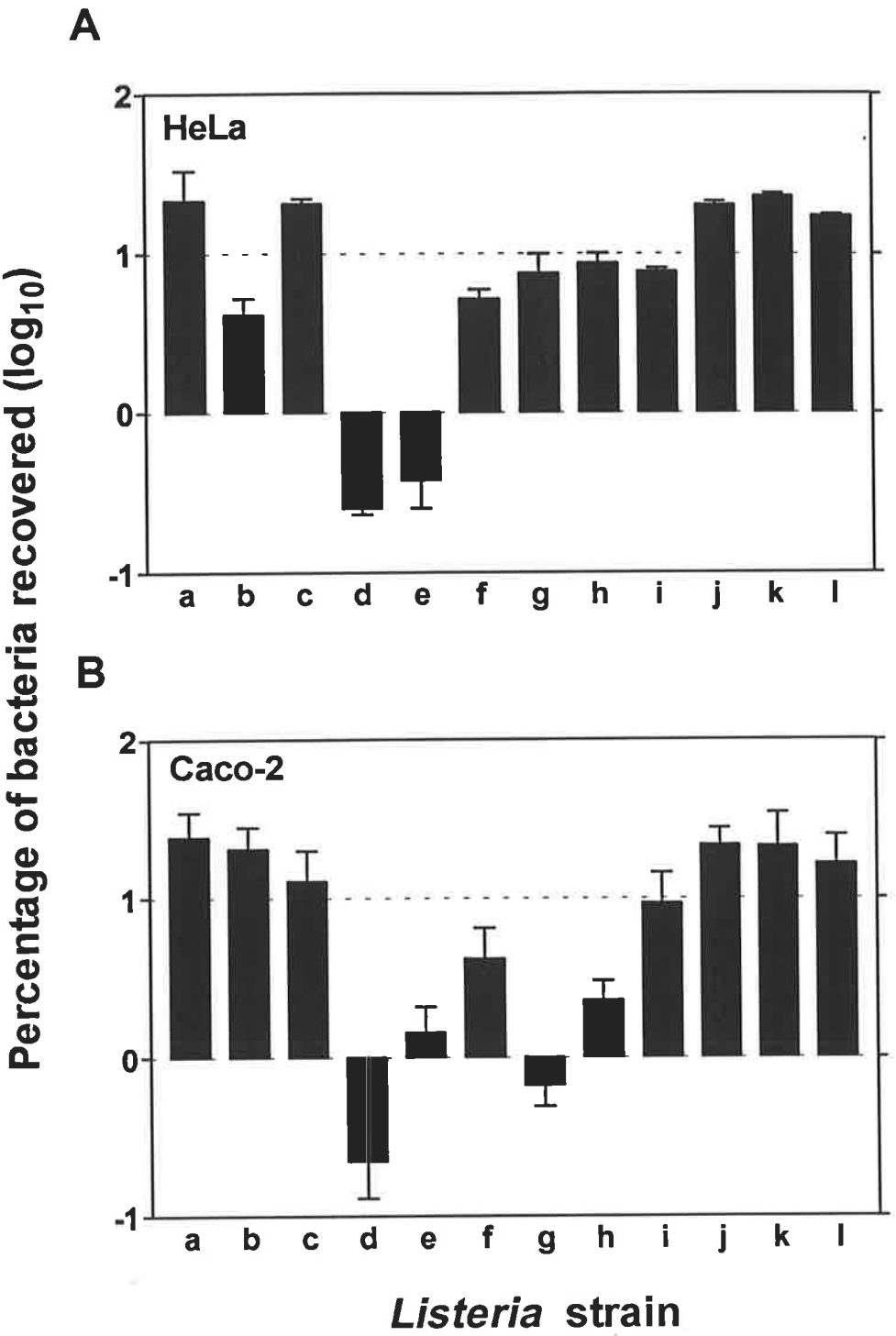


Figure 3.7: Internalisation of serotyped *L. monocytogenes* laboratory isolates by Caco-2 and HeLa cell monolayers exposed to bacteria at a MOI of 1:1. Results are expressed as percent bacterial recovery of viable bacteria from infected cell lysates. Bar: [a], DRDC8; [b], SLCC 2371; [c], SLCC 2372; [d], SLCC 2373; [e], SLCC 2374; [f], SLCC 2377; [g], SLCC 2378; [h], SLCC 2540; [i], SLCC 2755; [j], SLCC 5764; [k], EGD (Bubert), and [l], EGD (Kaufmann). Each column represents the mean ± SEM (log_{10}) of at least four independent experiments.

3.2.6 Utility of HeLa cell monolayers for assessment of *L. monocytogenes* pathogenicity

A number of studies have demonstrated a direct correlation between ability of *L. monocytogenes* strains to induce cell to cell spread in tissue culture monolayers and pathogenicity. Cell to cell spread can be demonstrated by formation of plaques in tissue culture monolayers or through the use of immunofluorescence microscopy. In this study, immunofluorescence microscopy (Dabiri *et al.*, 1990; Domann *et al.*, 1992; Niebuhr *et al.*, 1993), was used as a means of confirming cell to cell spread in monolayers infected with a variety of *Listeria* spp. Cell lines (J774, Caco-2, and HeLa) inoculated at an MOI of 1:1 with DRDC8 or SLCC 5764, demonstrated classical features indicative of bacterial induced actin polymerisation and cell to cell spread (Figure 3.8, 3.9, and 3.10, respectively). Moreover, these features were more evident in J774 and HeLa cells, and suggests more extensive bacterial parasitism within these cell lines. However, HeLa cells also revealed distinct patterns of filamentous actin, a feature not observed for either Caco-2 or J774 cells. Non-internalised strains of *Listeria* spp. and *L. monocytogenes* LLO19, were unable to extensively multiply within cells nor polymerise actin or engage in cell to cell spread. This data suggested the HeLa cell line was appropriate for distinguishing pathogenic vs non-pathogenic *Listeria* spp. based on ability to mediate internalisation and intracellular parasitism.

3.3 Discussion

Over the last decade, major advances have been made in understanding the role of virulence factors involved in the pathogenesis of *Listeria*. Development of tissue culture models of infection provided the catalyst by which these rapid advances were achieved. In particular, the Caco-2 colon carcinoma cell line has been routinely used in studies associated with *L. monocytogenes* infection (Gaillard *et al.*, 1987; Dabiri, *et al.*, 1990; Kathariou *et al.*, 1990; Quinn *et al.*, 1993; Karunasagar *et al.*, 1994). Caco-2 cells have been considered to be significantly more sensitive to *L. monocytogenes* internalisation when compared to other human epithelial cell lines including HeLa, Henle 407 and Hep-2 (Gaillard *et al.*, 1991; Pine *et al.*, 1991). This sensitivity is assumed to be the effect of unique features associated with Caco-2 morphology (Pinto *et al.*, 1983; Rousset, 1986).

Results presented in this thesis show that permissiveness of *L. monocytogenes* to invade either HeLa or Caco-2 cell lines was directly dependent upon the assay conditions used. To establish

Figure 3.8: Immunofluorescence photomicrographs (1000×) of J774 cell monolayers after inoculation with *Listeria* spp at a MOI of 1:1. Monolayers were incubated with rabbit *Listeria* O Antiserum Poly (serotypes 1 and 4), followed by goat anti-rabbit IgGAM (H+L) and fluorescein isothiocyanate (FITC) conjugate prior to examination. Panel: [a], *L. monocytogenes* DRDC8; [b], *L. monocytogenes* SLCC 5764; [c], *L. monocytogenes* LLO19; [d], *L. innocua*; [e], uninfected control. Arrowheads indicate pseuodopod-like structures indicative of cell to cell spread of *L. monocytogenes*.

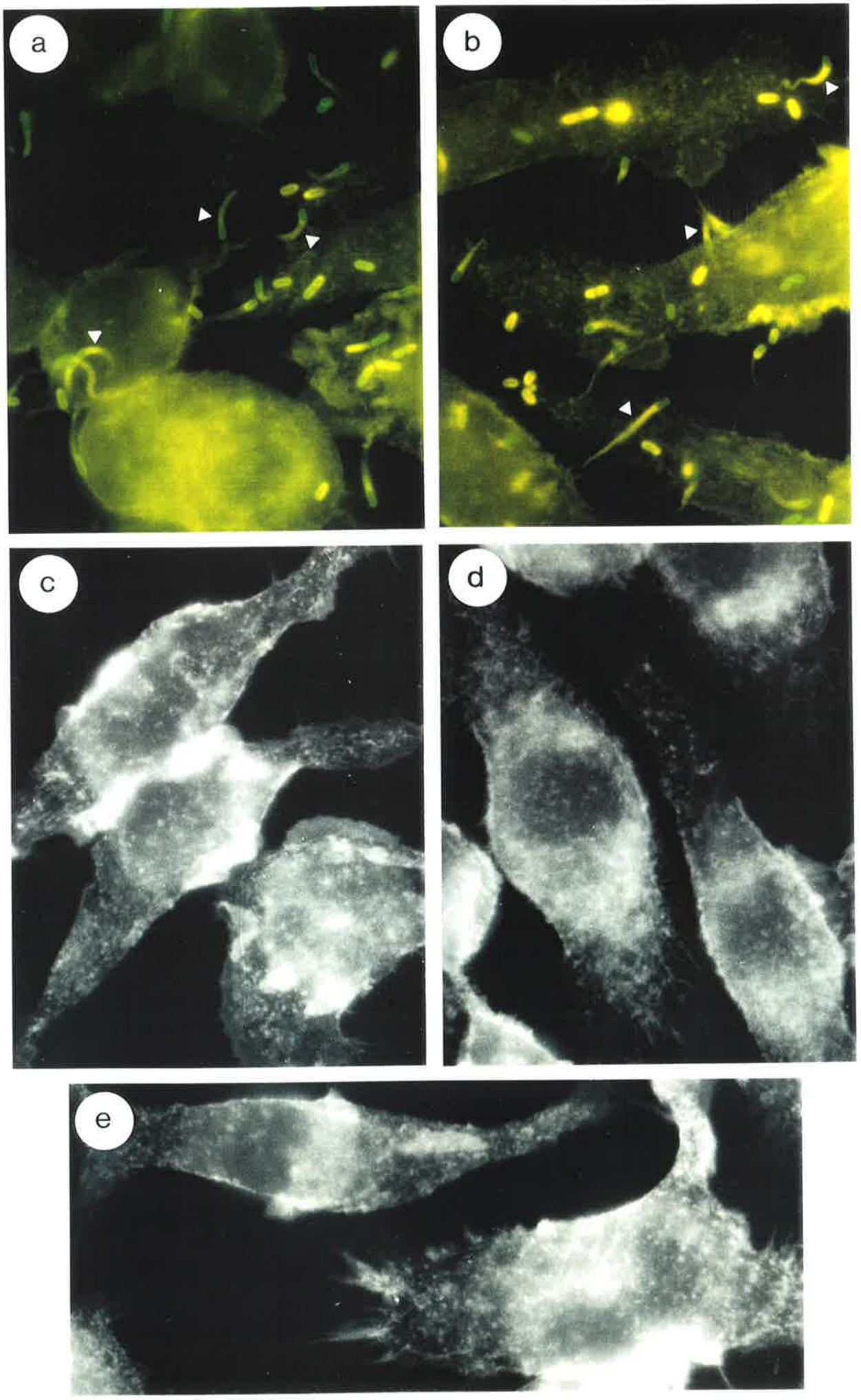

a
b
c
d
e

Figure 3.9: Fluorescence photomicrographs (1000×) of Caco-2 cell monolayers after inoculation with *Listeria* spp. at a MOI of 1:1. Monolayers were stained with phalloidin rhodamine conjugate prior to examination. Panel: [a], *L. monocytogenes* DRDC8; [b], *L. monocytogenes* SLCC 5764; [c], *L. monocytogenes* LLO19; [d], *L. innocua*; [e], uninfected control. Arrowheads indicate pseuodopod-like structures indicative of cell to cell spread of *L. monocytogenes*.

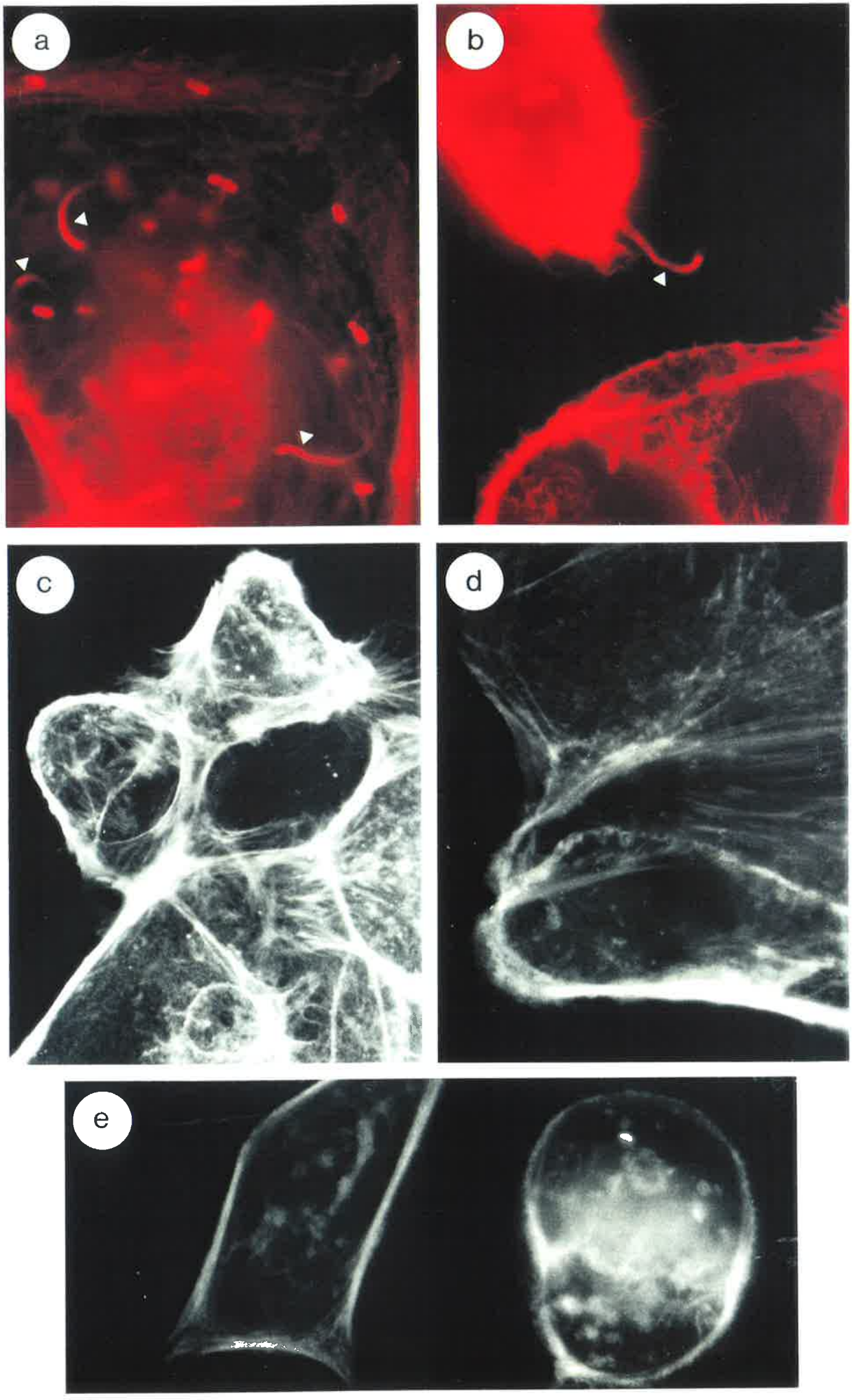
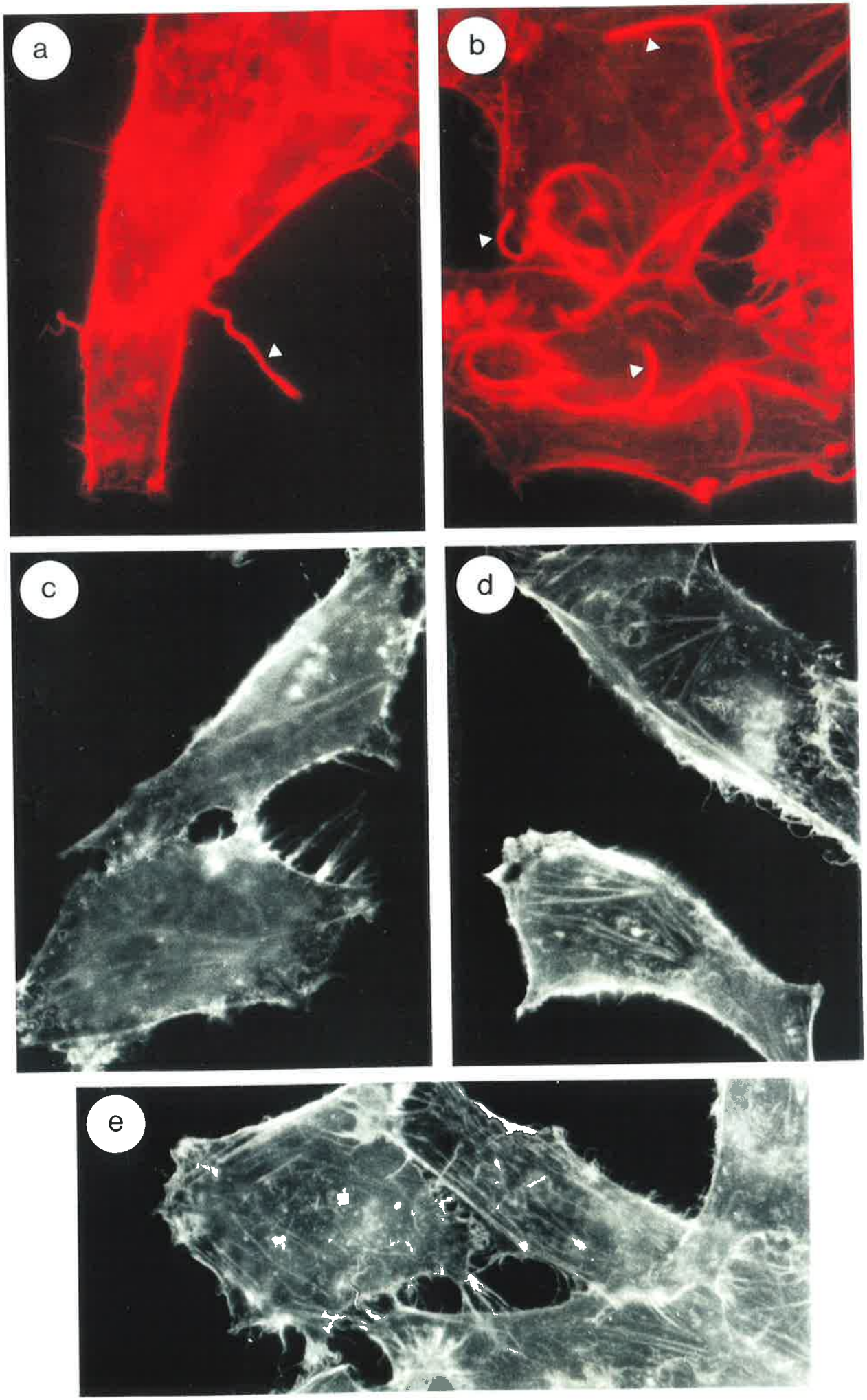

a
b
c
d
e

Figure 3.10: Fluorescence photomicrographs (1000×) of HeLa cell monolayers after inoculation with *Listeria* spp. at a MOI of 1:1. Monolayers were stained with phalloidin rhodamine conjugate prior to examination. Panel: [a], *L. monocytogenes* DRDC8; [b], *L. monocytogenes* SLCC 5764; [c], *L. monocytogenes* LLO19; [d], *L. innocua*; [e], uninfected control. Arrowheads indicate pseuodopod-like structures indicative of cell to cell spread of *L. monocytogenes*.


a
b
c
d
e

significant bacterial internalisation of the HeLa cell line, a centrifugation step was incorporated into the infection protocol. These conditions enabled an assessment of the effect multiplicity of infection had on internalisation and multiplication of *L. monocytogenes* in HeLa and Caco-2 cells. In particular, significantly more bacteria were recovered from monolayers exposed to bacteria at low MOI's, irrespective of the cell line. Conversely, high MOI's resulted in low bacterial recovery from cell monolayers. One explanation for this result is that high MOI's lead to extensive bacterial internalisation and intracellular multiplication, and ultimately cell lysis. Intracellular bacteria were then exposed to inhibitory concentrations of gentamycin contained in the extracellular milieu, which prevented recovery of viable bacteria from cell lysates.

There is some debate concerning the effectiveness of gentamycin in tissue culture models of infection. In a recent study, gentamycin was shown to be pinocytosed by murine peritoneal macrophages (Drevets *et al.*, 1994). *L. monocytogenes* contained within phagosomes of gentamycin treated cells were killed, apparently as a result of fusion between the phagosomes and pinocytic endosomes. However, this phenomenon was restricted to macrophages exhibiting pinocytotic activity and has not been observed in epithelial cell lines. In these cell lines, gentamycin is normally unable to permeate through cell membranes or maintain bactericidal activity intracellularly, and is therefore routinely used in tissue culture models of infection to kill extracellular bacteria (Vaudaux and Waldvogel, 1979; Lissner *et al.*, 1983; Havell, 1986; Portnoy *et al.*, 1988; Nichterlein and Hof, 1991). Nevertheless, the bactericidal activity of gentamycin to *L. monocytogenes* released from heavily infected cells was overlooked in earlier studies that describe recovery and intracellular multiplication of this organism in Caco-2 monolayers exposed to a high MOI (Gaillard *et al.*, 1987; Gaillard *et al.*, 1991; Dramsi *et al.*, 1993b).

Two lines of evidence strongly indicate heavily infected cells lyse due to excessive structural stress imposed by high numbers of intracellular bacteria. Firstly, a direct correlation between membrane damage and the percentage of cells within the monolayer that host intracellular bacteria was observed. Secondly, culture supernatants derived from cell monolayers infected with bacteria, did not affect cellular integrity. Therefore, cytotoxic activity of bacterial enzymes or toxic by-products of bacterial growth was not responsible for the observed lysis of tissue culture monolayers. This is in contrast to other studies, that suggest the supernatants of *S. typhimurium* and *S. flexneri* cultures, contain free LPS molecules, which can result in

significant monolayer disruption. In addition, activity of listerial enzymes involved in pathogenesis, is usually controlled by a cellular tropism. For this reason, these enzymes are unlikely to induce direct monolayer disruption. For example, cytolytic activity of listeriolysin O, a pore forming haemolysin responsible for phagolysosome membrane disruption (Cossart and Mengaud, 1989), was restricted to the phagolysosome where an internal pH of 5.5 was necessary for optimal activity (Geoffroy *et al.*, 1987).

Interestingly, two recent studies have confirmed *L. monocytogenes* induced cell death of infected murine dendritic cells (Guzman *et al.*, 1996) and hepatocytes (Rogers *et al.*, 1996) through apoptosis. These findings have significant implications for non-specific mechanisms of defence against infections by this organism. Nevertheless, the process of programmed cell death is dependent on cell type and apparently does not occur in infected HeLa or Caco-2 cells. Therefore, *L. monocytogenes* induced apoptosis is unlikely to be responsible for the membrane disruption observed in this study.

Significant monolayer disruption noted after prolonged exposure (16 h to 24 h) of monolayers to high MOI of *L. monocytogenes* (Gaillard *et al.*, 1987; Pine *et al.*, 1991), was overcome by routinely limiting the incubation period to 4 hours with a MOI of 1:1. Under these conditions, a direct correlation between low MOI, and high bacterial recoveries from infected monolayers was observed. In this study, at least, careful selection of MOI was essential in order to provide a more appropriate method of determining an organisms ability to initiate uptake into tissue culture cells. Furthermore, this data demonstrates that HeLa and Caco-2 cells were equally efficient at discriminating between isolates capable of internalisation (*L. monocytogenes*) and isolates that could not (*L. ivanovii, L. seeligeri, L. welshimeri, L. grayi, L. innocua* and *L. monocytogenes* LLO19), a feature observed in other tissue culture models of infection (Gaillard *et al.*, 1987; Kuhn *et al.*, 1988; Pine *et al.*, 1991; Bhunia *et al.*, 1994). While these results correlate with previous communications that suggest Caco-2 cells were more susceptible to internalisation by *L. monocytogenes* (Gaillard *et al.*, 1987; Kathariou *et al.*, 1990; Gaillard *et al.*, 1991; Pine *et al.*, 1991), in my opinion, at least, this cell line was not the most suitable for the analysis of listerial internalisation. Any advantages of Caco-2 cells over HeLa cells can be overcome by employing a combination of a 15 min centrifugation step, short incubation periods, and low MOI. Using a modified infection process, this study has clearly demonstrated the utility of the HeLa cell line for use in a tissue culture model of *L. monocytogenes* infection.

The ability of an organism to mediate internalisation is not the only requirement for establishing an infection. Virulence of *L. monocytogenes* is also dependent on ability to spread from cell to cell. Consequently, the utility of HeLa cells as a suitable tissue culture model of infection was confirmed by assessment of *Listeria* pathogenicity using immunofluorescent microscopy to compare ability of *Listeria* spp. to engage in cell to cell spread. In addition to Caco-2 and HeLa cells, the phagocytic murine macrophage-like cell line J774 was included in this study, because these cells have been extensively used to study characteristics of *L. monocytogenes* infection (Tilney and Portnoy, 1989; Bielecki *et al.*, 1990; Kocks *et al.*, 1992; Neibuhr *et al.*, 1993; Raybourne and Bunning, 1994). As expected, *L. monocytogenes* contained within J774 cells displayed a high degree of filamentous actin polymerisation and cell to cell spread in comparison to infected Caco-2 and HeLa cell lines. Nevertheless, *L. monocytogenes* induced filamentous actin polymerisation and pseudopodia-like structures were more distinctive in HeLa cells as opposed to either J774 or Caco-2 cells, and this suggests a greater parasitism of pathogenic *L. monocytogenes* for the HeLa cell line. This observation may however reflect a greater rate of growth in HeLa cells.

Variation in bacterial growth rates within different cell lines has previously been described. Gaillard *et al.*, (1987), reported an intracellular doubling time of ~90 min for *L. monocytogenes* in Caco-2 cells, and Portnoy *et al.*, (1988), reported doubling times of ~60 min in a variety of cell lines including the epithelial cell line Henle 407. Since intracellular multiplication of *Listeria* is routinely used as a measure of bacterial virulence (Jones and Portnoy, 1994), it follows that enhanced intracellular multiplication of an organism is most likely reflected by an increase in intracellular parasitism towards the host cell. While this study did not seek to determine bacterial growth rates within HeLa and Caco-2 cells, this data could help explain why *L. monocytogenes* shows superior parasitism for HeLa cells. Caco-2 cells may be more sensitive to internalisation by *L. monocytogenes* (Gaillard *et al.*, 1987; Kathariou *et al.*, 1990; Gaillard *et al.*, 1991; Pine *et al.*, 1991), whereas HeLa cells apparently promote rapid intracellular growth of this organism.

The isogenic mutants LLO17 and LLO19, were included as negative controls. These strains contain a Tn*917* insertion in listeriolysin O (Hly), and the positive regulatory factor (PrfA), respectively. While Hly is an essential virulence determinant for *L. monocytogenes* pathogenesis (Gaillard *et al.*, 1986; Kathariou *et al.*, 1987a; Cossart *et al.*, 1989; Michel *et al.*,

1990), PrfA is responsible for the coordinate regulation of *hly*, and other virulence determinants from *L. monocytogenes* (Leimeister-Wächter *et al.*, 1990; Chakraborty *et al.*, 1992; Dramsi *et al.*, 1993b), and is essential for the establishment of infection (Freitag *et al.*, 1993). As expected, these mutants demonstrated significantly reduced internalisation potential, were unable to engage in cell to cell spread, and did not induce cell membrane disruption. This suggests *L. monocytogenes* does not shed toxic by-products during *in vitro* growth, that may contribute to cytopathogenicity of tissue culture cells.

Interestingly, the animal pathogen *L. ivanovii*, was unable to polymerise host cell actin and was significantly less invasive then *L. monocytogenes*. Similarly, *L. ivanovii* is unable to invade the mouse embryo fibroblast cell line 3T6 (Kuhn *et al.*, 1988). No evidence of cytopathogenicity for hybridoma and myeloma cell lines has been observed, even after prolonged infection (Bhunia *et al.*, 1994). However, other studies have reported internalisation of cell lines and polymerisation of host cell actin by this bacterium (Gaillard *et al.*, 1987; Karunasagar *et al.*, 1993). Although there is no obvious reason for these apparent abnormalities, strain variation among *L. ivanovii* isolates may contribute to the different responses observed.

In conclusion, the results of this study have shown the importance of MOI in tissue culture models of infection. Indeed, using the conditions described, HeLa and Caco-2 cells were equally efficient at discriminating between *Listeria* spp. capable of internalisation and those that were not. Furthermore, HeLa cells provided a more convenient means to distinguish pathogenic and non-pathogenic *Listeria* spp. using immunofluorescence microscopy. In contrast to Caco-2 cells, features of HeLa cells, including flat morphology, large cytoplasmic area and ease of culturing, make this the preferred cell line for use in the analysis of pathogenicity of *Listeria* spp. by tissue culture models of infection.

Chapter 4 Construction of a promoter::*lacZ* reporter gene fusion library in *L. monocytogenes*, and induction of β-galactosidase expression by environmental stimuli

4.1 Introduction

To establish an infection, bacterial pathogens must overcome extensive environmental change within a host and evade host immune defence mechanisms. Bacteria have developed strategies to sense environmental change and transform this into a signal to regulate expression of virulence determinants. This regulatory control means that virulence gene expression is limited to times of bacterial/host interaction. A number of concise reports have reviewed the mechanisms of bacterial infection and the significant role environmental factors play in control of virulence gene expression (Miller *et al.*, 1989; Mekalanos, 1992; Gross, 1993). To this end, a single environmental stimuli is usually responsible for the coordinate regulation of virulence determinants acting in concert at a particular stage of an infection. In fact, environmental conditions such as temperature, osmolarity, iron and calcium are known to regulate expression of well characterised genetic determinants responsible for the pathogenic mechanisms of a number of bacteria such as *E. coli, S. flexneri, V. cholerae, C. diphtheriae* and *Yersinia* spp. (see Section 1.15, Table 1.5).

The situation for *L. monocytogenes* is no different. Expression of all PrfA-dependent virulence genes (*plcA, hly, mpl, plcB, actA,* and *inlA*) are induced following a shift in growth temperature from 25°C to 37°C (Leimeister-Wächter *et al.*, 1992; Dramsi *et al.*, 1993b). Other environmental cues including heat stress, osmolarity, pH, and iron have been implicated in regulation, and these may reflect the presence of PrfA-independent promoters capable of initiating transcription under environmental conditions other then temperature.

This phenomenon of environmental control of bacterial virulence gene expression, can be exploited by constructing transcriptional promoter::reporter gene fusions, as a means to isolate novel virulence determinants. Transcription from the native promoter is determined by the activity of the product of the fused reporter gene. In this chapter, construction of a promoter::reporter gene transcriptional fusion library using a Tn*917* derived transposon containing promoterless *lacZ* and *cat86* genes in *L. monocytogenes* is described. Difficulties

associated with DNA mobilisation into *L. monocytogenes* is discussed. Furthermore, experimentation leading to the selection of suitable environmental stimuli for screening the transcriptional fusion library is presented.

4.2 Results

4.2.1 Introduction of Tn*917* derivatives into *L. monocytogenes* DRDC8

Even though Tn*917* derivatives are consistently used for mutagenesis of Gram-positive bacteria, the unwieldy size of most of these vectors has generally limited their application to bacteria which display increased natural competence for uptake of foreign DNA. In preliminary experiments, therefore, several methods were used to transfer mutagenesis vectors containing Tn*917* derivatives [pTV53 [16.9 kb] (Youngman *et al.*, 1985a), and pLTV1 [20.6 kb] or pLTV3 [22.1 kb] (Camilli *et al.*, 1990), diagrammatically represented in Figure 4.1], into *L. monocytogenes* DRDC8.

4.2.1.1 Protoplast transformation

Protoplast transformation has been used to mobilise plasmid DNA into *Listeria* spp. at high efficiency (Vicente *et al.*, 1987; Cossart *et al.*, 1989; Wuenscher *et al.*, 1991). Plasmids pLTV1 and pLTV3 were also introduced into *L. monocytogenes* by this method (Camilli *et al.*, 1990). Based on these studies, the protoplast technique was first selected to transform the Tn*917* derived mutagenesis vectors into *L. monocytogenes* DRDC8. Given that protoplasts are difficult to prepare, protoplast formation derived from a starting culture of logarithmic phase bacteria, was monitored by phase contrast microscopy. Protoplasts have a distinctive appearance and can be readily distinguished from normal bacterial cells using this technique. Few protoplasts were visualised even from several independent preparations. Consistent with this result, no Em^R, Lm^R, Tc^R transformants were recovered when these protoplast preparations were infected with pTV53 DNA. Consequently, other methods of plasmid DNA transfer were investigated.

4.2.1.2 Conjugation

Conjugation occurs naturally in Gram-positive bacteria, and recent studies report an increase in efficiency of DNA mobilisation into *L. monocytogenes* (Trieu-Cuot *et al.*, 1991; Trieu-Cuot *et al.*, 1993). In this study, the Em^R *E. coli/L. monocytogenes* shuttle vector pAT19 (6.6 kb), was used to demonstrate conjugal transfer of plasmid DNA from *E. coli* S17-1 (Sm^R) to

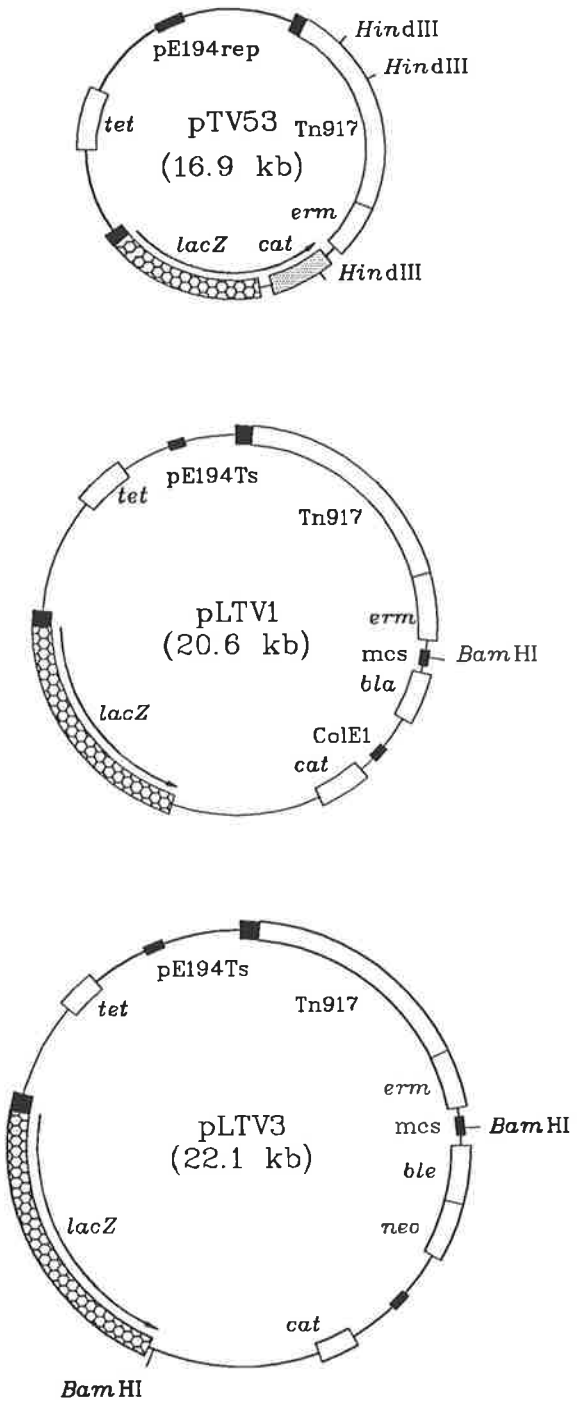


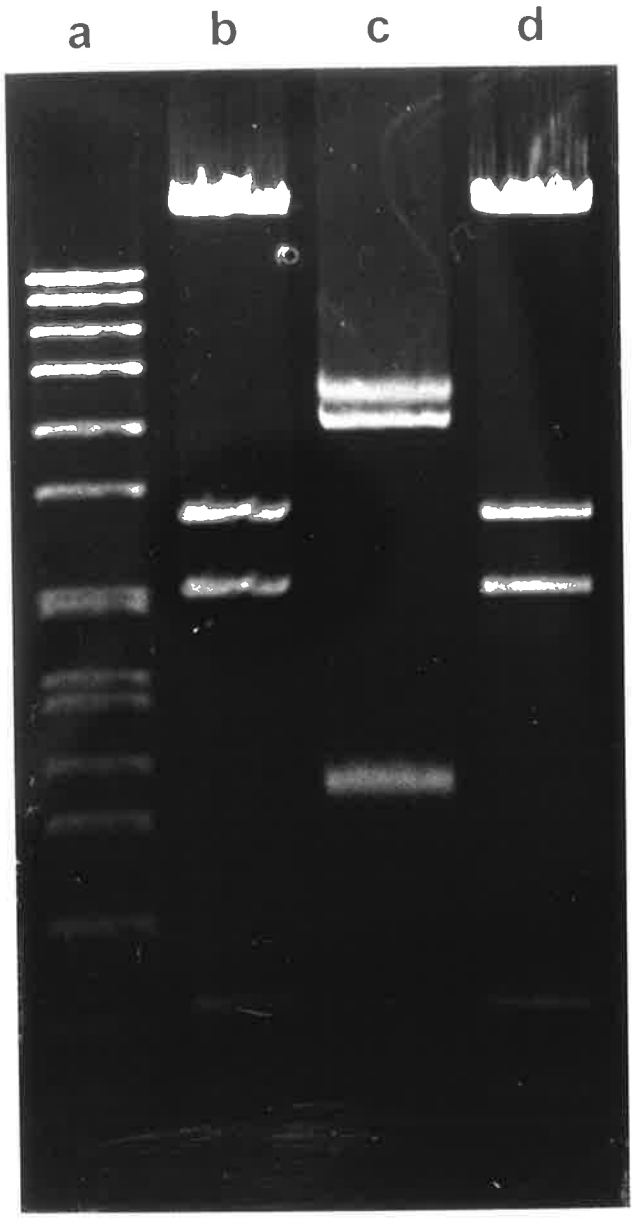
Figure 4.1: Schematic representation of temperature sensitive Gram-positive mutagenesis vectors that contain Tn*917* derived transposable elements. Abbreviations: Tn*917*, transposon Tn*917* isolated from *Streptococcus faecalis*; *erm*, erythromycin resistance cartridge; *cat*, chloramphenicol resistance cartridge (promoterless in plasmid pTV53); *tet*, tetracycline resistance cartridge; *neo*, neomycin phosphotransferase II gene; *ble*, bleomycin gene; *bla*, β-lactamase gene; *lacZ*, promoterless β-galactosidase gene; mcs, multiple cloning site; pE194rep, temperature-sensitive replicon from *Staphylococcus aureus* plasmid pE194; pE194Ts, mutanted temperature-sensitive pE194 replicon; ColE1, Gram-negative replicon. The *Hin*dIII (pTV53) and *Bam*HI (pLTV1 and pLTV3) restriction sites used for routine analysis of DNA constructs containing these vectors are indicated. Adapted from Youngman *et al.*, (1985a) [pTV53] and Camilli *et al.*, (1990) [pLTV1 and pLTV3].

L. monocytogenes LM002 (Rp^R). Transfer to *E. coli* HB101 (Sm^R) was included as a positive control. As expected, plasmid pAT19 was efficiently mobilised into HB101. Em^R, Sm^R bacteria were isolated at a frequency of 1.1×10^{-1} transconjugates/donor cfu. In contrast, the efficiency of transfer of this vector into *L. monocytogenes* LM002 was considerably reduced (2.3×10^{-6} Em^R, Sm^R transconjugates/donor cfu). These findings were comparable to a previous study that utilised a similar sized conjugative plasmid (Trieu-Cuot *et al.*, 1991). In this report, a 10.6 kb derivative of pAT19 was mobilised into *L. monocytogenes* at a frequency of 7.2×10^{-7} transconjugates/donor cfu. This data was encouraging and lead to the opportunity to apply the principle of conjugation to the transfer of mutagenesis vectors into *L. monocytogenes* DRDC8.

One significant advantage of conjugation, is that routine mobilisation of large plasmids is possible. However, the mutagenesis vectors used in this study are not naturally conjugative. Therefore, the construction of a mobilisable derivative of pLTV1, using a modification of the cloning strategy adopted by Sharma *et al.*, (1989) was considered. Essentially, this required the introduction of the mobilisable region (*mob*) from pSUP201-1 (Simon *et al.*, 1983, see Section 6.2.1.1, Figure 6.1), a derivative of the broad host range plasmid RP4, into a non-essential region of plasmid pLTV1. A 1.7 kb *Bam*HI fragment encoding the *mob* site was purified from pSUP201-1, cloned into the unique *Bam*HI site of pLTV1 (see Figure 4.1) and transformed into *E. coli* S17-1. *E. coli* Ap^R (derived from vector DNA), Sm^R transformants were isolated. However, conjugation of plasmid DNA from these transformants into *E. coli* SM10 (Km^R) by suitable filter mating experiments (Sharma *et al.*, 1989), could not be demonstrated. To investigate this problem, the *Pvu*II restriction profiles of pLTV1 DNA isolated from *E. coli* S17-1, and the control strains *E. coli* HB101 and *B. subtilis* PY1177 (Camilli *et al.*, 1990) were compared. The restriction profile of plasmid DNA isolated from S17-1 was significantly different compared to digested control DNA (Figure 4.2). One explanation for this result is that pLTV1 DNA is modified/rearranged in *E. coli* S17-1. This most likely prevented the isolation of a conjugative pLTV1 plasmid derivative containing *mob*.

Attempts to isolate a conjugative pLTV1 plasmid derivative were repeated using *E. coli* HB101/pRK2013 as the donor strain in filter matings with SM10. *E. coli* HB101 is a restriction/modification deficient bacterium unable to alter the configuration of pLTV1 (see Figure 4.2, Lane b). The conjugative vector pRK2013 (Figurski and Helinski, 1979), has

Figure 4.2: Agarose gel electrophoresis of *Pvu*II digested pLTV1 DNA. Lane: [a], *Eco*R1 digested Bacteriophage SPP-1 DNA; [b], *E. coli* HB101/pLTV1; [c], *E. coli* S17-1/pLTV1; [d], *B. subtilis* PY1177/pLTV1. A significant difference in the *Pvu*II restriction pattern of pLTV1 DNA isolated from *E. coli* S17-1, compared to the control strains was observed. This is suggestive of modification or rearrangement of pLTV1 DNA when maintained by the S17-1 strain.


a
b
c
d

a restricted host range, and its sole function is to *trans*-complement pLTV1 derivatives for mobilisation of DNA from HB101 into SM10. However, these experiments were also unsuccessful. The most likely explanation for this result is that pRK2013 was unstable in HB101 during filter mating experiments with SM10, which prevented the detection of derivatives of plasmid pLTV1 containing the *mob* site. These difficulties lead to the investigation of electroporation as a tool for mobilisation of mutagenesis vectors into *L. monocytogenes* DRDC8.

4.2.1.3 Electroporation

Electroporation offers a rapid, reliable, and efficient method for transferring plasmid DNA into bacteria. Moreover, this approach has been optimised for Gram-positive bacteria including *L. monocytogenes* (Alexander *et al.*, 1990; Park and Stewart 1990; Dunny *et al.*, 1991). The utility of electroporation for transforming plasmid DNA into *L. monocytogenes* was tested using the Gram-positive shuttle vector pAT19. Two different final concentrations of this vector (0.4 μg and 10 μg) was transformed in 10 μl volumes into 0.1 ml of a suspension of electrocompetent *L. monocytogenes* DRDC8 and SLCC 5764, prepared in sucrose magnesium electroporation medium, and 0.1 ml electrocompetent *E. coli* HB101 in a 10% (v/v) glycerol solution. Electroporation of pAT19 into HB101, was significantly more efficient using 0.4 μg of DNA (4×10^6 transformants/μg of DNA), when compared to 10 μg of DNA (7.5×10^3 transformants/μg of DNA). However, Em^R resistant DRDC8 and SLCC 5764 transformants were only consistently recovered when at least 10 μg of plasmid DNA was used (7.6×10^1 transformants/μg of DNA and 9×10^1 transformants/μg of DNA respectively). No Em^R DRDC8 transformants were recovered after electroporation with 0.4 μg plasmid DNA, while for SLCC 5764, 1.1×10^1 transformants/μg of DNA were recovered at this DNA concentration. The efficiencies of transformation of *L. monocytogenes* recorded in this study, are very low in contrast to a previous report (Alexander *et al.*, 1990). In this study, electroporation of *L. monocytogenes* with 0.4 μg of pGK12 DNA (4.3 kb), enabled the recovery of Em^R transformants at a transformation frequency of 4×10^6/μg pGK12 DNA.

Nevertheless, these results indicate that successful electroporation of plasmid DNA in *L. monocytogenes* requires a high concentration of DNA. When 0.1 μg to 2.5 μg of purified plasmid DNA (pTV53, pLTV1, or pLTV3) was electroporated into *L. monocytogenes*, no Em^R transformants were recovered. To circumvent this problem, a high yield of pTV53 DNA,

was prepared from a 100 ml culture of *B. megaterium* PV411/pTV53 grown aerobically at 30°C. The DNA pellet was resuspended in 50 µl of Milli-Q water. A 10 µg aliquot of pTV53 in 10 µl, was electroporated into *L. monocytogenes* DRDC8. The total bacterial suspension was cultured on selective media, and a single Em^R, Lm^R, Tc^R transformant was recovered at a transformation frequency of 1 x 10^{-1}/µg of DNA. DNA was prepared in Milli-Q water to reduced levels of contaminating salts, since these compounds are known to interfere with transformation (Dower, 1990).

The Em^R, Lm^R, Tc^R *L. monocytogenes* DRDC8/pTV53 transformant was characterised at the molecular level using several methods. Firstly, to confirm the Em^R, Lm^R, Tc^R transformant was indeed *L. monocytogenes* and not a resistant contaminant, a 417 bp portion of the listeriolysin O determinant *hly,* was amplified with synthetic oligonucleotides p234 and p319 (see Section 2.24 and Table 2.5), from chromosomal DNA purified from these strains using the method described by Fitter *et al.*, (1992). The location of the oligonucleotide pair relative to the *hly* virulence gene cluster is diagrammatically represented in Figure 4.3. An *hly* specific 417 bp amplified DNA product was obtained from three single colonies of the Em^R, Lm^R, Tc^R *L. monocytogenes* isolate DRDC8/pTV53 (Figure 4.4). *L. monocytogenes* strain DRDC8 and plasmid pLIS3, containing the *hly* gene (Cossart *et al.*, 1989) were included as positive controls. Template DNA from the non pathogenic *L. innocua* strain did not result in an amplification of *hly* specific DNA. Restriction analysis of the PCR products using *Hin*dIII, confirmed the specificity of the reaction. The *Hin*dIII restriction site within the 417 bp *hly* DNA fragment is apparently highly conserved among all *L. monocytogenes* strains (Thomas, 1995). Secondly, plasmid DNA isolated from three single colonies derived from the Em^R, Lm^R, Tc^R transformant was digested with *Hin*dIII and analysed by agarose gel electrophoresis (Figure 4.5). Three restriction fragments (12.2 kb, 3.6 kb and 1.1 kb), were represented in each independent preparation. These fragment sizes were consistent with the *Hin*dIII restriction fragment profile of pTV53 DNA isolated from the control strain *B. megaterium* PV411/pTV53. The Em^R, Lm^R, Tc^R transformant is referred to as *L. monocytogenes* DRDC8/pTV53 in the remainder of this thesis.

4.2.2 Induction of transposition of Tn*917-lacZ-cat86* from pTV53 into DNA of *L. monocytogenes* DRDC8

The mutagenesis vector pTV53 contains a temperature sensitive origin of replication (pE194rep). The plasmid is maintained by bacteria when grown at 30°C, but not when grown

Figure 4.3: Diagrammatic representation of a *Hin*dIII restriction map of the chromosomal located virulence gene cluster (*prfA*, *plcA*, *hly*, and *mpl*) from *L. monocytogenes* DRDC8 (Thomas, 1995). Nucleotide sequence of *hly* (Mengaud *et al.*, 1988), was used to design the synthetic oligonucleotides p234 and p319 (shown relative to *hly*). This oligonucleotide pair was intended to PCR amplify a 417 bp portion of *hly*, for rapid and specific identification of *L. monocytogenes* isolates using the method developed by Fitter *et al.*, (1992). The invariant *Hin*dIII restriction site located at the 3' end of *hly* (Thomas, 1995; and indicated by an asterisk) was used to confirm the specificity of the amplified 417 bp DNA product by restriction digestion. The 5.96 kb DNA fragment amplified by PCR using the synthetic oligonucleotides p408 and p409 is indicated (see Section 4.2.5). This DNA fragment was cloned into pBluescript KS to generate pCT006. The *Hin*dIII restriction fragment sizes (kb) from this region are indicated and correspond with Southern hybridisation analysis of this region of DRDC8 chromosomal DNA, probed with plasmid pCT006 (see Figure 4.11).

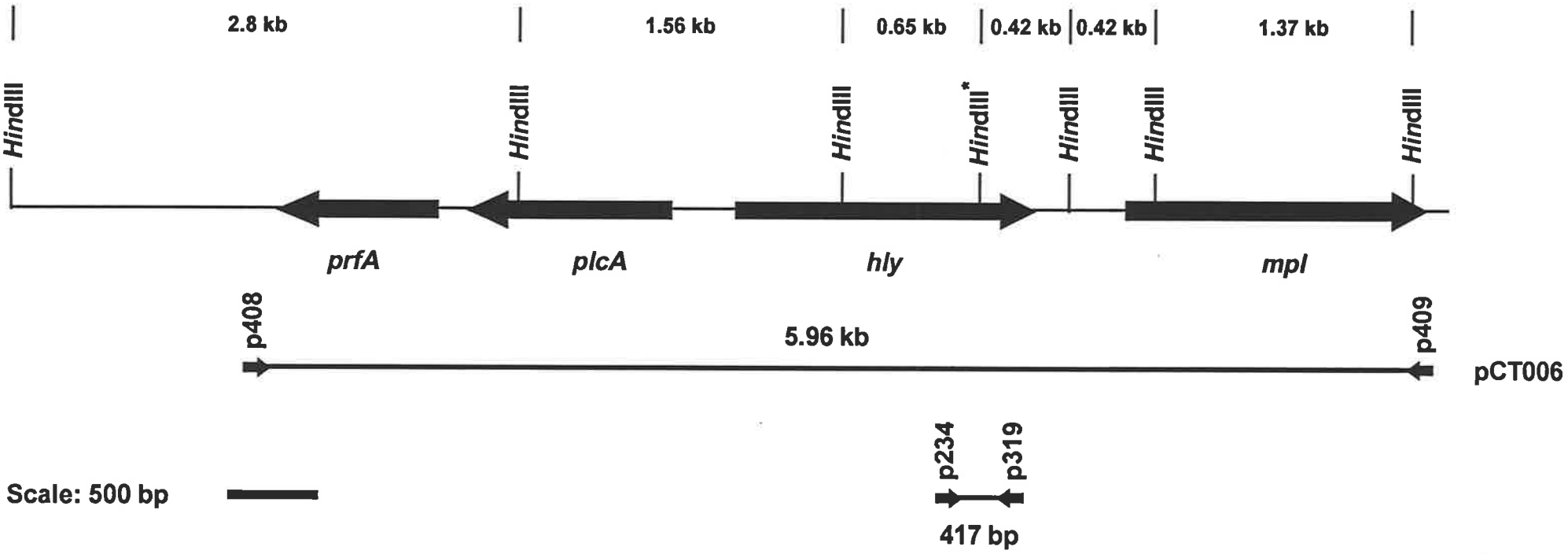

2.8 kb
1.56 kb
0.65 kb
0.42 kb
0.42 kb
1.37 kb
HindIII
HindIII
HindIII
HindIII*
HindIII
HindIII
HindIII
prfA
plcA
hly
mpl
p408
5.96 kb
p409
pCT006
p234
p319
417 bp
Scale: 500 bp

Figure 4.4: PCR analysis of chromosomal DNA isolated from individual colonies of a Em^R *L. monocytogenes* DRDC8/pTV53 electro-transformant using the specific *L. monocytogenes* *hly* synthetic oligonucleotides p234 and p319 (diagrammatically represented in Figure 4.3), using the thermal cycling protocol described by Fitter *et al.*, (1992) which is reproduced in Table 2.5. Lane: [a], non-pathogenic *L. innocua*; [b and c], DRDC8; [d to i], Em^R *L. monocytogenes* DRDC8/pTV53 from three individual isolates; [j and k], 10 ng plasmid pLIS3 [a 4 kb *Bam*HI *hly* clone (Cossart *et al.*, 1989)]. The 417 bp PCR amplified DNA products are indicated by an arrow. Lanes c, e, g, i, and k are *Hin*dIII restricted PCR product, which generates a 162 bp and 255 bp fragment (indicated by arrowheads) as predicted from the nucleotide sequence of the *hly* gene (Mengaud *et al.*, 1988).

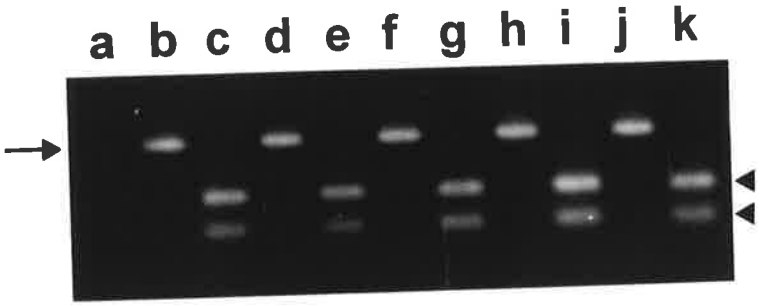
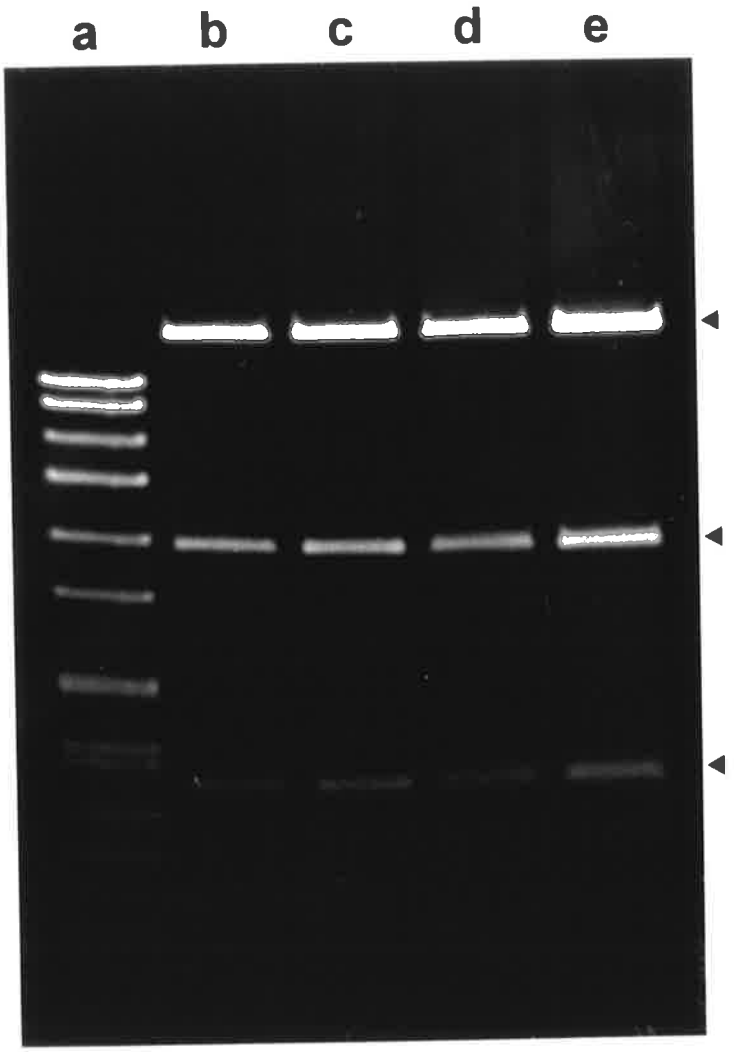

a b c d e f g h i j k

Figure 4.5: Agarose gel electrophoresis of *Hin*dIII digested pTV53 DNA isolated from individual colonies of a Em^R *L. monocytogenes* DRDC8/pTV53 electro-transformant. Lane: [a], *Eco*R1 digested Bacteriophage SPP-1 DNA; [b], *B. megaterium* PV411/pTV53; [c to e], Em^R *L. monocytogenes* DRDC8/pTV53. No difference in the *Hin*dIII restriction pattern of pTV53 DNA isolated from DRDC8 and control plasmid DNA isolated from the parental strain *B. megaterium* PV411/pTV53 was observed. Arrowheads indicate the 12.2 kb, 3.6 kb and 1.1 kb *Hin*dIII restriction fragments of pTV53 (see Figure 4.1).


a
b
c
d
e

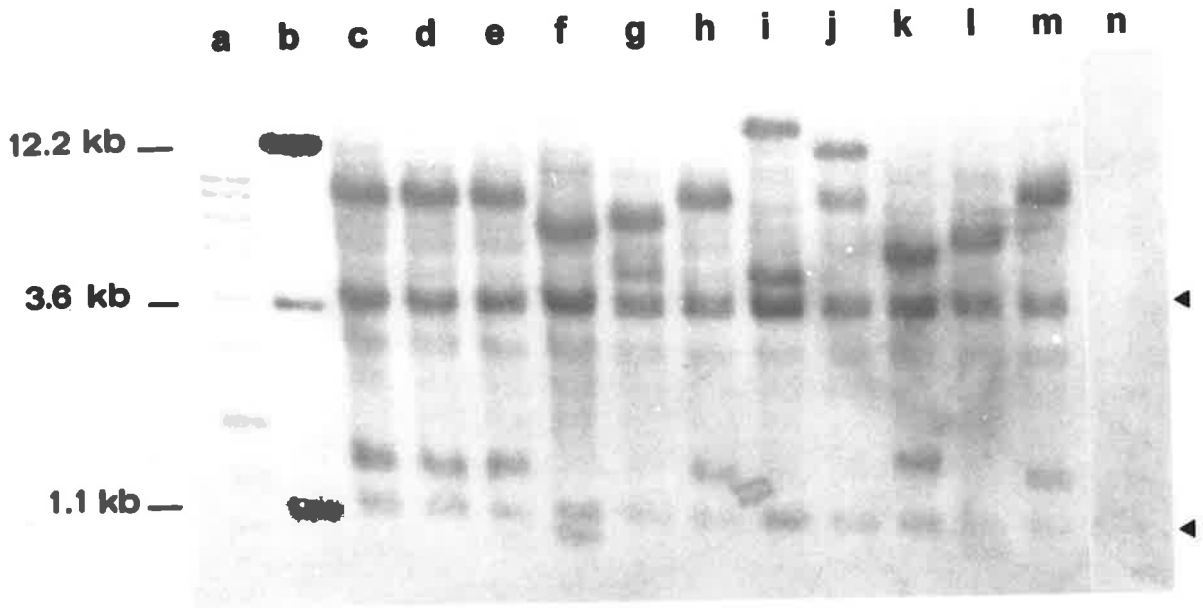
at elevated temperatures (41°C). Therefore, growth of *L. monocytogenes* DRDC8/pTV53 at 41°C in BHI broth containing Em and Lm will select for cells in which transposition of Tn*917-lacZ-cat86* from plasmid pTV53 to DRDC8 DNA has occurred. Small scale experiments performed in 500 ml shake flasks designed to determine the frequency of isolation of Tn*917-lacZ-cat86* transposon insertion into DRDC8, involved a single O/N culture grown at 30°C in BHI broth containing Em, Lm, and Tc, sub-cultured 1:800 in fresh BHI broth (200 ml) with Em and Lm, and grown to stationary phase at 41°C. When grown at the elevated temperature, 31% of Em^R, Lm^R colonies recovered were also Tc^R. This is indicative of stable maintenance of vector DNA or formation of plasmid cointegrates. To compensate for this limitation, two hundred 2 ml aliquots of BHI broth with Em, Lm, and Tc contained in serology tubes, were inoculated with single colonies of *L. monocytogenes* DRDC8/pTV53 and incubated O/N at 30°C. These cultures were used to inoculate fresh BHI broth containing Em and Lm. Cultures were grown to stationary phase at 41°C with aeration, prior to direct inoculation of appropriate dilutions of culture onto BHI agar containing Em and Lm. Using this approach, 90% of Em^R, Lm^R colonies screened were Tc^S, indicating that problems relating to plasmid cointegrate formation or stable maintenance of plasmid DNA was significantly resolved. The isolation of Em^R, Lm^R, and Tc^S colonies provided the reservoir for isolation of promoter::*lacZ* transcriptional fusion mutants in *L. monocytogenes*.

4.2.3 Phenotypic characterisation Tn*917-lacZ-cat86* induced mutants of *L. monocytogenes* DRDC8

Once a transposon mutagenesis library has been generated in a bacterial strain, it is important to establish the frequency and randomness of transposition. Using this information, the utility of the transposon for mutagenesis of the organism of interest can be predicted. This can be achieved by determining the frequency of insertion mutants in alleles with readily identifiable phenotypes, such as, haemolytic activity, dye binding, and auxotrophy.

A preliminary investigation into the randomness of Tn*917-lacZ-cat86* transposition into the *L. monocytogenes* chromosome was assessed by screening 4000 Em^R, Lm^R and Tc^S colonies for insertions resulting in auxotrophies. Thirteen auxotrophs were isolated on minimal medium at an efficiency of 0.33%. One explanation for the low frequency of recovery of auxotrophs concerns the composition of this medium, which is complex with seven amino acids (leucine, isoleucine, valine, methionine, arginine, cysteine, and glutamine) and several vitamins required to support growth of *L. monocytogenes* (Premaratne *et al.*, 1991). Therefore, only mutants

Figure 4.6: Southern hybridisation analysis of *Hin*dIII digested chromosomal DNA preparations from randomly selected Em^R, Lm^R, and Tc^S auxotrophic mutants of *L. monocytogenes* DRDC8 obtained by transposition with Tn*917-lacZ-cat86.* Auxotrophic mutants were selected by their inability to grow on Minimal medium which was previously developed by Premaratne *et al.*, (1991). DNA was probed with digoxigenin labelled *Hin*dIII digested pTV53. Lane: [a], *Eco*RI digested Bacteriophage SPP-1 DNA; [b], plasmid pTV53; [c], A03315; [d], A0448; [e], A1620; [f], A3418; [g], A3816; [h], A4534; [i], A5004; [j], A5101; [k], A5504; [l], A6413; [m], A6835; [n], DRDC8. Probe DNA hybridised with two invariant and two variant DNA fragments from each Em^R, Lm^R, and Tc^S auxotroph mutant, because three *Hin*dIII restriction sites exist within the transposon (see Figure 4.1). Lanes c to e, h, and m possess an identical hybridisation pattern and may represent DNA from daughter cells or a region of transposon "hotspotting". Arrowheads indicate 3.6 kb and 1.1 kb internal *Hin*dIII fragments of Tn*917-lacZ-cat86.* The sizes (kb) of the DNA fragments obtained following *Hin*dIII digestion of pTV53 control DNA are shown.


a b c d e f g h i j k l m n
12.2 kb
3.6 kb
1.1 kb

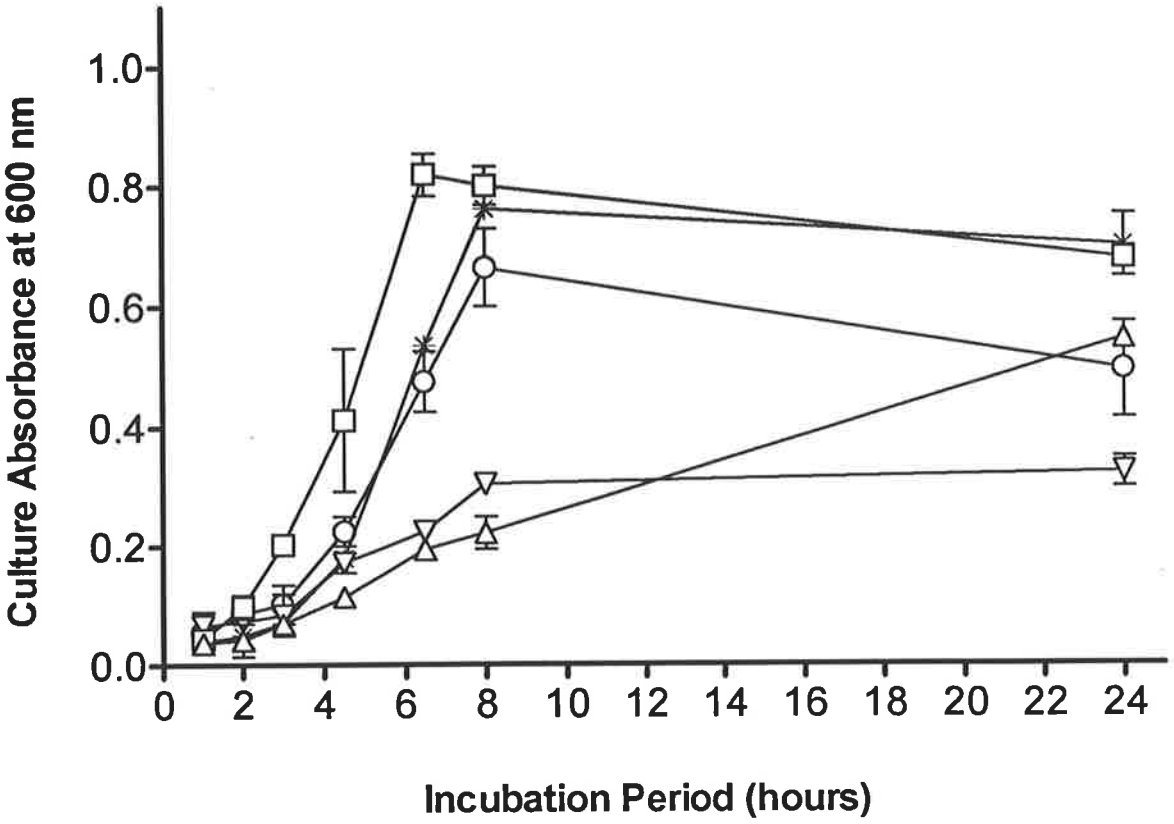


Figure 4.7: Growth of *L. monocytogenes* DRDC8 under iron and calcium stress. The optical density of bacterial cultures was measured at 600 nm over a 24 h incubation at 37°C. Iron and calcium stress were induced by addition of 2',2'-Dipyridyl and EGTA to growth media respectively. Where indicated, 0.5 mM $FeSO_4$ and 4 mM $CaCl_2$ were added to cultures 3 h post inoculation. [□], standard BHI broth; [Δ], BHI broth + 1 mM EGTA; [✳], BHI broth + 1 mM EGTA + 4 mM $CaCl_2$; [▽], BHI broth + 1 mM 2',2'-Dipyridyl; [○], BHI broth + 2',2'-Dipyridyl + 0.5 mM $FeSO_4$. Each graph represents the mean ± SEM of three independent experiments.

with insertions in genes encoding biosynthesis of growth factors which are not represented in the minimal medium, can be identified.

Nevertheless, Southern hybridisation analysis of *Hin*dIII digested chromosomal DNA of eleven randomly selected auxotrophic mutants were probed with DIG labelled pTV53 (Figure 4.6). In all chromosomal samples, four fragments hybridised to the probe DNA, including the conserved 1.1 kb and 3.6 kb fragments that represent internal *Hin*dIII fragments within the transposon (see Figure 4.1). The two variable fragments that hybridised to probe DNA, represents chromosomal DNA flanking the transposon insertion. *Hin*dIII digested pTV53 DNA was included as a positive control. DNA extracted from DRDC8, did not hybridise with probe DNA under the stringency conditions used. Furthermore, 45% (5/11) of the auxotrophic mutants probed contained an identical *Hin*dIII restriction profile (see Figure 4.6, Lanes c, d, e, h, and m). This may suggest that transposition is not random, but rather the transposon has a tendency to insert into particular "hotspot" regions along the *L. monocytogenes* chromosome. However, Southern hybridisation analysis does not preclude the possibility that mutants displaying an identical restriction profile are daughter cells. Characterisation of the auxotrophic phenotype of these mutants was not undertaken in this study, but is necessary before regions of potential transposition "hotspotting" by Tn*917-lacZ-cat86* can be confirmed or otherwise. To this end, isolation of insertion mutants in other loci possessing readily identifiable phenotypes would complement auxotrophy data. Candidate genetic loci include genes involved in haemolytic activity or cell to cell spread in tissue culture monolayers.

4.2.4 Isolation of promoter::*lacZ* transcriptional fusion mutants in *L. monocytogenes* which are responsive to temperature upshift and to changes in iron and calcium levels

4.2.4.1 Growth of *L. monocytogenes* is dependent on trace concentrations of iron and calcium

The trace elements iron and calcium are essential nutritional requirements for bacterial growth. Bacteria may also utilise these elements in regulation of gene expression. To investigate potential roles for these ions as environmental cues for gene expression, their influence on growth of *L. monocytogenes* was determined. *In vitro* growth of *L. monocytogenes* was shown to be dependent on iron and calcium (Figure 4.7). In the presence of the iron chelator 2',2'-Dipyridyl (1 mM) (Williams and Manning, 1991; Poole *et al.*, 1993; Adhikari *et al.*,

1995), or the calcium chelator EGTA (1 mM) (Garduno *et al.*, 1992; Van Leengoed and Dickerson, 1992; Laoudji *et al.*, 1994), cultures of *L. monocytogenes* DRDC8 were significantly restricted compared to cultures grown in standard BHI broth after at least 8 h incubation with aeration at 37°C. Growth was determined by measurement of culture optical density at 600 nm. However, when cultures containing ion chelators were supplemented after 3 h, with 0.5 mM $FeSO_4$ or 4 mM $CaCl_2$ respectively, growth was fully restored. Growth of *L. monocytogenes* SLCC 5764 and the environmental isolate 5708, were similar to DRDC8 in each culture condition used (data not shown).

4.2.4.2 Protein profiles of *L. monocytogenes* grown under conditions of iron and calcium limitation

Since iron and calcium influenced growth of *L. monocytogenes,* the effect of these cations on protein expression by this organism was examined. Protein profiles of whole cell lysates and supernatant extracts from *L. monocytogenes* DRDC8 and SLCC 5764 incubated in the presence of [^{35}S]-Methionine in BHI broth depleted of iron (1 mM 2',2'-Dipyridyl) or calcium (1 mM EGTA), were compared to protein profiles of bacteria grown in standard BHI broth (Figure 4.8). Analysis of protein profiles following SDS-PAGE and autoradiography, identified several proteins in cell lysates prepared from bacteria grown in cation deprived media which showed altered expression compared to proteins extracted from *L. monocytogenes* grown in BHI broth. Few proteins were visualised in the supernatant preparations reflecting a low level of secreted proteins from this bacterium. Nevertheless, this study confirms that iron or calcium limiting conditions alter protein expression by *L. monocytogenes*. Consequently, the effect of iron and calcium depletion, together with other physiological parameters such as growth temperature upshift, carbon dioxide, high osmolarity, and acidity, were used to identify promoters by upregulation of β-galactosidase activity of a bank of Tn*917* induced promoter::*lacZ* transcriptional gene fusion mutants.

4.2.4.3 Qualitative analysis of β-galactosidase activity produced by promoter::*lacZ* transcriptional fusion mutants by hydrolysis of the colorimetric substrate X-gal

The rationale for detecting transcriptional *lacZ* fusions to environmentally regulated *L. monocytogenes* promoters, is outlined in Figure 4.9. This approach relied on the fused native promoter to initiate expression of the *lacZ* reporter gene. The product of the *lacZ* gene, β-galactosidase, is readily detectable when expressed by bacteria during growth on solid media containing X-gal (40 μg/ml). Typical LacZ producing bacteria are readily distinguished from

Figure 4.8: SDS-PAGE analysis and autoradiography of [^{35}S]-Methionine incorporated whole cell and supernatant protein preparations of *L. monocytogenes* grown in BHI broth with iron or calcium limiting conditions induced by addition of 2',2'-Dipyridyl and EGTA to growth media respectively. Lane: [a], DRDC8 (standard BHI broth); [b], DRDC8 (BHI broth + 1 mM 2',2'-Dipyridyl); [c], DRDC8 (BHI broth + 1 mM EGTA); [d], SLCC 5764 (BHI broth + 1 mM 2',2'-Dipyridyl); [e], SLCC 5764 (BHI broth + 1 mM EGTA). Variations in protein expression compared to the control protein profile (Lane a) are indicated by arrowheads. Few extracellular proteins were detected in supernatant preparations, and this may suggest only a limited number of proteins are secreted in detectable concentrations by *L. monocytogenes*. Protein molecular weight markers are indicated by solid bars: Phosphorylase b (94 kDa), Bovine Serum Albumin (67 kDa), Ovalbumin (43 kDa), Carbonic Anhydrase (30 kDa), Soybean Trypsin Inhibitor (20.1 kDa), α-Lactalbumin (14.4 kDa).

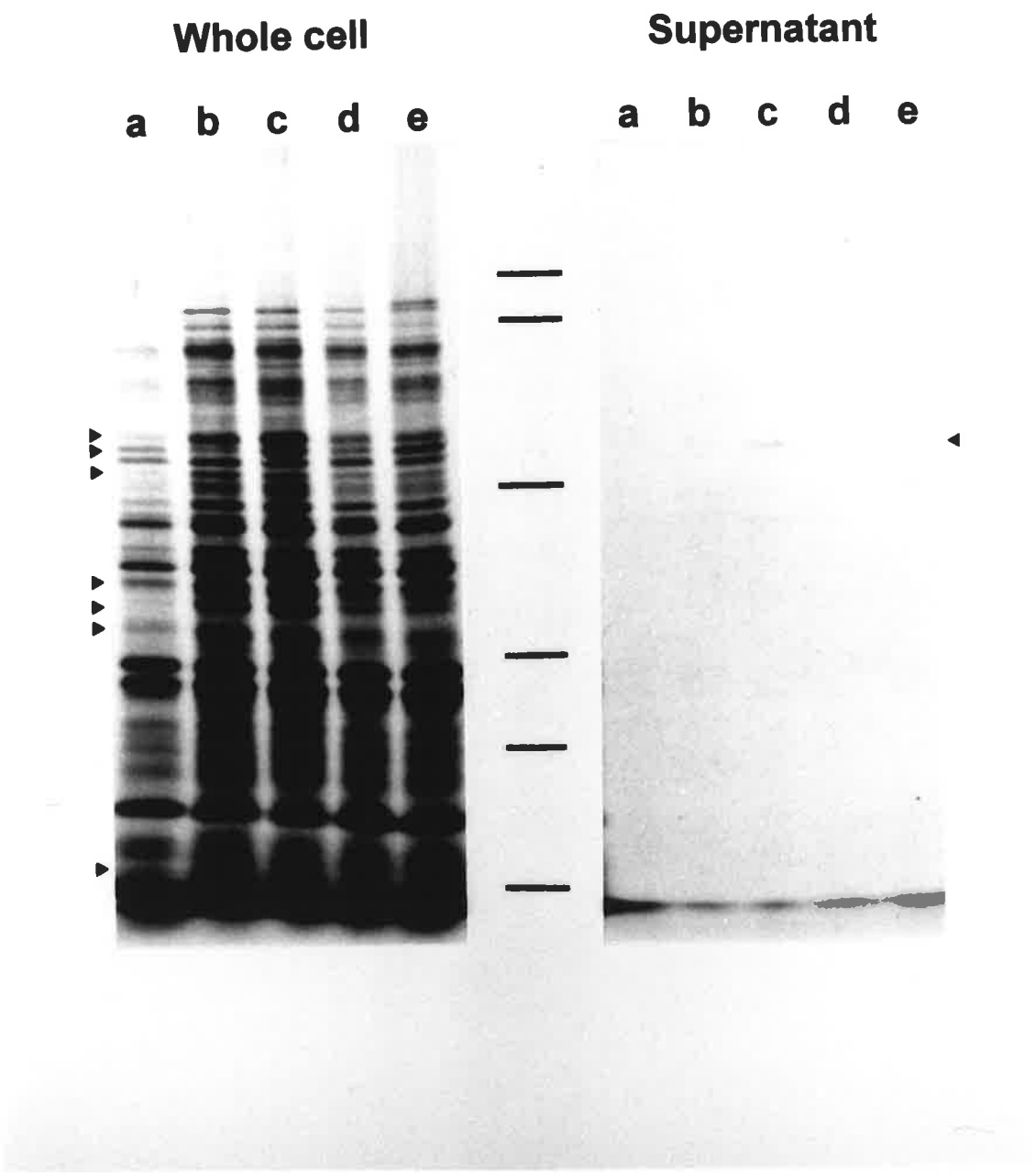
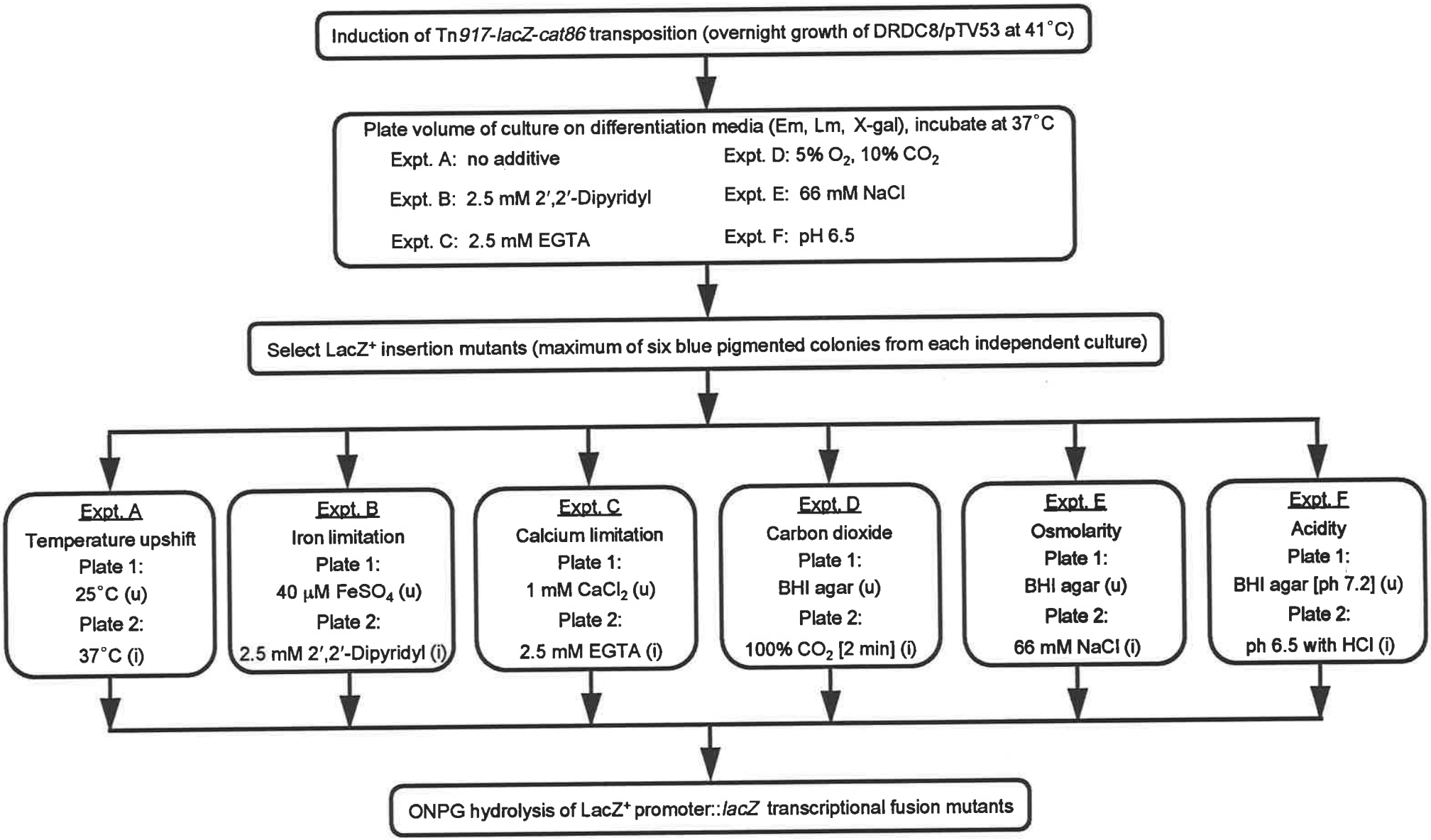

Whole cell
Supernatant
a b c d e
a b c d e

Figure 4.9: A schematic representation of the protocol used for preliminary screening of promoter::*lacZ* transcriptional fusion mutants in *L. monocytogenes* DRDC8. β-galactosidase activity was assessed by the ability to cleave the colorimetric substrate X-gal. In particular, the growth conditions used in this experimental design are described. Basic agar media consisted of Difco BHI with Em (10 μg/ml), Lm (25 μg/ml), and X-gal (40 μg/ml). Unless indicated all cultures were grown at 37°C for at least 36 h. Abbreviations: u, uninduced growth condition; i, induced culture condition.

Note: In Experiment D, conditions of 5% O_2 and 10% CO_2 were established using a Gas Generating Kit (Oxoid Ltd.) with an active catalyst.


Induction of Tn917-lacZ-cat86 transposition (overnight growth of DRDC8/pTV53 at 41°C)
Plate volume of culture on differentiation media (Em, Lm, X-gal), incubate at 37°C
Expt. A: no additive
Expt. B: 2.5 mM 2′,2′-Dipyridyl
Expt. C: 2.5 mM EGTA
Expt. D: 5% O2, 10% CO2
Expt. E: 66 mM NaCl
Expt. F: pH 6.5
Select LacZ+ insertion mutants (maximum of six blue pigmented colonies from each independent culture)
Expt. A
Temperature upshift
Plate 1:
25°C (u)
Plate 2:
37°C (i)
Expt. B
Iron limitation
Plate 1:
40 μM FeSO4 (u)
Plate 2:
2.5 mM 2′,2′-Dipyridyl (i)
Expt. C
Calcium limitation
Plate 1:
1 mM CaCl2 (u)
Plate 2:
2.5 mM EGTA (i)
Expt. D
Carbon dioxide
Plate 1:
BHI agar (u)
Plate 2:
100% CO2 [2 min] (i)
Expt. E
Osmolarity
Plate 1:
BHI agar (u)
Plate 2:
66 mM NaCl (i)
Expt. F
Acidity
Plate 1:
BHI agar [ph 7.2] (u)
Plate 2:
ph 6.5 with HCl (i)
ONPG hydrolysis of LacZ+ promoter::lacZ transcriptional fusion mutants

non-producers, as these colonies form a blue pigmentation. Using this preliminary screening strategy, strains containing promoter::*lacZ* transcriptional fusions were isolated, which responded to temperature upshift, iron limitation, calcium limitation, carbon dioxide, high osmolarity, or low pH, compared to growth in uninduced culture medium. All strains isolated were Em^R, Lm^R, and Tc^S and therefore, did not contain vector DNA.

4.2.4.4 Quantitative analysis of β-galactosidase activity produced by transcriptional promoter::*lacZ* fusion mutants using an ONPG hydrolysis assay

To examine the significance of individual transcriptional promoter::*lacZ* fusion mutants identified by an X-gal based preliminary screening procedure (see Section 4.2.4.3), expression of β-galactosidase in cell extracts of fusion mutants grown in the presence and absence of environmental stimuli was quantitatively determined using a modification of the ONPG hydrolysis assay described by Miller, (1972). The level of ONPG hydrolysis by β-galactosidase produced from a selection of fusion mutants is reported in Table 4.1. The laboratory broth culture conditions used to reproduce *in vivo* environmental cues of temperature upshift, iron limitation, calcium limitation, carbon dioxide, high osmolarity, and increased acidity, are presented as a footnote in Table 4.1. Fusion mutants which exhibited a >1.5 fold increase in β-galactosidase activity compared to the uninduced state were considered significant. A number of mutants, including T3619, expressed β-galactosidase activity at least nine fold greater than the uninduced cell extract. However, in general, the induced β-galactosidase activity observed for most promoter fusions examined, was in the order of two to three fold greater, over uninduced cultures. Using this technique, 100 fusion mutants were isolated in *L. monocytogenes* (summarised in Table 4.2), which identified promoters whose activity is influenced by environmental stimuli.

4.2.5 Preliminary molecular characterisation of transcriptional fusion mutants

To ensure that the Em^R, Lm^R, and Tc^S transcriptional fusion mutants were indeed due to Tn*917-lacZ-cat86* insertions, *Hin*dIII digested, chromosomal DNA preparations of nine randomly selected Em^R, Lm^R, and Tc^S colonies were probed with DIG labelled pTV53 (Figure 4.10). All preparations of DNA contained four fragments that hybridised to probe DNA. Seven of the nine mutants examined apparently contained unique insertions. This suggested the transposable element from pTV53, must have inserted randomly in target DNA. Four fragments were obtained because the transposable element contains three *Hin*dIII restriction endonuclease sites. Two identical fragments from each DNA preparation hybridised

Table 4.1 Expression of β-galactosidase activity in cell extracts from Tn*917-lacZ-cat86* induced fusion mutants.

Strain	Environmental condition	β-Galactosidase activity[a]		Induction ratio[b]
		Uninduced	Induced	
T042	Temperature[c]	22.80 ± 8.75	61.01 ± 10.54	2.68
T069		17.30 ± 9.02	44.63 ± 16.34	2.58
T071		11.26 ± 0.99	42.54 ± 13.98	3.78
T073		17.32 ± 10.58	52.38 ± 20.98	3.02
T087		23.82 ± 3.43	54.04 ± 14.50	2.27
T106		22.01 ± 7.48	42.38 ± 11.82	1.93
T138		42.11 ± 20.52	92.68 ± 2.17	2.20
T145		28.89 ± 4.68	71.31 ± 33.35	2.47
T221		15.52 ± 12.06	40.36 ± 10.25	2.60
T244		16.42 ± 5.01	41.64 ± 11.42	2.54
T288		33.69 ± 9.13	95.47 ± 27.82	2.83
T309		23.32 ± 9.93	59.96 ± 14.05	2.57
T390		68.36 ± 34.54	113.02 ± 50.94	1.65
T1619		9.74 ± 2.27	23.76 ± 5.25	2.44
T3619		0.21 ± 0.10	1.88 ± 0.72	8.95
I055	Iron stress[d]	4.16 ± 0.85	7.45 ± 2.55	1.79
I249		34.01 ± 9.10	82.17 ± 12.09	2.42
I259		24.83 ± 2.02	59.98 ± 15.45	2.42
C044	Calcium Stress[e]	2.88 ± 0.32	5.21 ± 1.40	1.81
C059		1.32 ± 1.19	2.51 ± 1.3	1.90
C185		2.33 ± 0.59	6.08 ± 1.36	2.61
CD90	Carbon dioxide[f]	39.55 ± 1.85	65.99 ± 7.64	1.67
CD95		42.69 ± 4.17	72.32 ± 4.56	1.69
nd	Osmolarity[g]	-	-	-
nd	Acidity[h]	-	-	-

[a] β-Galactosidase activity was assessed by hydrolysis of ONPG expressed as mean Miller units ± SEM from at least three independent experiments

[b] Induction ratio expressed as induced/uninduced

[c] Strains grown in BHI broth at 25°C (uninduced) or 37°C (induced)

[d] Strains grown in BHI broth containing 40 μM $FeSO_4$ (uninduced) or 2.5 mM 2',2'-Dipyridyl (induced)

[e] Strains grown in BHI broth containing 1 mM $CaCl_2$ (uninduced) or 2.5 mM EGTA (induced)

[f] Strains grown in BHI broth (uninduced) or BHI broth aerated with 100% gaseous CO_2 for 2 min and sealed with parafilm wrap (induced)

[g] Strains grown in BHI broth (uninduced) or BHI broth containing 66 mM NaCl (induced)

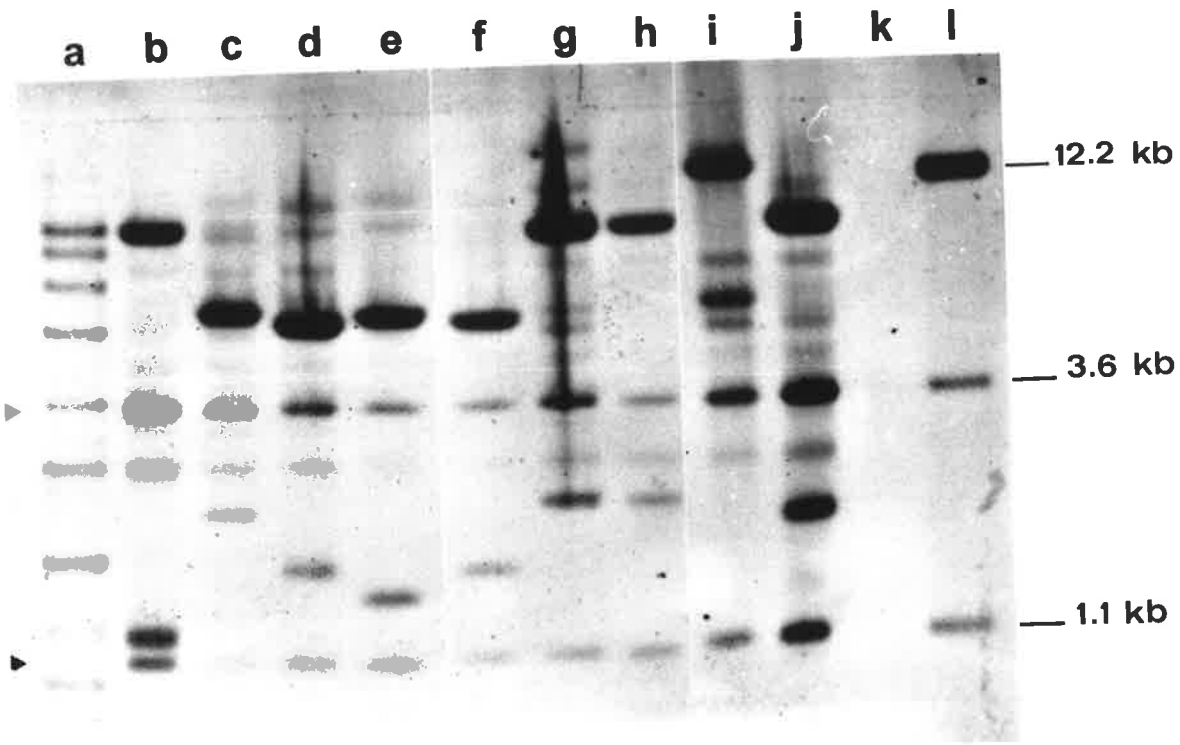
[h] Strains grown in BHI broth (pH 7.2, uninduced) or BHI broth [pH 6.5, adjusted with concentrated HCl (induced)]

nd No transcriptional fusion mutants detected

Table 4.2 Environmentally influenced transcriptional promoter::*lacZ* fusion mutants in *L. monocytogenes* DRDC8, obtained from insertion of Tn*917-lacZ-cat86*.

Environmental signal	Number of transcriptional promoter::*lacZ* fusion mutants isolated
Temperature	84
Iron	8
Calcium	6
Carbon dioxide	2
Osmolarity	0
pH	0

Figure 4.10: Southern hybridisation analysis of *Hin*dIII digested chromosomal DNA preparations of randomly selected Em^R, Lm^R, and Tc^S promoter::*lacZ* transcriptional fusion mutants of *L. monocytogenes* DRDC8 obtained by Tn*917-lacZ-cat86* mutagenesis. DNA was probed with digoxigenin labelled *Hin*dIII digested pTV53. Lane: [a], *Eco*RI digested Bacteriophage SPP-1 DNA; [b], C044; [c], C185; [d], I055; [e], I249; [f], T042; [g], T069; [h], T221; [i], T1619; [j], T3619; [k], DRDC8; [l], plasmid pTV53. Probe DNA hybridised with two invariant and two variant DNA fragments from each Em^R, Lm^R, and Tc^S fusion mutant, because three *Hin*dIII restriction sites exist within the transposon (see Figure 4.1). Arrowheads indicate 3.6 kb and 1.1 kb internal *Hin*dIII fragments of Tn*917-lacZ-cat86.* The sizes (kb) of the DNA fragments obtained following *Hin*dIII digestion of pTV53 control DNA are shown.

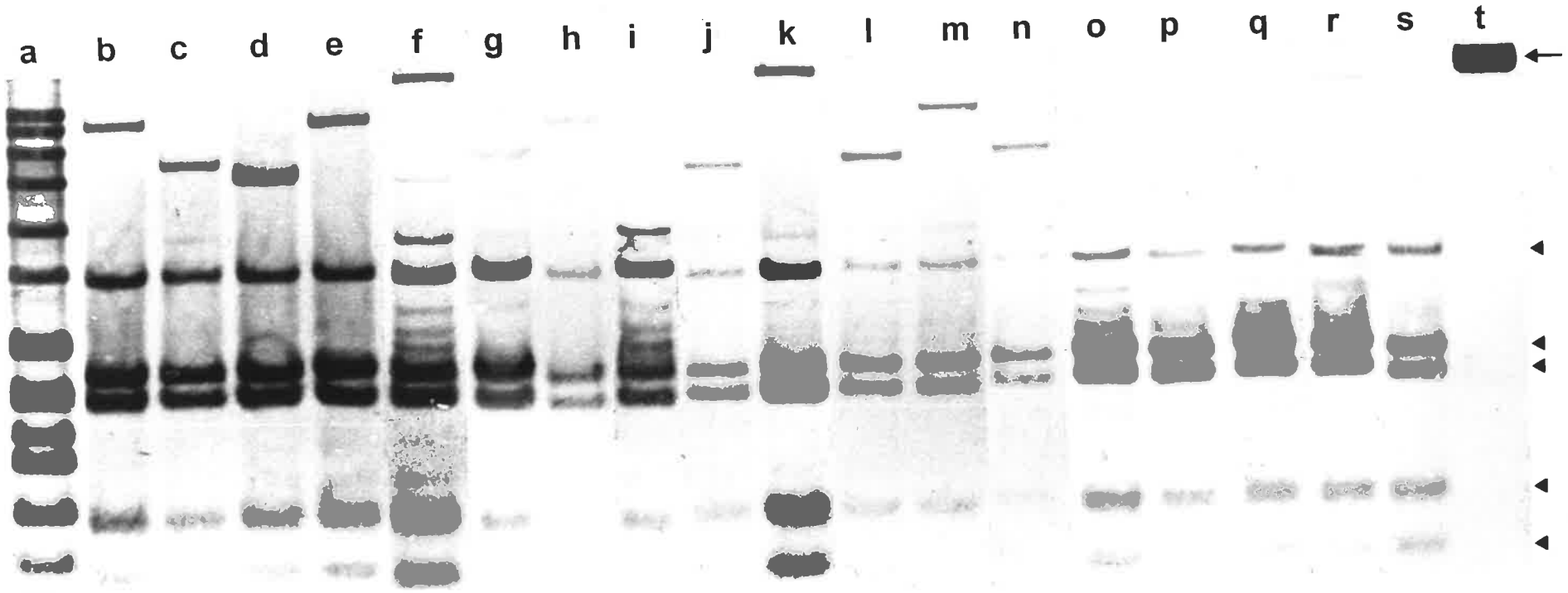

a
b
c
d
e
f
g
h
i
j
k
l
12.2 kb
3.6 kb
1.1 kb

to probe DNA corresponding to the internal 3.6 kb and 1.1 kb *Hin*dIII restriction fragments from pTV53, located within Tn*917-lacZ-cat86* (see Figure 4.1). The two variable fragments that hybridise to probe DNA, represent chromosomal DNA flanking the transposon insertion. *Hin*dIII digested pTV53 DNA was included as a positive control. DNA extracted from DRDC8, did not hybridise with probe DNA under the stringency conditions used.

To avoid selection of previously characterised virulence determinants of *L. monocytogenes* (reviewed by Portnoy *et al.*, 1992a; Sheehan *et al.*, 1994), fusion mutants were screened by Southern hybridisation analysis, for transposon insertions within the 5.96 kb virulence region containing *prfA, plcA, hly,* and *mpl.* Within this region of chromosomal DNA, a previous report identified a characteristic restriction fragment length polymorphism based on *Hin*dIII digestion of *L. monocytogenes* DRDC8 DNA (Thomas, 1995) (schematically shown in Figure 4.3). The 5.96 kb PCR amplified DNA fragment (using the synthetic oligonucleotides p408 and p409) cloned into pBluescript KS to give rise to pCT006, is also indicated. If Tn*917-lacZ-cat86* transposed into this region, a change in the *Hin*dIII restriction pattern of mutant strains would result. These alterations could then be detected by Southern hybridisation using plasmid pCT006 as probe DNA. Thus, the DNA restriction profile of *Hin*dIII digested chromosomal DNA isolated from wild type DRDC8 and promoter::*lacZ* fusion mutants, were compared by Southern hybridisation with DIG labelled *Hin*dIII restricted pCT006 as probe DNA. Probe DNA hybridised to six *Hin*dIII restriction fragments from DRDC8 chromosomal DNA [0.42 kb (doublet), 0.65 kb, 1.37 kb, 1.56 kb, and 2.8 kb] (Figure 4.11, Lane s), consistent with a previous report (Thomas, 1995). Significantly, all *Hin*dIII restriction profiles from at least 40 fusion mutants screened (a random selection are shown in Figure 4.11), were identical to the *Hin*dIII restriction profile of wild type DRDC8. This confirms that all transposon insertions described in this study were independent of loci established as virulence determinants of *L. monocytogenes.*

The 12.2 kb *Hin*dIII DNA fragment from the control plasmid pTV53 which hybridised to pCT006 DNA, is representative of the homology between *lacZ* DNA present in Tn*917-lacZ-cat86* and a portion of *lacZ* on the pBluescript KS cloning vector involved in α-complementation for blue/white colour selection of DNA recombinants. Interestingly, homology to probe DNA was also observed in DNA from each fusion mutant, represented by hybridisation of probe DNA to a large variable *Hin*dIII DNA fragment not observed in DRDC8. It is likely these *Hin*dIII restriction fragments harbour the promoterless *lacZ* gene

Figure 4.11: Southern hybridisation analysis of *Hin*dIII digested chromosomal DNA preparations of randomly selected Em^R, Lm^R, and Tc^S promoter::*lacZ* fusion mutants of *L. monocytogenes* DRDC8 obtained by Tn*917-lacZ-cat86* mutagenesis. DNA was probed with digoxigenin labelled *Hin*dIII digested pCT006. Lane: [a], *Eco*RI digested Bacteriophage SPP-1 DNA; [b to r], randomly selected transcriptional promoter::*lacZ* fusion mutants; [s], DRDC8; [t], plasmid pTV53. Probe DNA hybridised to six identical *Hin*dIII DNA fragments isolated from both fusion mutants and control strain DRDC8. This suggests Tn*917-lacZ-cat86* has not inserted into the virulence region of DRDC8. The six identical fragments in each lane are indicated by arrowheads [0.42 kb (doublet), 0.65 kb, 1.37 kb , 1.56 kb, and 2.8 kb *Hin*dIII fragments] (see Thomas, 1995 and Section 4.2.1.3, Figure 4.3). The arrow indicates the 12.2 kb *Hin*dIII fragment from pTV53 DNA, containing the promoterless *lacZ* gene (see Figure 4.1).


a
b
c
d
e
f
g
h
i
j
k
l
m
n
o
p
q
r
s
t

from Tn*917-lacZ-cat86*. The variable size of the *Hin*dIII restriction fragments is suggestive of a relatively random transposition by Tn*917-lacZ-cat86* into DNA of *L. monocytogenes* DRDC8.

4.3 Discussion

The primary goal of this study was to introduce, into *L. monocytogenes,* a mutagenesis vector which contains a Tn*917* derivative that would enable isolation of promoter::*lacZ* transcriptional fusions in this organism. A feature of these mutagenesis vectors is their unwieldy size which does reduce the efficiency of DNA transfer into bacteria. To overcome this limitation, several methods were employed to introduce vector DNA into *L. monocytogenes* DRDC8.

In my hands at least, protoplast transformation and conjugation of mutagenesis vectors into *L. monocytogenes* was not successful. Preparation of protoplasts is routinely difficult, and is dependent on a number of parameters, including resistance of the organism to lysozyme (Luchansky *et al.*, 1988). In particular, it was likely that insufficient protoplast formation lead to the failure of this method. On the other hand, although preliminary experiments were able to demonstrate efficient conjugal transfer of the *E. coli/L. monocytogenes* shuttle vector pAT19, from *E. coli* S17-1 to *L. monocytogenes*, attempts to transfer the 20.6 kb Tn*917* mutagenesis vector (pLTV1) containing *mob,* into *L. monocytogenes,* were also unsuccessful. One possible reason for this failure may have involved the consistent modification and/or rearrangement of pLTV1 DNA in *E. coli* S17-1. However, it is likely a similar approach to transformation of DNA into *L. monocytogenes* will prove successful in future studies, so long as a suitable donor *E. coli* strain, deficient in the host restriction/modification system, is available.

In view of these difficulties, electroporation was used as a means to transfer plasmid DNA into *L. monocytogenes* DRDC8. Several protocols have been reported that optimised DNA transfer by electroporation into *L. monocytogenes* and other Gram-positive bacteria (Alexander *et al.*, 1990; Park and Stewart, 1990; Dunny *et al.*, 1991) These researchers claimed this approach provided a rapid, reliable, and efficient method for the introduction of plasmid DNA into bacteria. In this study, the Gram-positive shuttle vector pAT19 was used as a test vector to optimise electrotransformation of DRDC8. Results of this study indicated a requirement for high concentrations of DNA, before transformants were recovered, albeit at a

very low frequency of transformation. In contrast, Alexander *et al.*, (1990), reported frequencies of transformation using an equivalent sized plasmid, that were at least 10^4-fold higher than frequencies achieved in this study. These differences, may reflect variations in the susceptibility of the two *L. monocytogenes* strains used in the two independent studies to electroporation (P.W. Andrew, personal communication)[1]. In the same way, variations have been observed in a comparison of electroporation efficiencies between different *Listeria* spp. (Alexander *et al.*, 1990). Moreover, the nature of the cell wall may also contribute to the relative inefficiency of electroporation of Gram-positive organisms, since efficiencies can be improved by growth of bacteria in sub-inhibitory concentrations of penicillin prior to electroporation (Park and Stewart, 1990). Nevertheless, using a modification of the method described by Alexander *et al.*, (1990), a single Em^R, Lm^R, and Tc^R *L. monocytogenes* DRDC8/pTV53 transformant was isolated following electroporation of 10 μg of plasmid DNA into DRDC8, albeit at a unacceptably low efficiency of transformation.

The Tn*917* derived mutagenesis vector, pTV53 (Youngman *et al.*, 1985a), used to electrotransform DRDC8, contains a promoterless *lacZ* and *cat86* gene suitable for the construction of transcriptional promoter::reporter gene fusions in bacteria. Tn*917* derivatives have been used in a wide range of Gram-positive organisms, such as *L. monocytogenes* (Camilli *et al.*, 1990; Mengaud *et al.*, 1991a), *Bacillus* spp. (Love *et al.*, 1985; Atkinson *et al.*, 1990; Tao and Vary, 1991), and *Lactococcus lactis* (Israelsen and Hansen, 1993). These reports indicate that the frequency of transposition is high, which is in good agreement with the transposition frequency observed in this study using *L. monocytogenes* DRDC8. However, in my hands at least, it was necessary to induce at least 200 independent cultures of DRDC8/pTV53 at the restrictive temperature (41°C), before strains were cured of vector DNA. This was a problem in preliminary induction experiments which initially resulted in a high recovery of Tc^S strains. One explanation for this is if the temperature sensitive origin of replication (pE194rep) is not absolutely restricted at 41°C, allowing maintenance of the vector. Interestingly, a mutant form of pE194 is available, referred to as pE194Ts (cited in Youngman, 1987). This replicon is reported to be significantly more temperature sensitive reducing the incidence of stable plasmid maintenance. For this reason it has been incorporated into recent mutagenesis vectors including pLTV1 and pLTV3 (Camilli *et al.*, 1990).

[1] P.W. Andrew, Department of Microbiology and Immunology, University of Leicester, Leicester, United Kingdom

Although Tn*917* is considered to integrate randomly into the bacterial chromosome, "hotspotting" has been reported (Youngman *et al.*, 1984; Camilli *et al.*, 1990; Israelsen and Hansen, 1993). In particular, Camilli *et al.*, (1990), identified a putative hotspot region in genes associated with adenosine biosynthesis in *L. monocytogenes*. Furthermore, data from Southern hybridisation analysis of chromosomal DNA from a random selection of auxotrophic mutants probed with the mutagenesis vector pTV53, revealed over 45% of mutants contained an identical *Hin*dIII restriction fragment profile (see Section 4.2.3). This is particularly striking given wild type *L. monocytogenes* require no less than 13 chemical supplements for growth on synthetic minimal medium (Premaratne *et al.*, 1991). Consequently, Tn*917-lacZ-cat86* induced auxotrophy may be restricted to insertions in a limited number of essential biosynthetic operons in *L. monocytogenes*. Alternatively, the complex composition of the minimal medium would bias selection of certain auxotrophs. In addition, Southern hybridisation of insertion mutants is unable to define whether strains which yield an identical *Hin*dIII restriction fragment profile result from "hotspotting" or are daughter strains. To resolve this issue, insertion mutants need to be characterised at the molecular level to identify the site of transposon insertion. To further confirm transposon "hotspotting" or otherwise, investigation of the frequency of insertion of Tn*917-lacZ-cat86* in genetic loci responsible for haemolytic activity (*hly*) and cell to cell spread in tissue culture monolayers (lecithinase operon) should be undertaken. However, this aspect of work is peripheral to the scope of this project. Nevertheless, using the mutagenesis vector pTV53, a Tn*917-lacZ-cat86* insertion library was successfully generated in DRDC8 for use in the isolation of transcriptional fusions to environmentally regulated promoters.

Trace elements such as iron and calcium are essential nutritional requirements for growth of all life forms. Thus, upon infection, pathogenic bacteria have developed scavenging mechanisms to compete for all available ion sources. Furthermore, bacteria utilise this environmental change as a cue to regulate expression of virulence determinants as a prerequisite to establishment of infection. In this study, growth of *L. monocytogenes* in media deprived of iron and calcium was significantly restricted. Cowart, (1987), also observed a dependency of *L. monocytogenes* growth *in vitro* on iron concentration. Additional reports describe the growth limiting effect on *L. monocytogenes* of other environmental factors including temperature, sodium chloride, and acidity (McClure *et al.*, 1989; Farber *et al.*, 1989). Furthermore, SDS PAGE analysis of cell lysates from *L. monocytogenes* DRDC8 and SLCC 5764, indicated a significant number of proteins were expressed in response to growth

in iron or calcium limiting conditions (see Section 4.2.4.2). It is anticipated that a portion of these proteins may contribute to virulence of *L. monocytogenes.* Many reports have indicated that growth conditions including temperature, sodium chloride, potassium chloride, iron, pH, and heat shock enhanced the virulence of *L. monocytogenes* in experimentally infected mice (Sword, 1966; Cowart and Foster, 1981; Czuprynski *et al.*, 1989; Sokolovic and Goebel, 1989; Stephens *et al.*, 1991; Park *et al.*, 1992; Khan *et al.*, 1993; Morange *et al.*, 1993; Myers *et al.*, 1993). One likely explanation for this phenomena is that particular growth conditions stimulate expression of virulence determinants in this bacterium. This preliminary investigation, provided the opportunity to use iron and calcium limitation, together with temperature, carbon dioxide, osmolarity, and acidity as environmental cues to screen a library of Tn*917-lacZ-cat86* fusion mutants in *L. monocytogenes* DRDC8, for resident promoters whose activity is regulated by these physiologically significant signals.

The role of Ca^{2+} in the biology of living cells has attracted widespread attention among researchers. In both eukaryotic and prokaryotic systems, calcium dependency for cell growth and division has been described (Aranha *et al.*, 1986; Onoda and Oshima, 1988; Norris *et al.*, 1991; and references therein). Furthermore, calcium has been implicated in various assorted functions in bacteria including chemotaxis (Ordal, 1977), virulence (Brubaker, 1983; Ludwig *et al.*, 1988; Gygi *et al.*, 1992; Van Leengoed and Dickerson, 1992), transport of sugars and proteins (Bradbeer *et al.*, 1986), and stability of the envelope (Schindler and Osborn, 1979; Nikaido and Vaara, 1987). EGTA has routinely been used as a chelator of free Ca^{2+} ions to assess the function of Ca^{2+} dependent bacterial processes (Garduno *et al.*, 1992; Gygi *et al.*, 1992; Van Leengoed and Dickerson, 1992; Laoudji *et al.*, 1994; and references cited in Norris *et al.*, 1991), and was used in this study to establish a calcium depleted environment. However, high concentrations of EGTA is capable of binding other essential trace elements (Youatt, 1993).

Similarly, iron is an essential nutritional requirement for growth of most bacteria (Neilands, 1981). Bacteria have developed several iron dependent mechanisms for sequestering this trace element from the environment (reviewed by Crosa, 1989; Otto *et al.* 1992; Payne, 1993). The chelating agent 2′,2′-Dipyridyl has been routinely used to establish an iron limiting environment for the *in vitro* analysis of growth and regulation of protein expression by bacteria (Postle, 1990; Van Hove *et al.*, 1990; Williams and Manning, 1991; Angerer *et al.*, 1992; Dai *et al.*,

1992; Poole *et al.*, 1993; Adhikari *et al.*, 1995). Therefore, 2′,2′-Dipyridyl was employed in this study to prepare iron limiting medium for growth of *L. monocytogenes.*

A preliminary screening procedure using the substrate X-gal to identify β-galactosidase activity, enabled the isolation of promoter::*lacZ* transcriptional fusion mutants that exhibited increased β-galactosidase activity during growth under environmental stimulus. Furthermore, all fusion mutants were Em^R, Lm^R, and Tc^S, which indicated the mutagenesis vehicle was not maintained by this organism. To examine the significance of promoter::*lacZ* transcriptional fusion mutants, a quantitative analysis of β-galactosidase activity of individual transcriptional fusion mutants was performed by a modification of the ONPG hydrolysis assay previously described (Miller, 1972). This assay identified 100 insertion mutants which contained a *lacZ* fusion to promoters whose activity was influenced by either temperature upshift, iron limitation, calcium limitation, or carbon dioxide. Significantly more promoter fusions were isolated displaying an increase in activity in response to temperature upshift. This is not suprising given the role of temperature in the global regulation of protein expression (Maurelli, 1989).

In addition, Southern analysis of chromosomal DNA from representative Em^R, Lm^R, and Tc^S fusion mutants probed with DIG labelled pTV53, indicated that in each case, resistance was due to a single transposon insertion. Moreover, chromosomal DNA of mutant strains probed with DIG labelled pCT006, exhibited an identical *Hin*dIII restriction fragment profile to wild type DRDC8. This suggested that Tn*917-lacZ-cat86* did not insert into the virulence cassette of *L. monocytogenes*, and avoided the potential of identifying a previously characterised virulence determinant of this organism. Together, this information means that selection of the most interesting fusion mutants based on levels of β-galactosidase expression when grown under inducing conditions, should allow the isolation of novel genes from *L. monocytogenes.* In particular, the following chapters describe the molecular and functional characterisation of an EGTA induced transcriptional fusion mutant, *L. monocytogenes* C185.

Chapter 5 Isolation of *ctpA*, an EGTA induced stress associated gene from *Listeria monocytogenes* encoding a putative P-type ATPase involved in copper transport

5.1 Introduction

Transposon Tn*917*, is a useful tool for construction of insertion mutants in Gram-positive bacteria. This has lead to the development of a series of Tn*917* delivery vectors which utilise the pE194 derived temperature sensitive replicon first described by Iordanescu, (1976). These vectors have been routinely used to analyse the genetics of Gram-positive organisms, including genes involved in sporulation from *Bacillus* spp. (Zuber and Losick, 1983; Youngman *et al.*, 1985b; Tao and Vary, 1991). Recent reviews which describe these studies, discuss the generation of transcriptional fusions to promoterless reporter genes (*lacZ* and *cat86*) mediated by Tn*917* (Youngman *et al.*, 1984; Youngman *et al.*, 1985a; Youngman, 1987; Youngman *et al.*, 1989).

In this study, the 16.9 kb mutagenesis vector pTV53 (Youngman *et al.*, 1985a) was used to establish a Tn*917* insertion library in *L. monocytogenes*. Unlike other Tn*917* derivatives, pTV53 does not contain the pBR322 replicon. Even though this may make recovery of *L. monocytogenes* chromosomal DNA flanking the transposon difficult, this vector has the advantage of being considerably smaller then pLTV1 (20.6 kb) and pLTV3 (22.1 kb). The comparatively small size of pTV53 facilitated electroporation into *L. monocytogenes* DRDC8, an environmental isolate. A promoter::*lacZ* transcriptional fusion library was established and enabled the isolation of insertion mutants in genes that were under the regulatory control of environmental stimuli including temperature upshift, iron limitation, calcium limitation, and carbon dioxide. Construction of promoter::*lacZ* fusions mediated by Tn*917* derivatives has previously demonstrated the utility of this mutagenesis system for isolation of genes influenced by environmental cues including stationary phase (Tao and Vary, 1991), amino acid availability (Atkinson *et al.*, 1990), nitrogen limitation (Atkinson and Fisher, 1991), and DNA damage (Cheo *et al.*, 1991).

A method for rapid isolation of *L. monocytogenes* chromosomal DNA flanking the Tn*917-lacZ-cat86* insertions isolated in this study was not available. In order to overcome this

limitation, a cloning strategy was developed to efficiently allow the isolation of DNA flanking transposon insertions. This chapter describes the approach used to characterise DNA flanking a promoter::*lacZ* transcriptional fusion mutation contained in *L. monocytogenes* C185. Based on this preliminary analysis, isolation of *ctpA*, an EGTA induced stress associated gene from *L. monocytogenes* is described. CtpA has significant amino acid identity to bacterial P-type adenosine triphosphatases involved in copper transport. Preliminary analysis of *ctpA* expression suggested that copper is involved in the regulation of this gene. Furthermore, nucleotide sequence analysis of DNA flanking *ctpA*, and temperature induced over-expression of CtpA in *E. coli* was used to confirm the coding region of this gene.

5.2 Results

5.2.1 Cloning DNA flanking the Tn*917-lacZ-cat86* transposon insertions in transcriptional promoter::*lacZ* fusion mutants from *L. monocytogenes*

Cosmid or plasmid library construction are routinely used to recover DNA flanking sites of transposon insertion in a variety of bacteria. However, when applied to cloning *L. monocytogenes* DRDC8 chromosomal DNA, these methods were unsuccessful. In view of this, it was unlikely a similar approach would permit the isolation of DNA flanking Tn*917-lacZ-cat86* insertions in the DRDC8 chromosome. To facilitate cloning of DNA flanking transposon insertions from selected *L. monocytogenes* fusions, a novel strategy was designed (see Figure 5.1). The aim of this strategy involved isolation of the promoterless *lacZ* gene in association with chromosomal DNA located immediately upstream of the left inverted repeat (LIR) region of Tn*917-lacZ-cat86* from transcriptional fusion mutants on a *Bam*HI fragment. Chromosomal DNA isolated from insertion mutants was partially digested with *Bam*HI to achieve large DNA fragments prior to purification from agarose gels. These DNA fragments were cloned into the *Bam*HI site of pBR322 and transformed into Lac$^-$ *E. coli* CC118. Positive clones were identified during growth on media containing X-gal (40 μg/ml), by their ability to produce β-galactosidase. Expression of the promoterless *lacZ* gene in these constructs was dependent on the presence of a functional promoter upstream of *lacZ*. Either the tetracycline resistance promoter contained on pBR322 or a promoter located on cloned chromosomal DNA flanking the LIR region of Tn*917-lacZ-cat86*, was responsible for expression of the promoterless *lacZ* gene in positive clones.

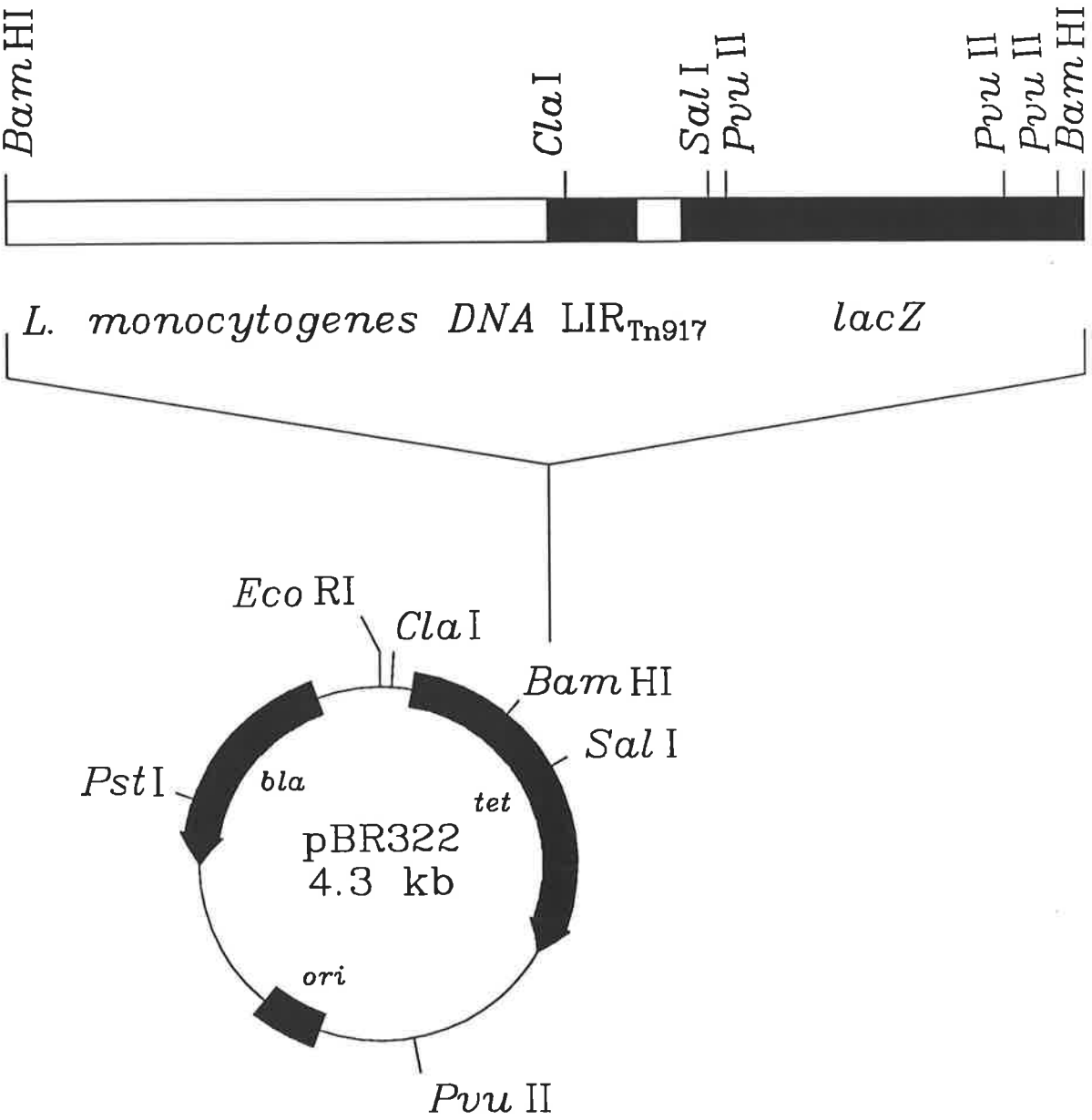


Figure 5.1: Schematic representation of the strategy used to clone *Bam*HI chromosomal DNA fragments containing the functional promoterless *lacZ* gene from Tn*917-lacZ-cat86* transcriptional fusion mutants of *L. monocytogenes.* The low copy number vector, pBR322 was used as the cloning vehicle. Selected transformants containing *L. monocytogenes* chromosomal DNA displayed a Lac^{+}, Ap^{R}, Tc^{S} phenotype. Abbreviations: LIR_{Tn917}, left inverted repeat region from Tn*917-lacZ-cat86*; *lacZ,* β-galactosidase gene from Tn*917-lacZ-cat86*; *bla,* ampicillin resistance cartridge; *tet,* tetracycline resistance cartridge; *ori,* pBR322 origin of replication

Using this approach, six *Bam*HI clones derived from four independent fusion mutants induced by temperature upshift (T390 and T3619), and cation limitation (C044 and C185) were isolated. Each conferred a $LacZ^+$, Ap^R and Tc^S phenotype to *E. coli* CC118 (Table 5.1). Other Ap^R and Tc^S transformants were also obtained which did not express β-galactosidase. This population apparently harboured pBR322, but it was likely the cloned *Bam*HI restricted *L. monocytogenes* chromosomal DNA fragments did not contain the *lacZ* gene. Nevertheless, plasmid pCT200, which contains chromosomal DNA from the transcriptional fusion mutant *L. monocytogenes* C185, was selected for detailed molecular characterisation. The findings of this analysis are presented in the proceeding sections.

5.2.2 Preliminary characterisation of pCT200 and cloning of the fusion joint between Tn*917-lacZ-cat86* and *L. monocytogenes* chromosomal DNA

The first step in characterising the insertion mutant in *L. monocytogenes* C185, involved identification of the fusion joint between Tn*917-lacZ-cat86* and *L. monocytogenes* chromosomal DNA from the ~27.8 kb *Bam*HI DNA fragment contained on plasmid pCT200. This was achieved by Southern hybridisation analysis of *Pvu*II digested plasmid pCT200 and chromosomal DNA isolated from *L. monocytogenes* C185, probed with a 663 bp *Cla*I/*Pvu*II LIR/*lacZ* DNA fragment isolated from Tn*917-lacZ-cat86*. *Pvu*II was chosen to restrict the sample DNA because *lacZ* contains a unique *Pvu*II recognition sequence (Fowler and Zabin, 1978; Kalnins *et al.*, 1983). Probe DNA hybridised with a 2.8 kb *Pvu*II DNA fragment from pCT200 and *L. monocytogenes* C185 (Figure 5.2). Purified pBR322 and chromosomal DNA extracted from DRDC8, did not hybridise with probe DNA. The 2.8 kb *Pvu*II DNA fragment from pCT200 was agarose gel purified, and cloned into *Pvu*II digested pBR322 to yield pCT201. The spatial arrangement of plasmids pCT200 and pCT201, and the location of the *lacZ* DNA probe used to identify the fusion joint between Tn*917-lacZ-cat86* and *L. monocytogenes* chromosomal DNA is presented in Figure 5.3.

To confirm plasmid pCT201 contained the LIR region of Tn*917-lacZ-cat86*, a synthetic oligonucleotide (p740) complementary to the LIR region of Tn*917* (diagrammatically represented in Figure 5.3), was used in a dye-terminator sequence reaction with pCT201 template DNA. Analysis of nucleotide sequence identified the LIR region of Tn*917-lacZ-cat86* and the fusion junction in the *L. monocytogenes* chromosome (Figure 5.4). This data, in combination with preliminary observations which included identification of a $LacZ^+$ phenotype by *E. coli* CC118/pCT200 (see Table 5.1) and *lacZ* DNA on plasmid

Figure 5.2. Southern hybridisation analysis of *Pvu*II digested chromosomal DNA prepared from *L. monocytogenes* C185, and plasmid DNA prepared from *E. coli* CC118/pCT200. DNA was probed with a LIR/*lacZ* specific non-radioactive labelled 663 bp *Cla*I/*Pvu*II DNA fragment isolated from Tn*917-lacZ-cat86*. Lane: [a], *Eco*RI digested Bacteriophage SPP-1 DNA; [b], *L. monocytogenes* C185; [c], plasmid pCT200; [d], *L. monocytogenes* DRDC8; [e], plasmid pBR322. The arrowhead indicates a 2.8 kb *Pvu*II fragment that hybridised to probe DNA, and contained the site of transposon integration.

Table 5.1 Primary clones containing *L. monocytogenes* chromosomal DNA which flanked the LIR region of Tn*917-lacZ-cat86* isolated from the *lacZ* transcriptional gene fusion library.

L. monocytogenes *lacZ* fusion	Environmental signal	Number of Ap^R, Tc^S transformants	Number of $LacZ^+$ transformants (% $LacZ^+$)[a]	DNA insert size (kb)	Clone designation
C044	cation stress	2	1 (50)	8.9	pCT228
C185	cation stress	13	1 (7.7)	27.3	pCT200
I055	iron stress	24	0	-	-
I249	iron stress	12	0	-	-
T042	temperature	1	0	-	-
T069	temperature	6	0	-	-
T390	temperature	30	2 (6.7)	9.6, 9.6	pCT229, pCT230
T1619	temperature	1	0	-	-
T3619	temperature	3	2 (66.7)	27.2, 27.2	pCT231, pCT232

[a] Positive primary clones were screened for production of β-galactosidase during growth on solid media containing X-gal (40 μg/ml)

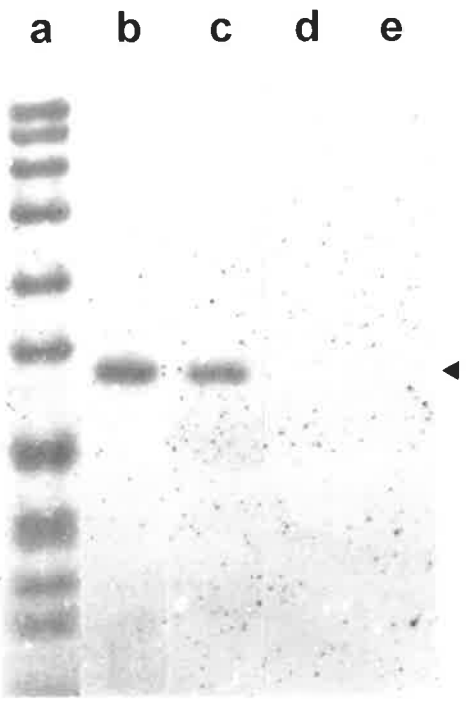
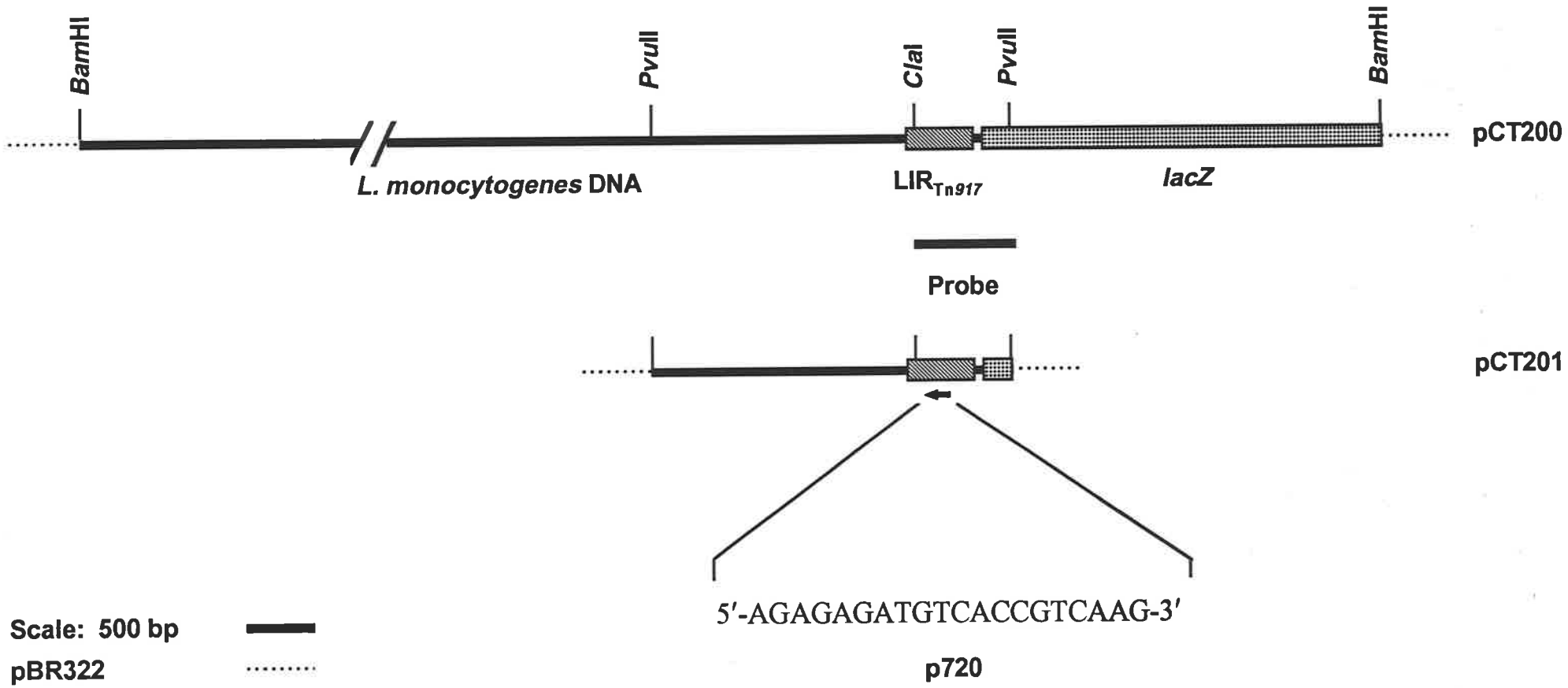

a
b
c
d
e

Figure 5.3. Schematic representation of the construction of plasmid pCT201, a pBR322 derived construct carrying a 2.8 kb *Pvu*II DNA fragment isolated from plasmid pCT200. The *Pvu*II DNA fragment contained the fusion junction between the LIR of Tn*917-lacZ-cat86* and chromosomal DNA from *L. monocytogenes* C185. The location of the LIR/*lacZ* probe used to identify the *Pvu*II DNA fragment by Southern hybridisation (see Figure 5.2), is indicated relative to plasmid pCT200. Synthetic oligonucleotide p720, used to generate nucleotide sequence derived from plasmid, pCT201 (see Figure 5.4), is shown relative to the LIR region of Tn*917*. This oligonucleotide confirmed the position of Tn*917-lacZ-cat86* integration into the *L. monocytogenes* DRDC8 chromosome.


BamHI
PvuII
ClaI
PvuII
BamHI
pCT200
L. monocytogenes DNA
LIR Tn917
lacZ
Probe
pCT201
5′-AGAGAGATGTCACCGTCAAG-3′
p720
Scale: 500 bp
pBR322

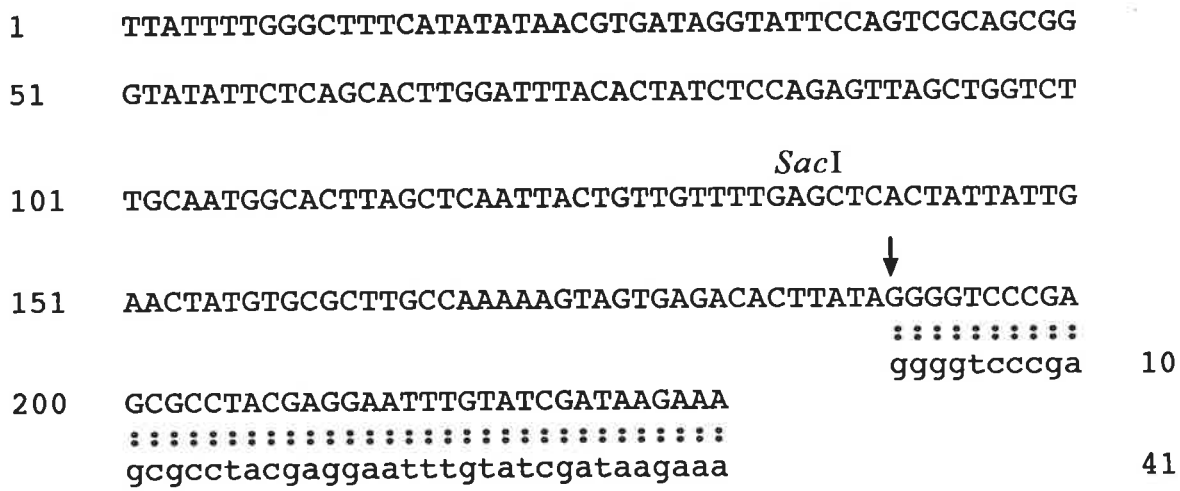


Figure 5.4: Partial nucleotide sequence of pCT201. Nucleotides are shown in uppercase letters numbered to the left hand side in the 5′ to 3′ direction. The position of Tn*917-lacZ-cat86* integration into the *L. monocytogenes* DRDC8 chromosome is indicated by an arrow. LIR_{Tn917} nucleotide sequence (Shaw and Clewell, 1985), shown in lowercase letters are numbered to the right hand side in the 5′ to 3′ direction. Nucleotide sequence identity is indicated by a colon. A *Sac*I restriction site is indicated beginning at nucleotide position 134.

pCT200 by Southern hybridisation analysis (see Figure 5.2), confirmed plasmid pCT201 harboured *L. monocytogenes* DNA flanking the LIR region of Tn*917-lacZ-cat86*. FASTA analysis (Pearson and Lipman, 1988) of DNA flanking the LIR indicated the transposon had inserted in DNA with similarity to that which encodes a P-type ATPase (data not shown).

5.2.3 Generation of chromosomal DNA constructs flanking the LIR and right inverted repeat (RIR) regions of Tn*917-lacZ-cat86* isolated from *L. monocytogenes* C185

5.2.3.1 Construction of DNA subclones flanking the LIR region

To facilitate nucleotide sequencing of chromosomal DNA flanking the LIR region of the transposon insertion in C185, the 2.8 kb *Pvu*II DNA fragment derived from pCT201, was subcloned into the *Sma*I restriction site of the high copy number, M13 based sequencing vectors pBluescript KS and pGEM-7Zf(+), to yield pCT202 and pCT203, respectively (Figure 5.5). Partial restriction mapping of plasmid pCT202, established useful restriction enzyme sites which were used to construct a series of subclones suitable for generation of complete nucleotide sequence data for this region. The spatial arrangement of these subclones relative to pCT202 and detailed descriptions of the plasmid construction is provided in Figure 5.6. The sequencing vector pGEM-7Zf(+) was used as host for all genetic manipulations. Furthermore, 0.32 kb of DNA immediately upstream of *L. monocytogenes* DNA previously cloned in the plasmids pCT202, pCT205, and pCT209 (see Figure 5.6) was also cloned. Using Southern hybridisation analysis, a 2.3 kb *Hpa*I DNA fragment from pCT200, which overlapped the 2.8 kb *Pvu*II DNA fragment contained in pCT202, was identified after hybridisation to a DIG labelled 0.58 kb *Hin*dIII DNA fragment isolated from pCT205 (Figure 5.7). This fragment contained on the plasmid pCT217, was further manipulated to isolate the 5′ located 0.32 kb fragment, which was cloned into pGEM-7Zf(+) to yield pCT226. The cloning rationale and spatial arrangement of these clones is described in Figure 5.8.

5.2.3.2 Preparation of DNA subclones flanking the RIR region

In contrast to the strategy used to clone chromosomal DNA flanking the LIR region (see Section 5.2.1), direct selection of DNA flanking the RIR region of the transposon insertion in C185 was not possible, as this DNA was not fused with a promoterless reporter gene or any readily selectable marker. To overcome this problem, chromosomal DNA contained on the plasmids pCT202, pCT204, and pCT211 (see Figure 5.6), which immediately flanked the

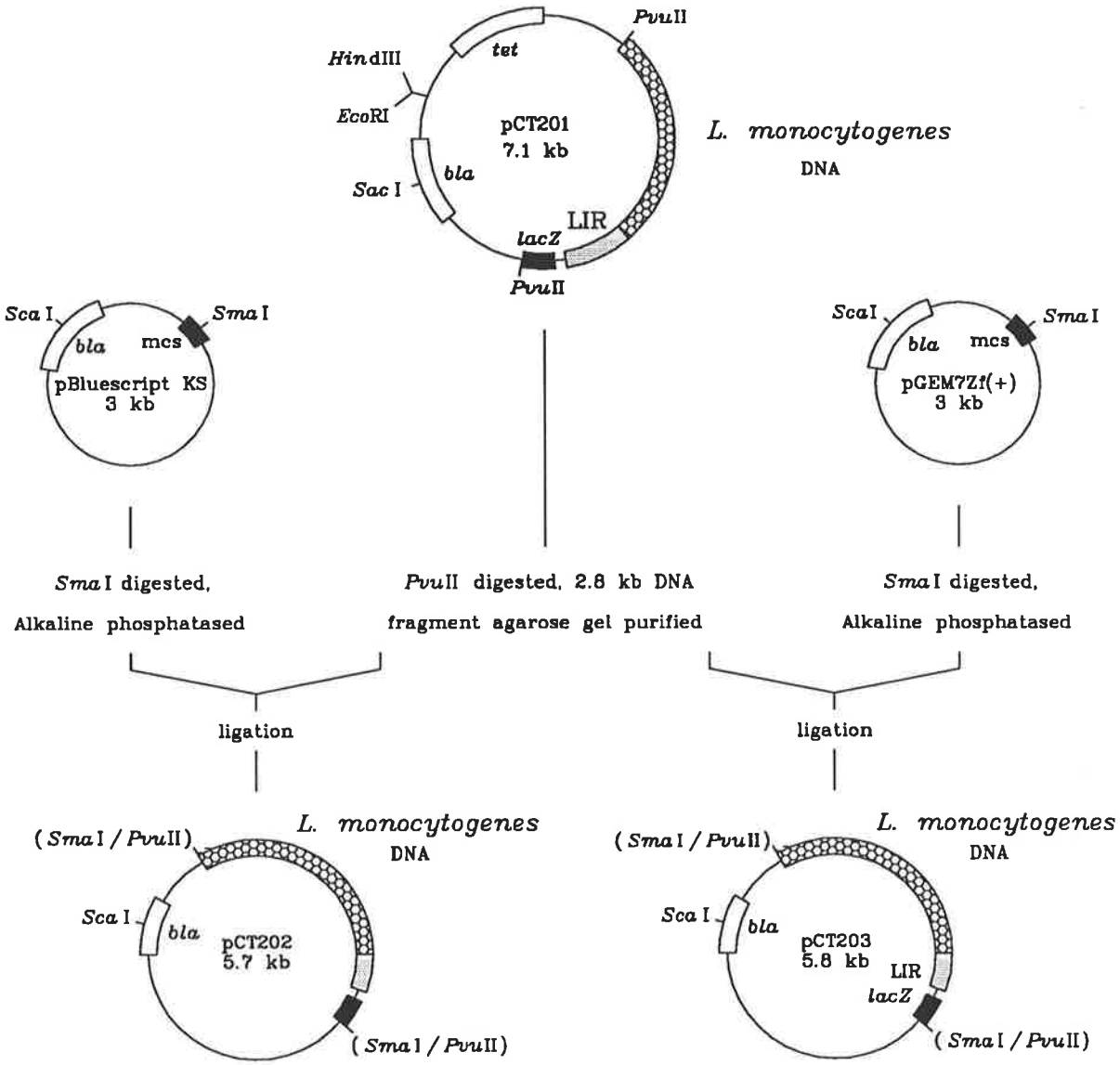


Figure 5.5: Construction of plasmids, pCT202 and pCT203. A 2.8 kb *Pvu*II DNA fragment from pCT201 which harbours the fusion junction between the LIR of Tn*917-lacZ-cat86* and chromosomal DNA from *L. monocytogenes* C185, was subcloned into the *Sma*I restriction site of high copy number, M13 based sequencing vectors pBluescript KS and pGEM-7Zf(+), respectively. Restriction enzyme sites in parentheses have been destroyed during the cloning procedure. Abbreviations: *bla*, ampicillin resistance cartridge; *tet*, tetracycline resistance cartridge; mcs, multiple cloning site; LIR, left inverted repeat region from Tn*917-lacZ-cat86*; *lacZ*, promoterless β-galactosidase gene from Tn*917-lacZ-cat86*.

Figure 5.6: Schematic representation of pCT202 and construction of subclones derived from this construct for direct nucleotide sequence analysis. Cloned DNA fragments are indicated by a heavy line. Plasmid pGEM-7Zf(+) was used as the host vector for all manipulations. Restriction enzyme recognition sequences used in the cloning procedure, derived from vector [pBluescript KS (asterisks) or pGEM-7Zf(+) (crosshatch)] or insert DNA, are shown. Restriction enzyme sites indicated by parentheses were destroyed during the cloning procedure. The orientation of cloned DNA is indicated by the restriction enzyme sites located in the multiple cloning site of vector DNA.

Cloning rationale:

pCT205: Cloned a 0.58 kb *Hin*dIII DNA fragment from pCT202 into the *Hin*dIII site from pGEM-7Zf(+)

pCT209: Cloned a 0.4 kb *Pst*I DNA fragment from pCT202 into the *Sma*I site from pGEM-7Zf(+)

pCT210: Cloned a 1.73 kb *Pst*I/*Sac*I DNA fragment from pCT202 into the *Sma*I site from pGEM-7Zf(+)

pCT206: Cloned a 0.86 kb *Hin*dIII DNA fragment from pCT202 into the *Hin*dIII site from pGEM-7Zf(+)

pCT204: Cloned a 0.73 kb *Cla*I/*Hin*dIII DNA fragment from pCT202 into the *Cla*I/*Hin*dIII site from pGEM-7Zf(+)

pCT211: Religated linearised pCT204, after deletion of a 0.24 kb *Hpa*I/*Bam*HI DNA fragment

Note: Plasmids pCT207 and pCT208 were derived from a nested deletion of pCT203 (see Section 2.13), and contain a 0.76 kb and 1.07 kb 5′ terminal deletion of the 2.8 kb *Pvu*II DNA fragment, respectively.

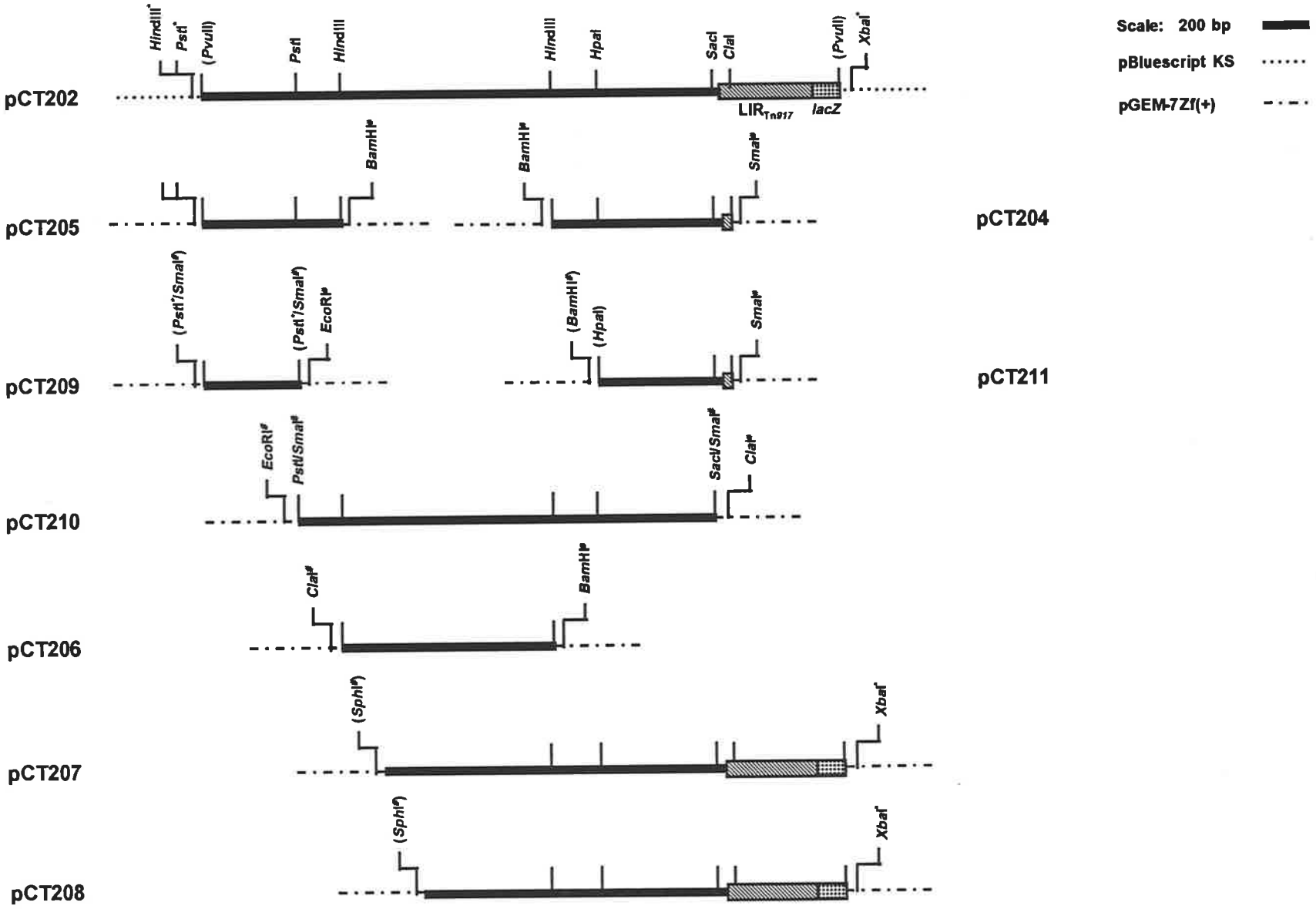

Scale: 200 bp
pBluescript KS
pGEM-7Zf(+)
pCT202
HindIII
PstI
(PvuII)
PstI
HindIII
HindIII
HpaI
SacI
ClaI
(PvuII)
XbaI
LIR$_{Tn917}$
lacZ
pCT205
BamHI
BamHI
SmaI
pCT204
pCT209
(PstI/SmaI)
(PstI/SmaI)
EcoRI
pCT211
(BamHI)
(HpaI)
SmaI
pCT210
EcoRI
PstI/SmaI
SacI/SmaI
ClaI
pCT206
ClaI
BamHI
pCT207
(SphI)
XbaI
pCT208
(SphI)
XbaI

Figure 5.7: Southern hybridisation analysis of *Hpa*I digested chromosomal DNA prepared from *L. monocytogenes* C185, and plasmid DNA prepared from *E. coli* CC118/pCT200. DNA was probed with a digoxigenin labelled 0.58 kb *Hin*dIII DNA fragment purified from plasmid, pCT205. Lane: [a], *Eco*RI digested Bacteriophage SPP-1 DNA; [b], *L. monocytogenes* C185; [c], plasmid pCT200; [d], plasmid pBR322; [e], plasmid pCT205. The arrow indicates a 2.3 kb *Hpa*I DNA fragment that hybridised to probe DNA. The arrowhead indicates the 0.58 kb *Hin*dIII DNA fragment from plasmid, pCT205, used as the probe.

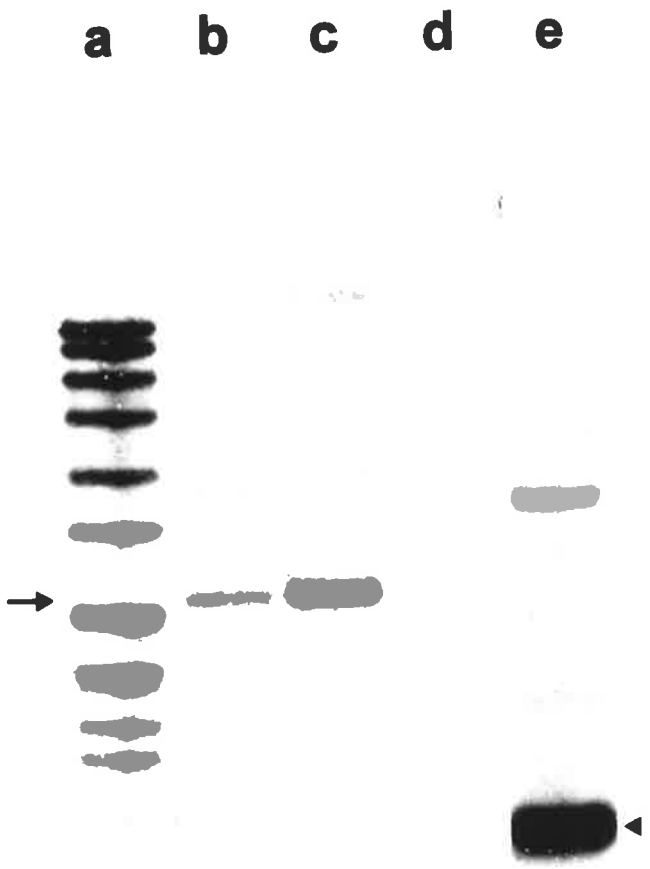
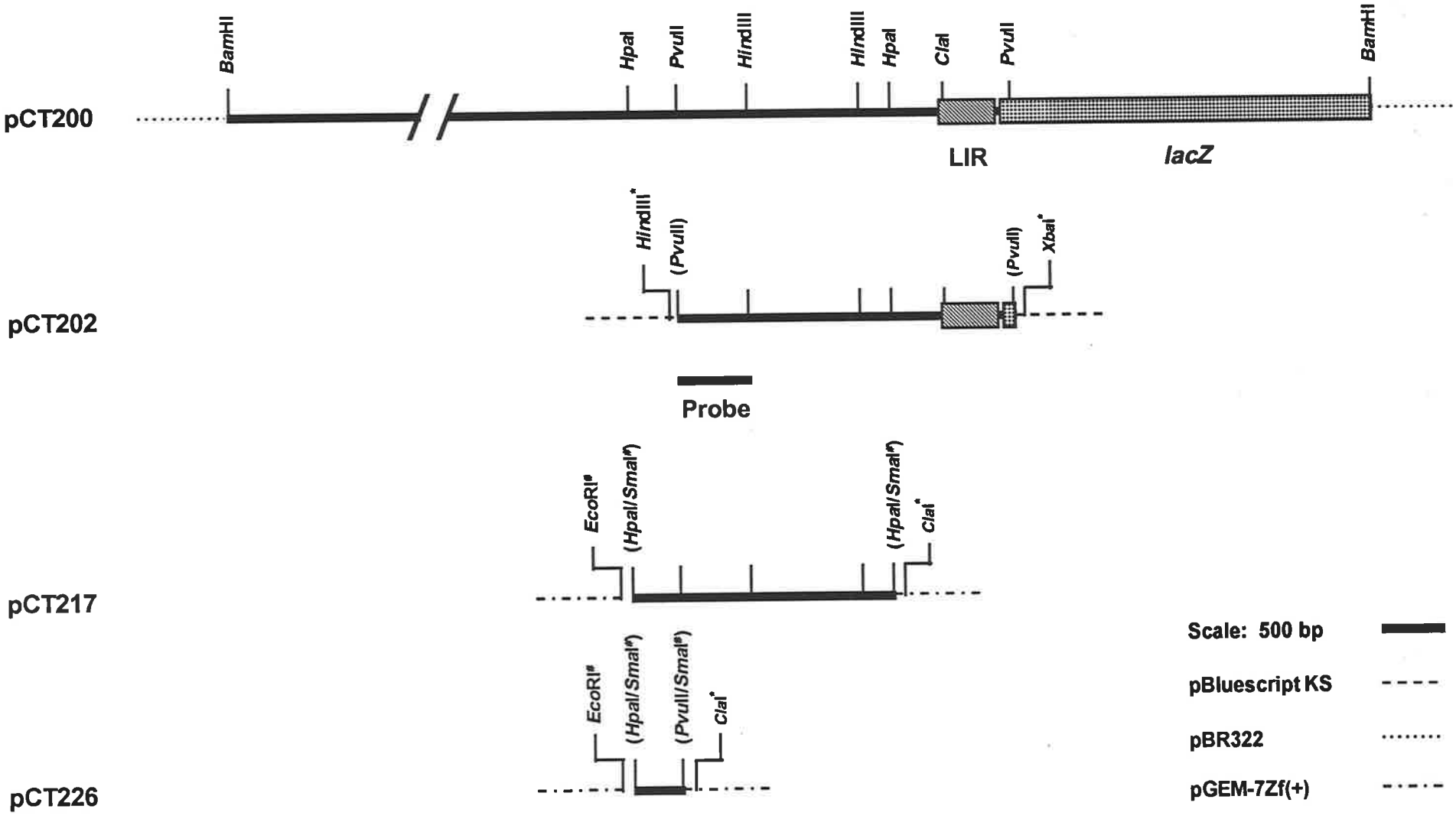

a
b
c
d
e

Figure 5.8: Schematic representation of the construction of plasmids pCT217 and pCT226. A 2.3 kb *Hpa*I DNA fragment from plasmid, pCT200, was subcloned into the *Sma*I restriction site of the high copy number, M13 based sequencing vector, pGEM-7Zf(+), to form pCT217. Plasmid pCT226, was constructed by subcloning the 0.32 kb *Pvu*II/*Eco*RI DNA fragment from pCT217 into the *Sma*I restriction site of pGEM-7Zf(+). The location of the *Hin*dIII restricted DNA probe isolated from plasmid pCT202, used to identify the *Hpa*I DNA fragment by Southern hybridisation (see Figure 5.7), is indicated. Restriction enzyme recognition sequences used in the cloning procedure, derived from vector [pBluescript KS (asterisks) or pGEM-7Zf(+) (crosshatch)] or insert DNA, are shown. Restriction enzyme sites indicated by parentheses were destroyed during the cloning procedure. The orientation of cloned DNA is indicated by the restriction enzyme sites located in the multiple cloning site of vector DNA.


pCT200
BamHI
HpaI
PvuII
HindIII
HindIII
HpaI
ClaI
PvuII
BamHI
LIR
lacZ
pCT202
HindIII*
(PvuII)
(PvuII)
XbaI*
Probe
pCT217
EcoRI#
(HpaI/SmaI#)
(HpaI/SmaI#)
ClaI*
pCT226
EcoRI#
(HpaI/SmaI#)
(PvuII/SmaI#)
ClaI*
Scale: 500 bp
pBluescript KS
pBR322
pGEM-7Zf(+)

fusion junction created by the LIR region of the transposon, was used as a probe in Southern hybridisation experiments to locate both fusion junctions flanking the LIR and RIR regions of the transposon on the same *Hin*dIII chromosomal DNA fragment.

Analysis of *Hin*dIII digested chromosomal DNA from DRDC8, identified a 5.3 kb DNA fragment which hybridised to a DIG labelled 0.73 kb *Cla*I/*Hin*dIII DNA fragment purified from pCT204 (Figure 5.9). This fragment was isolated in a heterogenous population of agarose gel purified, *Hin*dIII digested chromosomal DNA fragments, ligated into pGEM-7Zf(+) and transformed into *E. coli* DH5α. At least 1200 white transformants initially screened by blue/white differentiation, were replica plated and analysed by colony hybridisation using the DIG labelled 0.73 kb *Cla*I/*Hin*dIII DNA probe (Figure 5.10, Part A). Colonies that contained plasmid DNA which hybridised with the probe were detected at an efficiency of 0.92%. Southern hybridisation analysis was used to confirm plasmid DNA from two positive clones contained the 5.3 kb *Hin*dIII DNA fragment by hybridisation with the 0.73 kb *Cla*I/*Hin*dIII DNA probe (Figure 5.10, Part B). Partial restriction mapping of the 5.3 kb *Hin*dIII DNA fragment contained on the plasmid pCT212, established useful restriction enzyme sites which were used to construct a series of subclones suitable for generation of complete nucleotide sequence data for this region. The spatial arrangement of these subclones relative to pCT212 and detailed descriptions of the plasmid construction is shown in Figure 5.11.

5.2.3.3 Construction of pCT220, a clone connecting *L. monocytogenes* chromosomal DNA flanking both the LIR and RIR domains of Tn*917-lacZ-cat86*

Efforts to isolate a full length clone connecting DNA which flanked the LIR and RIR regions of the Tn*917-lacZ-cat86* insertion in *L. monocytogenes* C185 were unsuccessful. However, to overcome this problem, a two step *in vitro* cloning rationale was used to connect DNA fragments which flanked the repeat domains. This lead to the construction of plasmid pCT220. A detailed description and schematic representation of the construction of this plasmid is shown in Figure 5.12. An important feature of this strategy was that all restriction enzyme recognition sequences were completely conserved following recombinant manipulations. The orientation of DNA cloned into plasmid pCT220, was confirmed by *Hinc*II restriction digestion and dye-primer nucleotide sequence analysis (data not shown).

Figure 5.9: Southern hybridisation analysis of *Hin*dIII digested chromosomal DNA prepared from *L. monocytogenes* DRDC8. DNA was probed with a digoxigenin labelled 0.73 kb *Cla*I/*Hin*dIII DNA fragment purified from plasmid, pCT204. Lane: [a], *Eco*RI digested Bacteriophage SPP-1 DNA; [b], *L. monocytogenes* DRDC8; [c], plasmid pCT202; [d], plasmid pCT204; [e], plasmid pGEM-7Zf(+). The arrow indicates a 5.3 kb *Hin*dIII DNA fragment that hybridised to probe DNA and contains the DNA which flanked both the LIR and RIR fusion junctions of the transposon insertion from *L. monocytogenes* C185. The arrowhead indicates the 0.73 kb *Cla*I/*Hin*dIII DNA fragment from plasmid, pCT204, used as the probe.

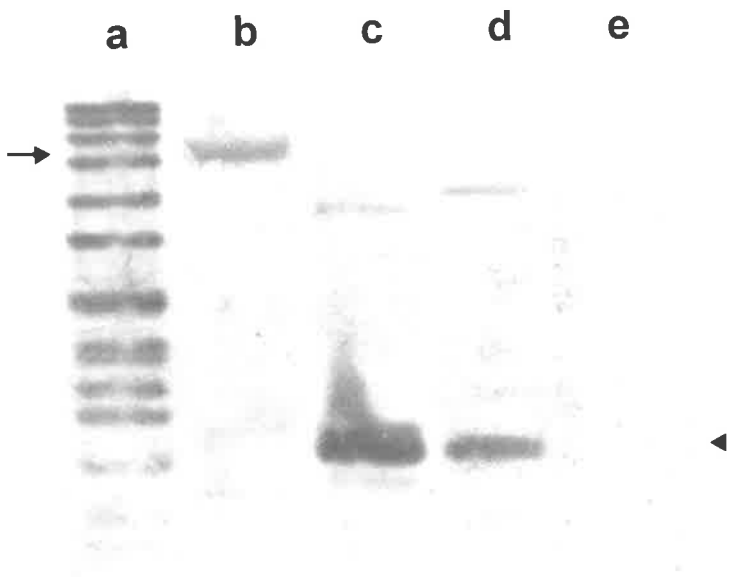

a
b
c
d
e

Figure 5.10

Plate A: Colony hybridisation of *E. coli* DH5α transformed with the high copy number, M13 based sequencing vector, pGEM-7Zf(+) which contained *Hin*dIII digested *L. monocytogenes* chromosomal DNA. Arrowheads indicate transformants that harbour pGEM-7Zf(+), containing a 5.3 kb *Hin*dIII chromosomal DNA fragment from *L. monocytogenes* DRDC8, that hybridised to probe DNA. The bold arrow indicates *E. coli* DH5α/pCT204, and the thin arrow indicates *E. coli* DH5α/pGEM-7Zf(+).

Plate B: Southern hybridisation analysis of *Hin*dIII digested plasmid DNA prepared from two *E. coli* DH5α transformants (from Figure 5.10, Plate A), which were identified by DNA hybridisation to the digoxigenin labelled 0.73 kb *Cla*I/*Hin*dIII DNA fragment purified from plasmid, pCT204. Lane: [a], *Eco*RI digested Bacteriophage SPP-1 DNA; [b], *L. monocytogenes* DRDC8; [c], plasmid DNA from *E. coli* transformant 1; [d], plasmid DNA from *E. coli* transformant 2; [e], plasmid pGEM-7Zf(+); [f], plasmid pCT202. The arrow indicates a 5.3 kb *Hin*dIII DNA fragment that hybridised to probe DNA. The arrowhead indicates a 4.8 kb *Hin*dIII DNA fragment from plasmid, pCT202, that contains the internal 0.73 kb *Cla*I/*Hin*dIII DNA fragment used as the probe.

Filters were probed with the digoxigenin labelled 0.73 kb *Cla*I/*Hin*dIII DNA fragment purified from plasmid, pCT204.

Plate A

Plate B

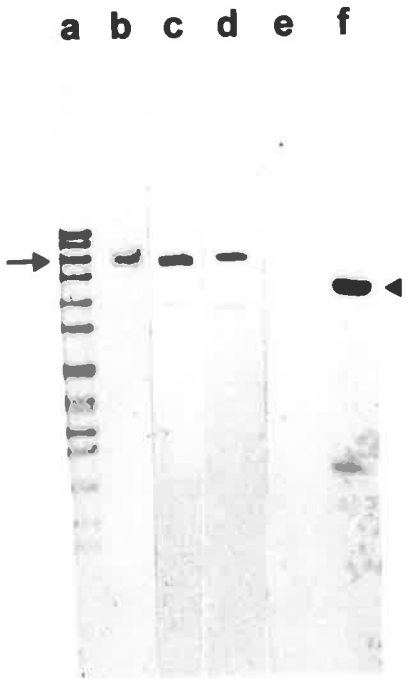


Figure 5.11: Schematic representation of the construction of plasmid pCT212 and derivatives of this plasmid used to generate nucleotide sequence of *L. monocytogenes* chromosomal DNA flanking the RIR region of Tn*917-lacZ-cat86*. Cloned chromosomal DNA fragments from *L. monocytogenes* are indicated by a heavy line. The location of the *Cla*I/*Hin*dIII restricted DNA probe used to identify the *Hin*dIII DNA fragment by Southern hybridisation (see Figure 5.9), contained on plasmid pCT212, is indicated relative to plasmid pCT202. Restriction enzyme recognition sequences used in the cloning procedure, derived from vector [pBluescript KS (asterisks), pGEM-7Zf(+) (crosshatch), and pGEM-3Zf(+) (cross)] or insert DNA, are shown. Restriction enzyme sites indicated by parentheses were destroyed during the cloning procedure. The orientation of cloned DNA is indicated by the restriction enzyme sites located in the multiple cloning site of vector DNA.

Cloning rationale

pCT212: Cloned a 5.3 kb *Hin*dIII DNA fragment from *L. monocytogenes* DRDC8 into the *Hin*dIII site from pGEM-7Zf(+)

pCT215: Cloned a 1.8 kb *Sac*I DNA fragment from pCT212 into the *Sac*I site from pGEM-7Zf(+)

pCT227: Cloned a 0.9 kb *Pst*I/*Sac*I DNA fragment from pCT212 into the *Pst*I/*Sac*I site from pGEM-3Zf(+)

pCT214: Cloned a 3.0 kb *Sac*I DNA fragment from pCT212 into the *Sac*I site from pGEM-7Zf(+)

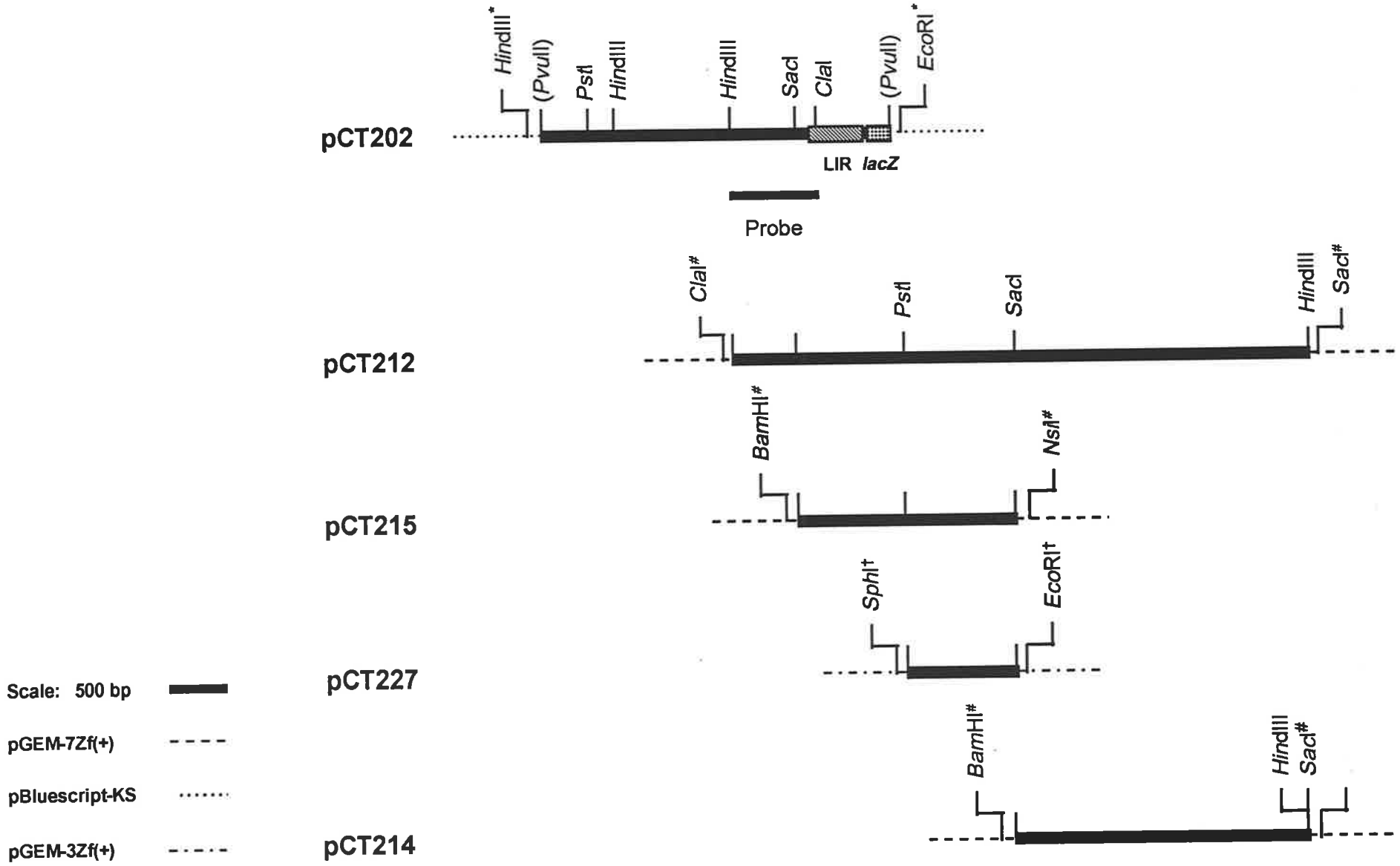
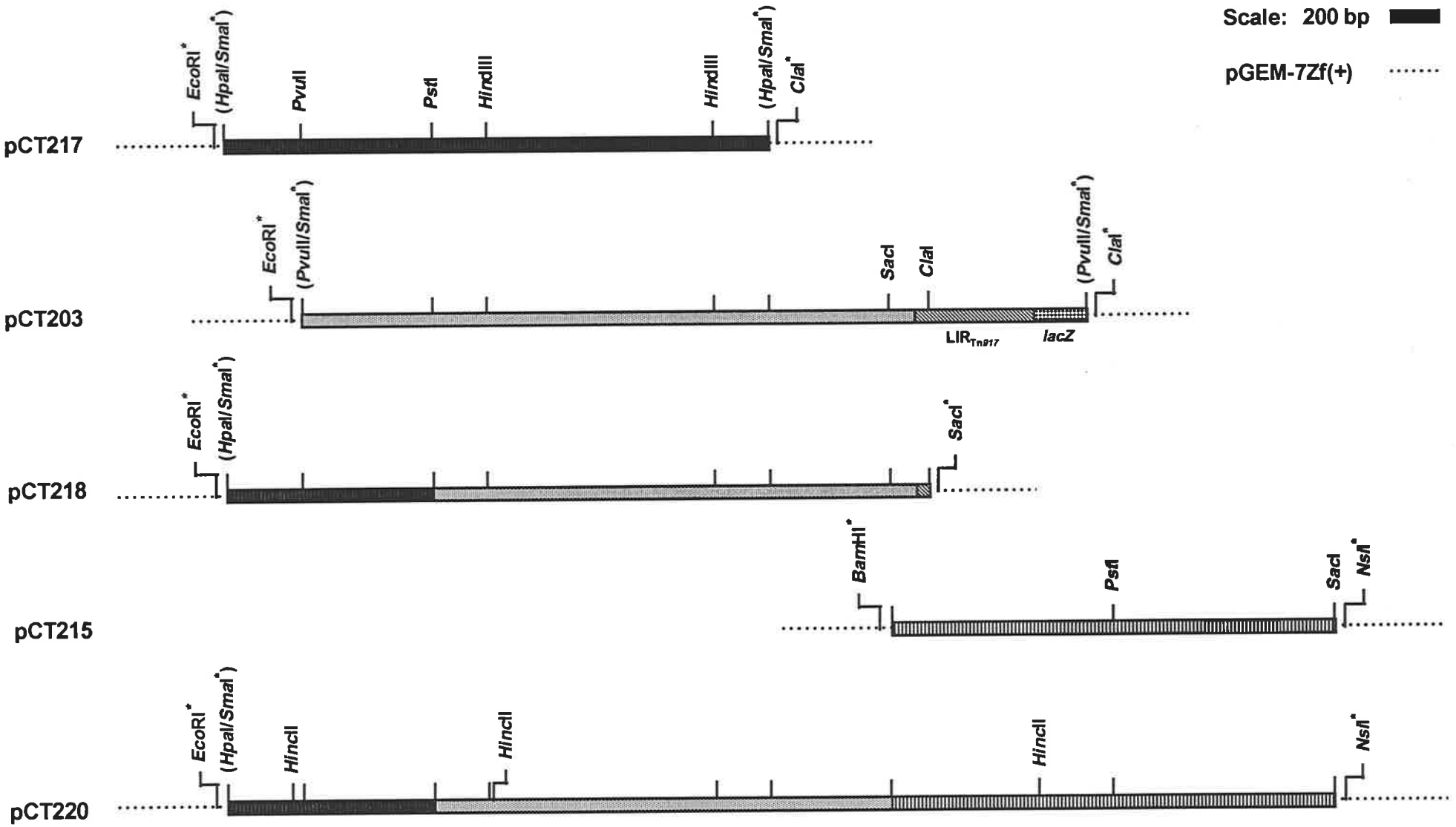

pCT202
HindIII*
(PvuII)
PstI
HindIII
HindIII
SacI
ClaI
(PvuII)
EcoRI*
LIR
lacZ
Probe
pCT212
ClaI#
PstI
SacI
HindIII
SacI#
pCT215
BamHI#
NsiI#
pCT227
SphI†
EcoRI†
pCT214
BamHI#
HindIII
SacI#
Scale: 500 bp
pGEM-7Zf(+)
pBluescript-KS
pGEM-3Zf(+)

Figure 5.12: Schematic representation of the two step construction of plasmid, pCT220. Step 1: a 1.8 kb *Cla*I/*Pst*I DNA fragment purified from plasmid, pCT203, was subcloned into a 3.7 kb *Cla*I/*Pst*I fragment purified from plasmid, pCT217. Step 2: a 1.8 kb *Sac*I DNA fragment from plasmid, pCT215, was subcloned into a 5.4 kb *Sac*I fragment from plasmid, pCT218. DNA fragments derived from plasmids pCT203, pCT215, pCT217, and pCT218, used to construct pCT220 are represented by different patterns. Restriction enzyme recognition sequences used in the cloning procedure, derived from vector [pGEM-7Zf(+) (asterisks)] or insert DNA, are shown. Restriction enzyme sites indicated by parentheses were destroyed during the cloning procedure. The orientation of cloned DNA is indicated by the restriction enzyme sites located in the multiple cloning site of vector DNA, and was confirmed by nucleotide sequence analysis and *Hinc*II restriction digestion.


Scale: 200 bp
pGEM-7Zf(+)
pCT217
EcoRI*
(HpaI/SmaI*)
PvuII
PstI
HindIII
HindIII
(HpaI/SmaI*)
ClaI*
pCT203
EcoRI*
(PvuII/SmaI*)
SacI
ClaI
(PvuII/SmaI*)
ClaI*
LIR$_{Tn917}$
lacZ
pCT218
EcoRI*
(HpaI/SmaI*)
SacI*
pCT215
BamHI*
PstI
SacI
NsiI*
pCT220
EcoRI*
(HpaI/SmaI*)
HincII
HincII
HincII
NsiI*

5.2.4 Nucleotide sequence analysis of chromosomal DNA flanking the Tn*917-lacZ-cat86* insertion from *L. monocytogenes* C185

Nucleotide sequence analysis of DNA flanking Tn*917-lacZ-cat86* in *L. monocytogenes* C185 was performed using the constructs described in Section 5.2.3, as DNA templates for automated sequencing reactions. A schematic representation of sequence reactions used to generate the complete nucleotide sequence, and DNA templates used for each reaction, is shown in Figure 5.13. Also included are the synthetic oligonucleotides (see Section 2.22, Table 2.6), specifically designed to generate overlapping nucleotide sequence. Figure 5.14 shows the nucleotide sequence of 4280 bp and corresponding translated amino acid sequence derived from a series of overlapping subclones established from plasmids pCT202 and pCT203. A single open reading frame, initially termed *orfA*[1], spanning nucleotides 543 to 2504, was identified. *orfA* encodes a protein of 653 aa with a predicted M_r value of 69,463. The region encompassing *orfA*, is contained on the plasmid pCT220 and is schematically represented in Figure 5.15. Also shown are common restriction enzyme recognition sites for subsequent modification of *orfA*, and the site of the transposon insertion, mapped to nucleotide 2495.

5.2.5 Analysis of DNA translation products

5.2.5.1. Comparison of OrfA polypeptide to sequences contained within protein databases

The amino acid sequence predicted from *orfA* was analysed for sequence similarity to related proteins present in the Genpept, Swissprot, and Pir protein data bases using FASTA alignment algorithms. The polypeptide encoded by *orfA*, showed significant identity to bacterial and eukaryotic P-type ATPases involved in cation transport (Table 5.2). These proteins, characterised by a phospho-aspartate intermediate, are responsible for ATP-driven translocation of cations through biological membranes and are known to confer resistance to toxic heavy metal ions (Pedersen and Carafoli, 1987; Silver *et al.*, 1989). OrfA displayed most sequence identity to copper transporters isolated from *Synechococcus* spp. (PacS) (Kanamaru *et al.*, 1993), and *E. hirae* (CopA) (Odermatt *et al.*, 1993). Moreover, significant identity over a 300 aa overlap was also observed to proteins associated with copper metabolism disorders in humans, Menkes syndrome (Chelly *et al.*, 1993; Mercer *et al.*, 1993; and Vulpe *et al.*, 1993) and Wilson disease (Bull *et al.*, 1993). Given that the *orfA* protein product may be involved in

[1] The initial designation of *orfA*, was changed to *ctpA* (copper transport protein) in Section 5.2.5.1 to account for amino acid similarity to copper transport P-type ATPase proteins.

Figure 5.13: A schematic representation of the sequence reactions used to generate the complete nucleotide sequence of the 4.3 kb fragment of chromosomal DNA from *L. monocytogenes*, contained on plasmid, pCT220. Restriction enzyme sites indicated by an asterisk have been destroyed during the cloning procedure. Synthetic oligonucleotides specifically designed to generate nucleotide sequence overlap are shown in parentheses and their respective nucleotide sequence is listed in Table 2.6.

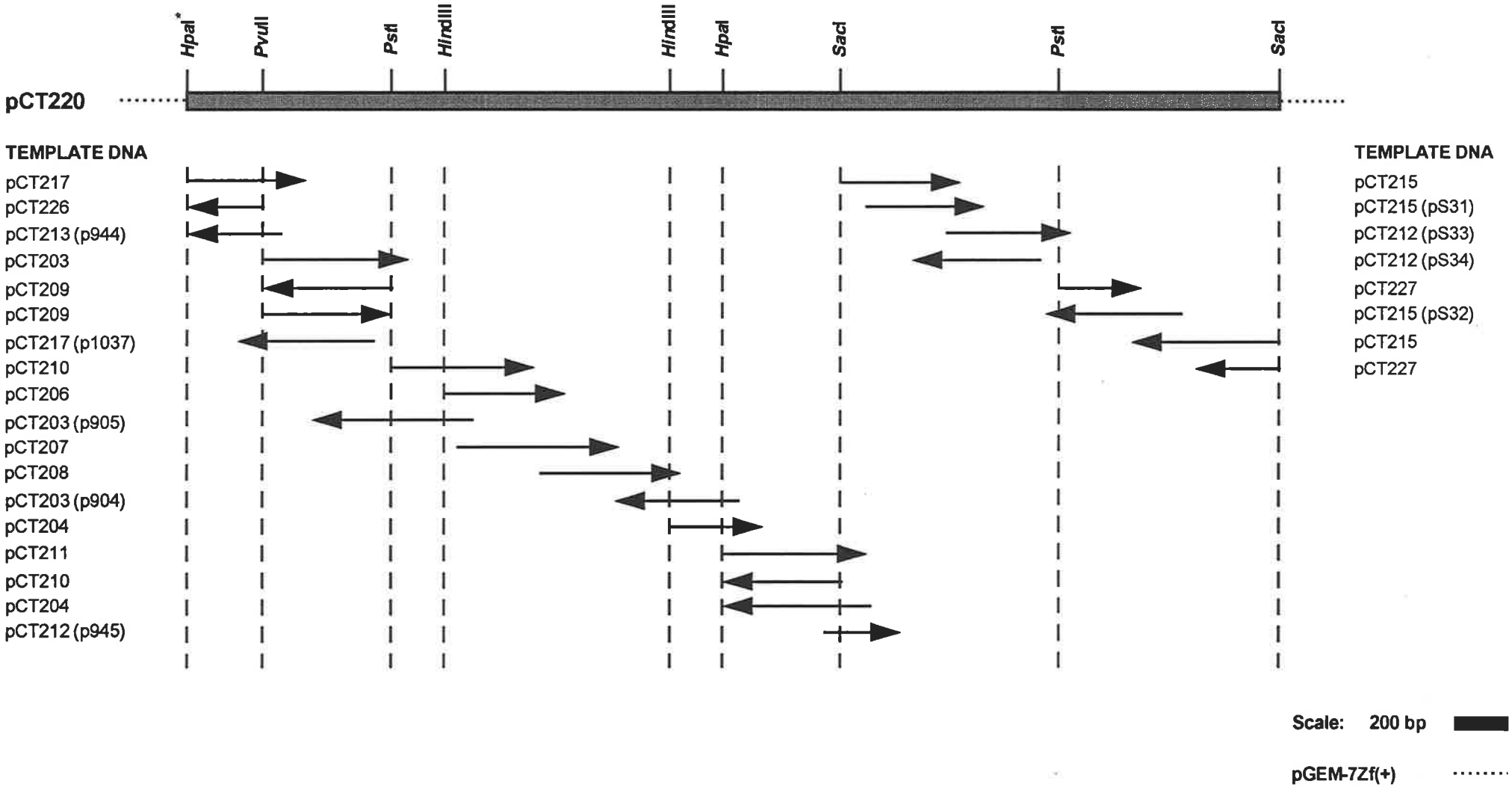

HpaI*
PvuII
PstI
HindIII
HindIII
HpaI
SacI
PstI
SacI
pCT220
TEMPLATE DNA
pCT217
pCT226
pCT213 (p944)
pCT203
pCT209
pCT209
pCT217 (p1037)
pCT210
pCT206
pCT203 (p905)
pCT207
pCT208
pCT203 (p904)
pCT204
pCT211
pCT210
pCT204
pCT212 (p945)
TEMPLATE DNA
pCT215
pCT215 (pS31)
pCT212 (pS33)
pCT212 (pS34)
pCT227
pCT215 (pS32)
pCT215
pCT227
Scale: 200 bp
pGEM-7Zf(+)

Figure 5.14: Nucleotide sequence of the 4.3 kb fragment of chromosomal DNA from *L. monocytogenes*, contained on plasmid, pCT220. The nucleotides are numbered to the right hand side in the 5′ to 3′ direction. The deduced protein sequence of the open reading frame, *orfA*, is given below the DNA sequence in the single letter amino acid code and is numbered to the right hand side. The putative regulatory elements of *orfA* located upstream of this gene predicted from primer extension analysis of *orfA* mRNA (see Figure 5.21) including the ribosome binding site (rbs), transcriptional initiation sites (P1, P2 and P3), –10 regions, and –35 regions upstream of the start codon are indicated. The position of the synthetic oligonucleotide (p1037) used in Primer Extension analysis is shown. The translational termination codon is indicated by an asterisk and repeat sequences (see Figure 5.22) potentially involved in transcriptional termination of *orfA* are presented. Relevant restriction sites useful for subsequent modification of *orfA* are highlighted.

*Hpa*I

```
GTTAACTTGATCTTTAACCGGAAAGATGCCTCATCTTGTCTTGAAAAAGTTATTTTTCCA      60

GATTTTGGAGTGGATGCGGATTTACCACTCAATCAGGATGTGTCTATTTTGATTGATACA     120

TCTAAGTCAGGCGAGTTTATATATTCATGTGGGATGAATATGTTCCATGGAAAAATCATC     180

ATCAAATAGAATCACAAATTAAATACTAATATACCCTAAGGAGGATATAAAATGTTCGGA     240

TCAAAAATTAATAATAAAGTAGAAGTAGTTGTTGACGGAGGTTACTCTCCTAGTAAATTT     300
```

*Pvu*II

```
AAGTTAAAAGCTGGAGAGCCAGCTGAAGTCTCTTTTACTCGTGTCTCTGATAAAGGTTGT     360

GCTCAGCAAATTATCTTCAATGGAGAACTTCGAAATTTACCCTTAAATGAATCTGTCACT     420
```

−35 region −10 region P3 P2

```
TTTAATTTCACTCCAGTTGAAAAAGGACGTCATAATTGGTCTTGCGGTATGAAAATGATC     480
```

−35 region −10 region

```
CTGGGGAGCTATTCAGTTAAATAAAAAACTATTTTTAATGTAGTTACAAAAGGAGATACT     540
```

P1 rbs

OrfA start ← primer

```
TTATGTCAATAAAAAATCGTTTTATCATAGGTGTCATCGGATCAGTCCCATTGCTTATCA     600
  M  S  I  K  N  R  F  I  I  G  V  I  G  S  V  P  L  L  I  N      20

ATATGTTTATGAGCTTGGGCGGTTCCATGCTTGGAGGCGATAAATATGGTGTTTGGATTC     660
  M  F  M  S  L  G  G  S  M  L  G  G  D  K  Y  G  V  W  I  L      40
```

*Pst*I

```
TGTTTGCCTTTGGCTCATTAGTTTACTGGTTCTCAGGATTGCCATTCTTGCGTACTGCAG     720
  F  A  F  G  S  L  V  Y  W  F  S  G  L  P  F  L  R  T  A  V      60

TCGCTTCGTTCAAAAATCATCATGCCAATATGGACACGCTTGTTGGATTAGGCACAACTA     780
  A  S  F  K  N  H  H  A  N  M  D  T  L  V  G  L  G  T  T  I      80

TTGCATATGTTTACAGTCTTTACGCTATGTTTGCTCGTCCAAATGAAACATATTTCGAAG     840
  A  Y  V  Y  S  L  Y  A  M  F  A  R  P  N  E  T  Y  F  E  A     100
```

*Hin*dIII

```
CTGTTGCTGTTGTTATTACATTAATCTTGTTAGGATCATATTTTGAGGAACGTATGAAAG     900
  V  A  V  V  I  T  L  I  L  L  G  S  Y  F  E  E  R  M  K  A     120

CTTCAGCTTCATCTGCTGTTGACAAATTGATGGGTTTGCAAGCAAAAGATGCAGAAGTTC     960
  S  A  S  S  A  V  D  K  L  M  G  L  Q  A  K  D  A  E  V  L     140

TGCGTGATGGTGAGTTTATTAAACTTCCTATTGAAGAAATTATTGTAGGTGATTTAATCC    1020
  R  D  G  E  F  I  K  L  P  I  E  E  I  I  V  G  D  L  I  R     160

GCGTCAAACCAGGTGAAAAAGTTGCTGTTGATGGCCAAATTGTTGAAGGAACTTCTACAT    1080
  V  K  P  G  E  K  V  A  V  D  G  Q  I  V  E  G  T  S  T  L     180

TAGATGAATCTATGGTAACTGGAGAATCCATGCCTGTAGAAAAGGGCCCTGGCGATAATG    1140
  D  E  S  M  V  T  G  E  S  M  P  V  E  K  G  P  G  D  N  V     200

TAATTGGAGCAACCCTTAATAACACTGGGTCATTTACATTCGAAGTTACCAAAGTTGGGG    1200
  I  G  A  T  L  N  N  T  G  S  F  T  F  E  V  T  K  V  G  A     220
```

```
CTGATACCATGTTGTCAAATATTGCTGAAATGGTTCGTCATGCACAAAACTCCCGAGCAC   1260
  D  T  M  L  S  N  I  A  E  M  V  R  H  A  Q  N  S  R  A  P    240

CTATACAAAAAACTGTTGATCGGATTTCAAATATTTTTGTACCTATAGTTTTAATGATTT   1320
  I  Q  K  T  V  D  R  I  S  N  I  F  V  P  I  V  L  M  I  S    260

CAATTTTAACTTTTATTGTATGGTATGTGTTTCTAGGATCAACTCTTGTCACTGCGATGA   1380
  I  L  T  F  I  V  W  Y  V  F  L  G  S  T  L  V  T  A  M  I    280

TATTTTCGGTATCAGTTATGATTATTGCTTGTCCATGTGCATTAGGGATCGCAACTCCAA   1440
  F  S  V  S  V  M  I  I  A  C  P  C  A  L  G  I  A  T  P  T    300

CAGCATTGATGGTTGGAACCGGACGTTCTGCTAAACTGGGAATTTTGATAAAAAATGCTG   1500
  A  L  M  V  G  T  G  R  S  A  K  L  G  I  L  I  K  N  A  E    320

AGGTTCTTGAAGCGACCCACGATATAAAAACCGTCGTCATGGATAAGACTGGAACAATTA   1560
  V  L  E  A  T  H  D  I  K  T  V  V  M  D  K  T  G  T  I  T    340

CTGTTGGCAAACCACAAGTGACCGATATTATCTCTATCGGAAGAATTAGTGAGAACGAGA   1620
  V  G  K  P  Q  V  T  D  I  I  S  I  G  R  I  S  E  N  E  I    360

TTCTGCGAATCGCTGCTGGACTTGAGGATTCATCAGAACACCCATTAGCTTTAGCAGTAA   1680
  L  R  I  A  A  G  L  E  D  S  S  E  H  P  L  A  L  A  V  I    380

TTAATGAAGCAAAGGACAAGAAAATTACTCCTGCCGTAGCTAAAAATTTCACTGCTATTT   1740
  N  E  A  K  D  K  K  I  T  P  A  V  A  K  N  F  T  A  I  S    400

                  HindIII
CTGGTAAAGGGGTACAAGCTTTGATTGATGGTAAGCAGGCTTTTATTGGTAATGATCGTT   1800
  G  K  G  V  Q  A  L  I  D  G  K  Q  A  F  I  G  N  D  R  L    420

TATCCGATGACTTTAACATGACAGATGATCTTAAGGTTAAAATGACATCTTTACAGGCGC   1860
  S  D  D  F  N  M  T  D  D  L  K  V  K  M  T  S  L  Q  A  Q    440

AGGCGAAAACTGTGGTATTAGTTGGTTACGATGGTCAAATAATTGCTTTAATTGGGATTC   1920
  A  K  T  V  V  L  V  G  Y  D  G  Q  I  I  A  L  I  G  I  Q    460

AAGATGCACCTAAGTCCAGCTCTAAAGCTGCTATCAGGGCAATGCAAAAATCAGGATTTC   1980
  D  A  P  K  S  S  S  K  A  A  I  R  A  M  Q  K  S  G  F  H    480

            HpaI
ACACTGTAATGTTAACTGGGGACAACCGTTTGGTCGCACAAGCCATAGCAGATGATATTG   2040
  T  V  M  L  T  G  D  N  R  L  V  A  Q  A  I  A  D  D  I  G    500

GGATTGACGAGGTCATAGCAGATGTTATGCCTGGGGACAAAGCACAACATATTAGAAAGT   2100
  I  D  E  V  I  A  D  V  M  P  G  D  K  A  Q  H  I  R  K  L    520

TGCAAGAAAAAGGAGCAGTCGCCTTTGTAGGTGATGGAATCAATGATGCCCCTGCATTAT   2160
  Q  E  K  G  A  V  A  F  V  G  D  G  I  N  D  A  P  A  L  S    540

CCACGGCAACAGTAGGTATTGCTATGGGATCGGGGAGTGATATTGCAATTGAATCTGGAG   2220
  T  A  T  V  G  I  A  M  G  S  G  S  D  I  A  I  E  S  G  G    560

GTATTGTACTAGTCAAAAATGATTTGATGGATGTTGTAACCTCTTTAGTATTAGCACGAA   2280
  I  V  L  V  K  N  D  L  M  D  V  V  T  S  L  V  L  A  R  K    580

AAACATATAGTCGGATTTTGATTAACTTATTTTGGGCTTTCATATATAACGTGATAGGTA   2340
  T  Y  S  R  I  L  I  N  L  F  W  A  F  I  Y  N  V  I  G  I    600
```

```
TTCCAGTCGCAGCGGGTATATTCTCAGCACTTGGATTTACACTATCTCCAGAGTTAGCTG    2400
  P  V  A  A  A  G  I  F  S  A  L  G  F  T  L  S  P  E  L  A  G     620

                                                  SacI
GTCTTGCAATGGCACTTAGCTCAATTACTGTTGTTTTGAGCTCACTATTATTGAACTATG    2460
  L  A  M  A  L  S  S  I  T  V  V  L  S  S  L  L  L  N  Y  V     640

                                                 OrfA Stop
TGCGCTTGCCAAAAAGTAGTGAGACACTTATAGGTAATAGTTAGTATTTGTAATATACAA    2520
  R  L  P  K  S  S  E  T  L  I  G  N  S  ***

AAGTGACGCTTTTAATTATAATAGTAATTTTCCTTTTTCAAAATTTGAATCAGAGTCTTA    2580

AATACTAGGCATACAATAAGAGAACCAAAAATCAGAACTACATCCAGTAGTTCTGATTTT    2640

TGATTCTACAATATTTAGTGTTGGTGGAAAATCTACAGATTTTCTACTGACACTTTTTTT    2700

GTAAAAAAAGATAGAAAAAAGAGATAGCCTAACTGGTGGAATTTAGTCGTCTAAAACAAA    2760

                  EcoRV
ACCATACCAAGAAAGGATATCTCCATGTATCATTGTACTAGAAAATTGTTAGGTTTAACA    2820

GATGAAAATTTATTCTTCGAAGAAGAATGGTTAGAGACAGTAGAAGAAGATGGTTTTCGC    2880

ACCAATCTTATCCATGCTAAACTTAGCTATATACTAAGCCATTGCCGTAAATGTGGCATC    2940

AAAAATGAAGGACAAATCATTAAGAATGGCTCACATAAAACAAAAGTCCAACTTTTGCCT    3000

TACCGAGCAACCAAAACAGAATTACGCTTGGTACGTACGCGCTTTTACTGTAAAGAATGT    3060

  SalI
CAGTCGACATTCAACGCTCAGACCAATTTGGTAGATGAAAACTGCTATCTCTCAAAGGAA    3120

TTAAAGGTACAAATTGCCTTGGAACTAGCGAAGAATACAATAAAAAAAGAATTGCCAATC    3240

GCTATTTTGTATCAGACGTAATCGTTTTGCGTGTCCTGCACACCTGTTTAAAAACGTATC    3300

ATCCTCGTTTTGATACGTTACCTTCGGTCCTTTGTTTTGATGAGTACAAATCGATGAAGT    3360

  PstI
CCTGCAGTGGAAAAAATGAGTTTTGTTTTTATGAATGGGCAGACCCAGCAATTAATCGGT    3420

GTATTGGAGAATCGTCGCCTTACTTTCTTAAAACCATATTTCCTTAATTTCACACGAAAG    3480

CACGAGCGAACGTAAAATACGTTGTGATGGATATGAATGCCCCTTATTTTGAACTTGTAA    3540

AAGCGGTCTTCCCAAACGCCAAAATCGTGACCAATCGCTTTCACATTGTCAAACAAATCA    3600

CTCGTACGTTAAATCAGTTGTGAATCAAAACAATGAACAGGTTTCAAAAAACGGAACCGA    3660

                                                  SphI
CAAAGTATTGACGATTGAAGCGATTCTGGAAATTACTCCTTACGCATGCCTATGATCTGG    3720

ATAGTTCCGATTATCAATATGATCGATTCTTTCGCAGACCAATGACGCAAAAAGCGATGG    3780

TCGATGAGCTTTTGAGTTACGACGAGCAATTAACCAGGGCTTACGAGACTTGCCAGCTCC    3840

TCCTCTATCACTTTAAACACAAGGACAACCAAAGTTTTTTCGATACGATCAACAGCTTAG    3900
```

ATCAATGCCTTCCACAATGGTTTTGTAAGAAGCTGACGTTTTTAAATAAATACAAATTAG 3960

GGATTCAATATGCTTTAAAACCCAGGTATAGTAATGGTGCATTGGAAAGGACAAATAATA 4020

AGATAAAAGTGATAAAACGAAGTCTACGGCTATCGAAATTTCCACAACTTTCGAGCAAGA 4080

ATTTATCTCATTCAAGGCTTGATCTTCCAAGTAAAACAAAAACCAGTTAAGCACTCTGCC 4140

TAACTGGTTATGACTAAATTTTCAGTTGTCTCTCTACCAGCACTAAATGTCGAAGAGCCA 4200

AAAATCTGAGCCAGAATGAGGTTCGTTCTGACCCAGATTTGATTTTGCACAACTCATCAG 4260

*Sac*I

TCCTTATTAACAAAGAGCTC 4280

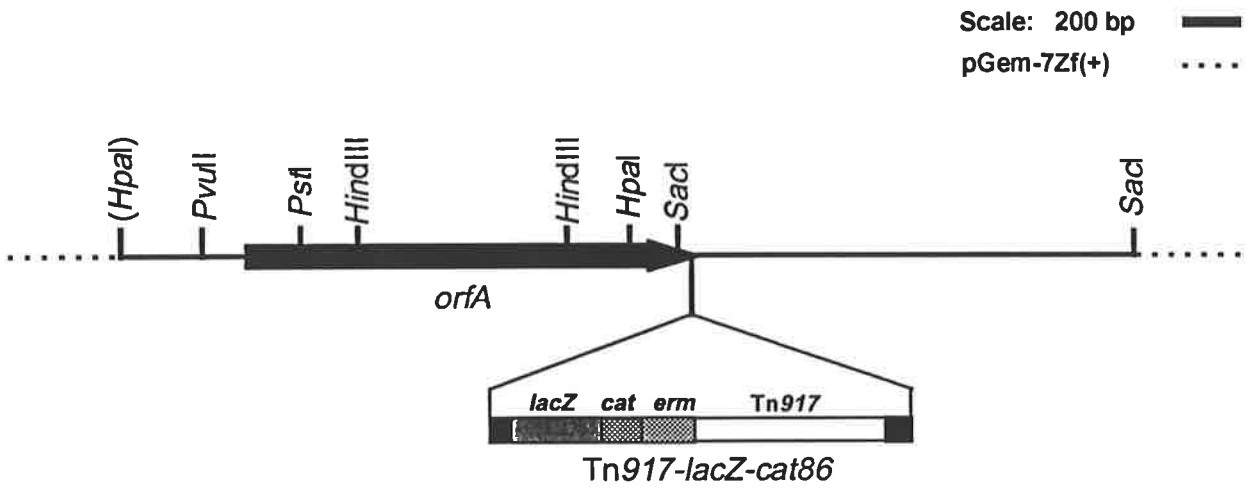


Figure 5.15: Diagrammatic representation of plasmid, pCT220, containing the *orfA* gene. The site of insertion of Tn*917-lacZ-cat86* in the C185 chromosome is indicated. This insertion was mapped to nucleotide 2495, nine base pairs from the termination codon of *orfA*. Restriction enzyme sites in parentheses have been destroyed during the cloning procedure. The Tn*917* transposon derivative is not drawn to scale.

Table 5.2 Analysis of sequence similarities of the OrfA ATPase with other cation-translocating ATPases.

ATPase source	Cation specificity	Length (amino acids)	% identities (overlapping amino acids)	Reference
PacS *Synechococcus* sp.	Cu^{2+}	747	48.4 (639)	Kanamaru *et al.*, (1993)
CopA *E. hirae*	Cu^{2+}	727	42.7 (648)	Odermatt *et al.*, (1993)
CtpB *M. leprae*	nd	780	38.2 (652)	Fsihi and Cole, (1995)
CtpA *M. leprae*	nd	750	37.7 (663)	Fsihi and Cole, (1995)
CadA *S. aureus*	Cd^{2+}	804	35.3 (620)	Chikramane and Dubin, (unpublished)
CadA *B. firmus*	Cd^{2+}	723	35.0 (592)	Ivey *et al.*, (1992)
CadA *S. aureus*	Cd^{2+}	727	34.2 (631)	Nucifora *et al.*, (1989)
hpCopA *H. pylori*	Cu^{2+}	611	34.0 (617)	Ge *et al.*, (1995)

Representation of a search of all accessible protein sequences for homology to the OrfA protein using the FASTA alignment algorithims (Pearson and Lipman, 1988). Sequence identities and length of overlapped regions are shown after the introduction of gaps to optimise the alignment. Only those sequences with significant identity over a region of at least 600 amino acid overlap are reported.

nd not determined

copper transport, this gene was subsequently designated *ctpA* (copper transport protein). This nomenclature will be used in all proceeding references to this open reading frame.

The CLUSTAL W multiple alignment package (Thompson *et al.*, 1994) was used to generate multiple protein sequence alignments of CtpA with a selection of bacterial P-type ATPases (Figure 5.16). Key structural domains present in this family of proteins including 1) Ion transduction domain, 2) Ion channel, 3) Aspartyl kinase domain, and 4) Hinge and ATP binding domain were also identified in CtpA. Consensus sequences which are essential for function of P-type ATPases in cation metabolism were located within structural domains and are indicated in Figure 5.16 (Nucifora *et al.*, 1989; Silver *et al.*, 1989, and references therein). Furthermore, the consensus sequence SEHPL downstream of the phosphorylation site in the Aspartyl kinase domain observed in all putative Cu-transporting ATPases (Tanzi *et al.*, 1993), was also identified in CtpA beginning at amino acid residue 371. It has been suggested this region is involved in copper binding and translocation of this metal through the membrane (Lutsenko and Kaplan, 1995).

5.2.5.2 Characterisation of nucleotide sequence downstream of *ctpA*

A 1.8 kb region of DNA directly downstream of *ctpA* has been partially sequenced (see Figure 5.14). To date, no complete open reading frame has been identified in this region. Nevertheless, the predicted amino acid sequence of all six reading frames was compared for sequence similarity to proteins contained within the Genpept, Swissprot, and Pir protein data bases using FASTA alignment algorithms (Table 5.3). The translated sequence from frame +2 and frame +3, showed significant identity to proteins associated with insertion elements (IS). When frame +2 amino acid sequence was examined, at least 33% of amino acid residues were identical to the transposase proteins isolated from *E. faecium,* IS element IS*1251* (Handwerger *et al.*, 1995), and *S. pneumoniae* IS element IS*1167* (Zhou *et al.*, 1995), while Frame +3 amino acid sequence displayed at least 24% amino acid residue identity to IS*1181* (Derbise *et al.*, 1994) and IS*1251*. The other four reading frames contained no extensive homology to proteins listed in the data bases.

The CLUSTAL W multiple alignment package, was used to generate multiple protein sequence alignments of bacterial transposases with the translated sequence from *L. monocytogenes* frame +2 (Figure 5.17) and frame +3 (Figure 5.18). Significantly, frame +2 was aligned with internal regions of transposase proteins, while frame +3 sequence was aligned

Figure 5.16: Alignments of the key functional domains of CtpA from *L. monocytogenes* with related proteins. Conserved amino acid sequences associated with 1, Ion transduction domain; 2, Ion channel; 3, Aspartyl kinase domain; and 4, Hinge and ATP binding domain are shown. Asterisks indicate identical residues in all sequences represented. Amino acid residues shown in bold typeface are absolutely conserved in all Cu^{2+}-transporting P-type ATPases (Lutsenko and Kaplan, 1995). Sequences were aligned using CLUSTAL W (Thompson *et al.*, 1994). Dashes (-) represent gaps introduced to optimise similarity.

Transduction Domain

L. monocytogenes CtpA	165	GEKVAVDGQIVEGTSTLDESMVTGESMPVEKGPGDN	200
Synechococcus sp. PacS	266	GEKVPVDGEVIDGRSTVDESMVTGESLPVQKQVGDE	301
E. hirae CopA	257	GEQVPTDGRIIAGTSALDESMLTGESVPVEKKEKDM	292
M. leprae AceA1	262	GQTIAADGLVIDGSATVSMSPITGEAKPVRVNPGAQ	297
B. firmus CadA	243	GQKIAMDGVVVSGYSAVNQTAITGESVPVEKTVDNE	278
S. aureus CadA	245	GEKIAMDGIIVNGLSAVNQAAITGESVPVSKAVDDE	280
Human Menkes Mcl	853	GGKFPVDGRVIEGHSMVDESLITGEAMPVAKKPGST	888
		* ** * *** **	

Ion Channel **Aspartyl Kinase Domain**

L. monocytogenes CtpA	291	CPCALGIATP	300	324	EATHDIKTVVMDKTGTITVGKPQVTDIISIGRISENEILRIAAGLEDS**SEHPL**	349
Synechococcus sp. PacS	391	CPCALGLATP	400	424	ELAQTIQTVILDKTGTLTQGQPSVTDFLAIGRDQQQTLLGWAASLENY**SEHPL**	449
E. hirae CopA	382	CPCALGLATP	391	415	EGAAHLNSIILDKTGTITQGRPEVTDV-----IGPKEIISLFYSLEHA**SEHPL**	440
M. leprae AceA1	387	CPCALGLATP	396	420	EATRAVDTVVFDKTGTLTTGQLKVSAVTAAPGWQANEVLQMAATVESASEHAV	445
B.firmus CadA	369	CPCALVISTP	378	402	EEMGALKAIAFDKTGTLTKGKPQVTDYNVLNKINEKELLSIITALEYRSQHPL	427
S. aureus CadA	372	CPCALVISTP	381	404	EKLGAIKTVAFDKTGTLTKGVPVVTDFEVLNQVEEKELFSIITALEYRSQHPL	429
Human Menkes Mcl	1000	CPCSLGLATP	1009	1033	EMAHKVKVVVFDKTGTITHGTPVVNQLTESNRISHHKILAIVGTAESN**SEHPL**	1058
		*** * **		* ***** * * * * * *		

Hinge and ATP Binding Domain

L. monocytogenes CtpA	511	PGDKAQHIRKLQEKG-AVAFVGDGINDAPALSTATVGIAM	549
Synechococcus sp. PacS	613	PDQKAAQVAQLQSRGQVVAMVGDGINDAPALAQADVGIAI	652
E. hirae CopA	600	PEEKANYVEKLQKAGKKVGMVGDGINDAPALRLADVGIAM	639
M. leprae AceA1	603	PEDKVDVIEQLRDRGHVVAMVGDGINDGPALARADLGMAI	642
B. firmus CadA	596	PQDKLDFIKQLRSEYGNVAMVGDGVNDAPALAASTVGIAM	635
S. aureus CadA	598	PQDKLDYIKKMQSEYDNVAMIGDGVNDAPALAASTVGIAM	637
Human Menkes Mcl	1281	PSHKVAKVKQLQEEGKRVAMVGDGINDSPALAMANVGIAI	1320
		* * * *** ** *** *	

Table 5.3 Comparison of amino acid translation products derived from the region of DNA flanking the 3′ end of *ctpA* to transposase proteins from Gram-positive insertion elements.

Organism (insertion element)	Gene symbol	% identical residues (overlapping amino acids)	Reference
	Frame 2 (sense strand)		
E. faecium (IS*1251*).	*orf*	35.2 (261)	Handwerger *et al.*, (1995)
S. pneumoniae (IS*1167*)	-	33.2 (217)	Zhou *et al.*, (1995)
L. delbrueckii (IS*L3*)	*isl3*	29.8 (299)	Germond *et al.*, (1995)
S. aureus (IS*1181*)	*orf439*	29.9 (278)	Derbise *et al.*, (1994)
L. mesenteroides (IS*1165*)	-	27.0 (274)	Johansen and Kibenich, (1992)
L. lactis (IS*1076*)	-	27.9 (172)	Huang *et al.*, (1993)
	Frame 3 (sense strand)		
S. aureus (IS*1181*)	*orf439*	24.3 (395)	Derbise *et al.*, (1994)
E. faecium (IS*1251*).	*orf*	27.9 (219)	Handwerger *et al.*, (1995)

Representation of a search of all accessible protein sequences for similarity to the translation product [frame 2 (sense strand) and frame 3 (sense strand)] from nucleotide sequence flanking the 3′ region of ***ctpA***, using the FASTA alignment algorithms (Pearson and Lipman, 1988). Sequence identities and length of overlapped regions (parentheses) are shown following the introduction of gaps to optimise the alignment. Only those sequences with significant identity over a region of at least 150 amino acids are reported.

```
E. faecium (IS1251)          157 PFR-TPLPKVLCFDEFKSVRGVSGAMSFIMMDGQTQRLLDIV
S. pneumoniae (IS1167)         3 ----------------------------FIAQDFEKLDIITVL
L. delbrueckii (ISL3)        152 TPSFDSLPEHLAFDEFRGVGR---KLHFICQDGEKHTIVAIL
S. aureus (IS1181)           154 IKPFNCLPEHIAMDEFKSVKNVTGSMSFIFIDNDTHDVIDIL
L. mesenteroides (IS1165)    153 PHASRRLPINLCFDEFRS---THGSMSFICIDADTHKSVKVL
L. monocytogenes (fr. +2)    252 P-RFDTLPSVLCFDEYKSMKSCSGKNEFCFYEWADPAINRCI
                                 **         *

E. faecium (IS1251)          198 ENRQLPFLKRYF-SHFSREIREAVEWIVIDMYAPYVSLVKKL
S. pneumoniae (IS1167)        18 EGRTQAIIRNHFL-RYDRVVRCRVKIITMDMFSPYYDLARQL
L. delbrueckii (ISL3)        190 ENRFKNTIIKYFL-QFPEIVRKTVRTVSMDLNCYYGDIVRQI
S. aureus (IS1181)           196 ENRTTRFLRAYFE-RFDLKNRQQVKTVTIDMYEPYVRLFRDL
L. mesenteroides (IS1165)    191 SDRLNRTIKQFFLSQYSTAEWAAVQRVIMDMNASYQAFVHEL
L. monocytogenes (fr. +2)    293 GESSPYFLKTIF--PFHTKARANVKYVVMDMNAPYFELVKAV
                                           *     *     *

E. faecium (IS1251)          239 FPKAQLIIDRFHIVQHIGRTFRNHRIKETNQLLKSKEQKHYQ
S. pneumoniae (IS1167)        59 FPCAKIVLDRFHIVQHLSRAMSRVRVQIMNQL--DRKSHEYK
L. delbrueckii (ISL3)        231 FPNAELVIDRFHMVQMVNRSFIGFRVQVMKQL--DKKSREYK
S. aureus (IS1181)           237 FPNAAIIFDRFHIVQHLNRELNKYRVQVMNEY-RNKKGPDYT
L. mesenteroides (IS1165)    233 FPNAELIIDRFHIIQLMGRTMDTIRTQCLKQL--DKHSREYK
L. monocytogenes (fr. +2)    333 FPNAKIVTNRFHIVKQITRTLNQL-IKTMNRFQKTEPTK-YR
                                 ** *     ***       *                  *   *

E. faecium (IS1251)          281 LGKQLKRYWKLLQKDERKLVYSS-LWRPGFKAHLTETDIVDR
S. pneumoniae (IS1167)        99 A---IKRYWKLIQQDSRKLSD-KHFYRPTFRMHLTNKEILDK
L. delbrueckii (ISL3)        271 L---LKRYWKLYMKKYKDLEGSKQFYDRCLKVPYTPAQIVDE
S. aureus (IS1181)           278 I---FKNNWKVLLMDTSKTIFSKYRWNKSFKAYKRSSDIVEF
L. mesenteroides (IS1165)    273 V---LKSLWRLLHKANPDAQKSRYLFG--LNEYSTEQNAIDI
L. monocytogenes (fr. +2)    373 ----LKRFWKLLLTHAYDLDSSDYQYDRFFRRPMTQKAMVDE
                                     *  *           *

E. faecium (IS1251)          322 LLKGSPALRVGYQLYQDFLYAVKERDYVSFEELLTNNIM--L
S. pneumoniae (IS1167)       137 LLSYSQDLKHHYQLYQLLLFHFQNKEPEKFFGLIEDNL-KQV
L. delbrueckii (ISL3)        310 GLKCNETLKNTYDFMQDFVYALADKDTKKINDLLDSNI-GQY
S. aureus (IS1181)           317 MLSKDDILRHSYELVQGLRKDLRLCNWPKFINRLNSVSKKSV
L. mesenteroides (IS1165)    310 GTDTFPAFKTAYETYIDLHDALMGRHADELKNIITNYQPNGT
L. monocytogenes (fr. +2)    411 LLSYDEQLTRAYETCQLLLYHFKHKDNQSFFDTINSLD-QCL
                                         *

E. faecium (IS1251)          362 PEGYQTTLRTFQKFLPQIKNALQ-QSYSNGPLECLNNHIKVL
S. pneumoniae (IS1167)       178 HPLFQTVFKTFLKDKEKIVNALQLP-YSNAKLEATNNLIKLI
L. delbrueckii (ISL3)        351 CERLKTTIRTLRKNRRAVINGAKMS-YSNGCLEGVNRKIKQI
S. aureus (IS1181)           359 SKGVWKAVKYYRKHQRMLRNTIYYPAFNNGAIEGINNKIKLI
L. mesenteroides (IS1165)    352 P--LDTAMHTLRKNLNGVINAAK-SSYSNGPIEGINRKIKEL
L. monocytogenes (fr. +2)    452 PQWFCKKLTFLNKYKLGIQYALK-PRYSNGALERTNNKIKVI
                                         *                 *   *   *   **
```

Figure 5.17: Alignment of the frame +2 amino acid sequence, deduced from translation of the 1.8 kb nucleotide sequence immediately downstream of *ctpA* from *L. monocytogenes*, with related proteins. Identical residues with respect to IS*1251*, are highlighted in bold typeface. Identical residues in all sequences shown are indicated by an asterisk. Sequences were aligned using CLUSTAL W (Thompson *et al.*, 1994). Dashes (–) represent gaps introduced to optimise similarity.

```
S. aureus (IS1181)          1 ----------MCNDTLELLRIKDENIKYINQEIDVIIKGK
E. faecium (IS1251)         1 ----------MSYTHLIKETLDILDLSVTFNENCLTKEKYKG
L. monocytogenes (fr. +3)  91 LVVNKTIPRKDISMYHCTRKLLGLTDENLFFEEEWLETVEEDG
                                          *   *

S. aureus (IS1181)         31 --K-ATVVNAVLTYKPSACYCCGVKNEGQIHKHGKRVSRITLL
E. faecium (IS1251)        33 --QICHIYRGNLIYTAQECIHCKHQIASDIVRWGTTTVRLLMN
L. monocytogenes (fr. +3) 134 --FRTNLIHAKLSYILSHCRKCGIKNEGQIIKNGSHKTKVQLL
                                * *    *  *       *   *

S. aureus (IS1181)         71 KTQGYNTYLNLAKQRFKCLECNGTFTAKTSIVDESCFISRCVT
E. faecium (IS1251)        74 DVSEYRTYLELKKQRFKCKACQRTFVADTSVAKKHCFISEKVR
L. monocytogenes (fr. +3) 175 PYRATKTELRLVRTRFYCKECQSTFNAQTNLVDENCYLSKELK
                                  * * *   ** *  *  ** * *      *  *

S. aureus (IS1181)         114 QKVIEEATKVKTEIDTAEDNCISPSTVSRIRTKAANSLRIKPF
E. faecium (IS1251)        117 WSVVTRLKKNTSMTEIAAQKNLSVSSVYCIMKRFYRPL-NPFR
L. monocytogenes (fr. +3)  218 VQIALELAKNTIKKELPIAILYQTSFCVSCTPVKRIIL-----
                                       *                      *

S. aureus (IS1181)         156 NCLPEHIAMDEFKSVKN-VTGSMSFIFIDNDTHDVIDILENRT
E. faecium (IS1251)        159 TPLPKVLCFDEFKSVRG-VSGAMSFIMMDGQTQRLLDIVENRQ
L. monocytogenes (fr. +3)  256 VLIRYLRSFVLMSTNRSPAVEKMSFVFMNGQTQQLIGVLENRR
                                           ***                  ***

S. aureus (IS1181)         198 TRFLRAYFE-RFDLKNRQQVKTVTIDMYEPYVRLFRDLFPNAA
E. faecium (IS1251)        201 LPFLKRYFS-HFSREIREAVEWIVIDMYAPYVSLVKKLFPKAQ
L. monocytogenes (fr. +3)  299 LTFLKPYFLNFTRKHERTNTLWIMPLILNLKRSSQTPKSPIAF
                                 **  **        *                    * *

S. aureus (IS1181)         237 IIFDRFHIVQHLNRELNKYRVQVMNEYR----NKKGPDYTIFK
E. faecium (IS1251)        243 LIIDRFHIVQHIGRTFRNHRIKETNQLLKSKEQKHYQLGKQLK
L. monocytogenes (fr. +3)  342 TLSNKSLVRISCESKQTGFKKRNRQSID---DSDSGNYSLRMP

S. aureus (IS1181)         275 NNWKVLLMDTSKTIFS--KYRWNKSFKAYKRS-------SDIV
E. faecium (IS1251)        286 RYWKLLQKDERKLV-Y--SSLWRPGFKAHLTE-------TDIV
L. monocytogenes (fr. +3)  382 MIWIVPIINMIDSFADQRKKRWSMSFVTTSNPGLTRLASSSSI
                                 *                  *   *
```

Figure 5.18: Alignment of the frame +3 amino acid sequence, deduced from translation of the 1.8 kb nucleotide sequence immediately downstream of *ctpA* from *L. monocytogenes*, with related proteins. Identical residues with respect to IS*1181*, are highlighted in bold typeface. Identical residues in all sequences shown are indicated by an asterisk. Sequences were aligned using CLUSTAL W (Thompson *et al.*, 1994). Dashes (–) represent gaps introduced to optimise similarity.

with the N-terminal region of transposase proteins. Furthermore, sequence similarity was restricted to transposases of Gram-positive origin. Moreover, alignment of the inverted repeats of the IS elements listed in Table 5.3, enabled the identification of putative inverted repeats at nucleotide position 2635 and 4206 immediately downstream of *ctpA* (Figure 5.19).

5.2.6 Induction of *ctpA* mRNA expression by metal ions

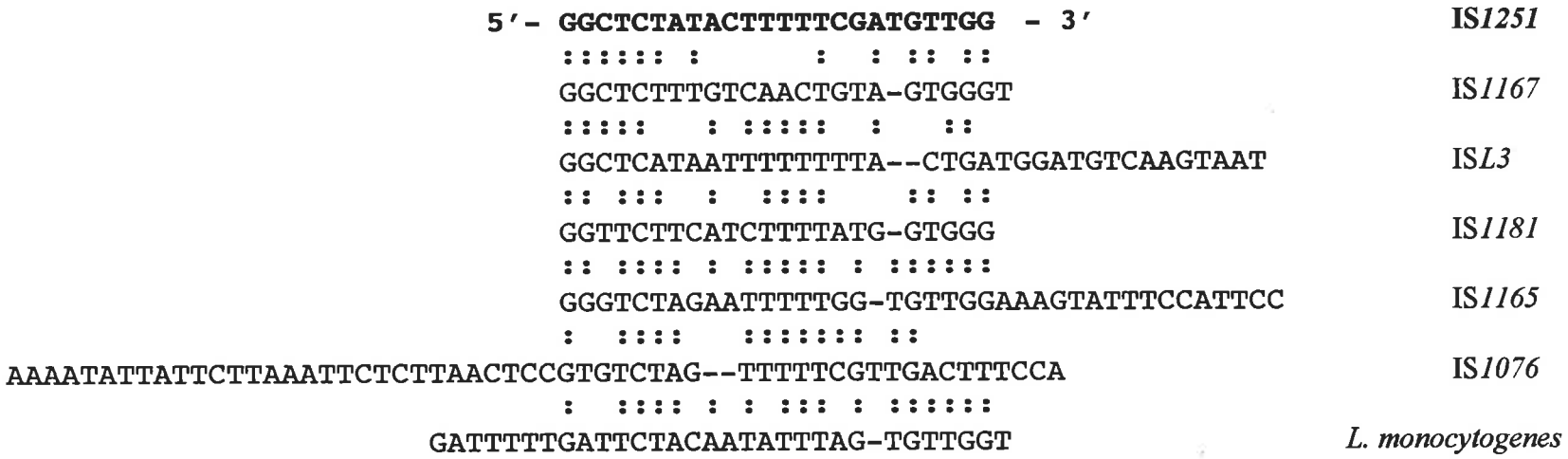
On the basis of sequence comparisons, CtpA is predicted to be a P-type ATPase from *L. monocytogenes*. This protein was most similar to a family of proteins involved in the transport of copper in both bacteria and eukaryotes. In view of this, examination of levels of *ctpA* mRNA under inducing conditions, was used to provide preliminary evidence that CtpA corresponds to an active copper transport system. In this analysis, the chelating agents EGTA and 8-hydroxyquinoline, were used to deplete the bacterial culture medium of copper. To determine whether *ctpA* mRNA levels respond to copper in the growth medium, total RNA from DRDC8 was prepared from cells grown to early logarithmic phase in BHI broth, followed by incubation in the same medium supplemented with each heavy metal ion at a sub-inhibitory concentration. RNA preparations were subjected to DNA:RNA slot blot hybridisation analysis using a DIG labelled 1.73 kb *PstI*/*Sac*I DNA fragment internal to *ctpA* as a probe. In the absence of added metal ions, *ctpA* specific RNA was barely detected (Figure 5.20, Lane 1), whereas in the presence of high and low concentrations of copper, *ctpA* signals were intense (Figure 5.20, Lane 2-4). *ctpA* specific RNA was not detected in the presence of other metal ions (Cd^{2+}, Hg^{2+}, Ni^{2+}, and Zn^{2+}) (Figure 5.20, Lane 5-8). Plasmid pCT220 was included as a control and hybridised strongly to probe DNA (Figure 5.20, Lane 9). From this result, both high (BHI + 4 mM $CuSO_4$) and low (BHI + 10 mM EGTA or 5 μM 8-hydroxyquinoline) concentrations of copper were specifically involved in the regulation of *ctpA* expression. This effect could be mediated by changes in mRNA stability and/or an upregulation of transcription.

5.2.7 Analysis of regulatory nucleotide sequences flanking *ctpA*

The *ctpA* promoter region was mapped by primer extension analysis of the 5′ end of the *ctpA* RNA transcript (Figure 5.21), using the synthetic oligonucleotide p1037 (highlighted in Figure 5.14). Oligonucleotide p1037, binds at nucleotide 584. Three transcriptional start sites for *ctpA* were identified at nucleotide 487 (P1 transcript), 470 (P2 transcript), and 469 (P3 transcript) (see Figure 5.14 and 5.21). Experiments which preclude the possibility that one or more of the primer extension products represent degradation artefacts were not carried out. Primer extension products were only detected when the RNA template used in the

Figure 5.19: Sequence similarity among terminal inverted repeats of IS*1251*, IS*1167*, IS*L3*, IS*1181*, IS*1165*, and IS*1076*, and the putative left (A) and right (B) inverted repeat from *L. monocytogenes* DNA. Nucleotides identical with respect to the terminal inverted repeats of IS*1251* (shown in bold typeface), are indicated by colons. Dashes (-) represent gaps introduced to optimise similarity.

A


5′- GGCTCTATACTTTTTCGATGTTGG - 3′
IS1251
GGCTCTTTGTCAACTGTA-GTGGGT
IS1167
GGCTCATAATTTTTTTTA--CTGATGGATGTCAAGTAAT
ISL3
GGTTCTTCATCTTTTATG-GTGGG
IS1181
GGGTCTAGAATTTTTGG-TGTTGGAAAGTATTTCCATTCC
IS1165
AAAATATTATTCTTAAATTCTCTTAACTCCGTGTCTAG--TTTTTCGTTGACTTTCCA
IS1076
GATTTTTGATTCTACAATATTTAG-TGTTGGT
L. monocytogenes

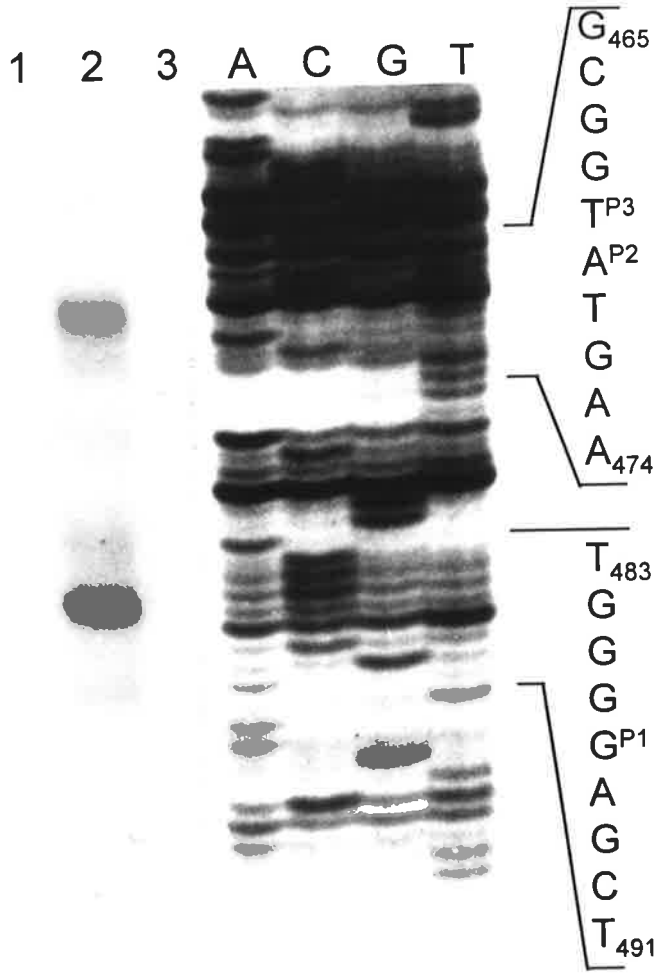
B


IS1251
3′- GGTTGTAGTAAACTG-TTTCTCGG - 5′
IS1167
TGGGTGATGTTTA-TAATATCTTGG
ISL3
TAAAGAACTGTTCGTAGTCATTCTAAACTG-TTTCTCGG
IS1181
GGGTGGT-GTAAACCA-CCTCTTGG
IS1165
CCTATCTTTGATAGGAAGTTGTGGTAAACTG-TTTGAAGG
IS1076
TGGAAAGTCAACGAAAAACT--AGACACGGAGTTAAGAGAATTTAAGAATAATATTTTTCAAAGT
L. monocytogenes
TGGTCGTGATTTACAG-CTTCTCGGTTTTTAG

Figure 5.20: Effect of heavy metals and cation chelators on the transcription of the *ctpA* gene. RNA (5 μg/preparation) was probed with a digoxigenin labelled 1.73 kb *Pst*I/*Sac*I DNA fragment internal to *ctpA*. Lane: [1], BHI broth medium (control); [2], 4 mM Cu^{2+}; [3], 10 mM EGTA; [4], 5 μM 8-hydroxyquinoline; [5], 2 mM Cd^{2+}; [6], 12.5 μM Hg^{2+}; [7], 9 mM Ni^{2+}; [8], 10 mM Zn^{2+}; [9], 1 μg pCT220 (plasmid DNA control containing the *ctpA* gene)


1
2
3
4
5
6
7
8
9

Figure 5.21: Primer extension analysis of *ctpA* promoter transcripts in *L. monocytogenes* DRDC8 grown in BHI broth in the presence of 4 mM $CuSO_4$. Sites of potential transcription initiation are shown (designated P1, P2, and P3). Transcript lengths were determined by dideoxy sequencing reactions using the plasmid, pCT220, as the DNA template containing the wild type *ctpA* gene. The letters above each lane indicate the dideoxynucleotide used to terminate each reaction. Lane: [1], *E. coli* DH5α:pGEM-7Zf(+); [2], *L. monocytogenes* DRDC8; [3], *E. coli* DH5α:pCT220.


1
2
3
A
C
G
T
G465
C
G
G
TP3
AP2
T
G
A
A474
T483
G
G
G
GP1
A
G
C
T491

reaction was isolated from *L. monocytogenes* DRDC8 grown in the presence of non-inhibitory concentrations of $CuSO_4$. Under these conditions, levels of specific *ctpA* mRNA were most abundant (see Section 5.2.6). These transcriptional start sites were located 56, 73, and 74 bp, respectively, from the putative translation initiation codon (ATG), (position 543 in Figure 5.14). The transcript originating from P1, was marginally more abundant than transcripts initiated from P2 and P3. At the spatial regions corresponding to the –10 and –35 regions of each transcript, the –10 regions share good similarity and the –35 regions have moderate similarity, when compared with consensus *E. coli* promoter regions (Hoopes and McClure, 1987) (Table 5.4). Furthermore, a putative ribosome binding site (AAGGAGA), was located 6 bp upstream of the ATG start codon (see Figure 5.14), and displayed high nucleotide identity to *E. coli* consensus sequences (Table 5.4).

In addition, 17 bp and 21 bp palindromic repeat sequences, located downstream of the *ctpA* stop codon, at nucleotide position 2648 and 2592, respectively, were identified using the University of Wisconsin Genetics Computer Group (GCG) StemLoop algorithm (Devereux *et al.*, 1984), using default parameters (Figure 5.22). These two repeat sequences potentially represent transcriptional terminators of *ctpA*.

5.2.8 Amino acid composition and codon usage of CtpA

An analysis of the amino acid composition of CtpA and selected P-type ATPases from both bacterial and eukaryotic origins is shown in Table 5.5. Also shown are the predicted isoelectric point values for each protein. The utilisation of amino acids among these proteins was relatively conserved. However, the degree of conservation of amino acid composition was more striking in proteins expressed by Gram-positive bacteria [CtpA (this study) and CadA (Lebrun *et al.*, 1994) from *L. monocytogenes*, and CopA (Odermatt *et al.*, 1993) from *E. hirae*]. Nevertheless, differences were observed. For example, cysteine residues were more predominant in the P-type ATPase associated with Menkes disease in humans (Chelly *et al.*, 1993; Mercer *et al.*, 1993; and Vulpe *et al.*, 1993), primarily due to multiple cysteine residues associated with the N-terminal repeat sequences thought to be responsible for initial cation binding. Furthermore, the amino acid composition of PacS (Kanamaru *et al.*, 1993) from the cyanobacterium *Synechococcus* spp., has a comparatively low proportion of lysine residues, but glutamine and tryptophan residues were more abundant than in the other proteins critically examined. In agreement with the overall amino acid composition, the predicted isoelectric

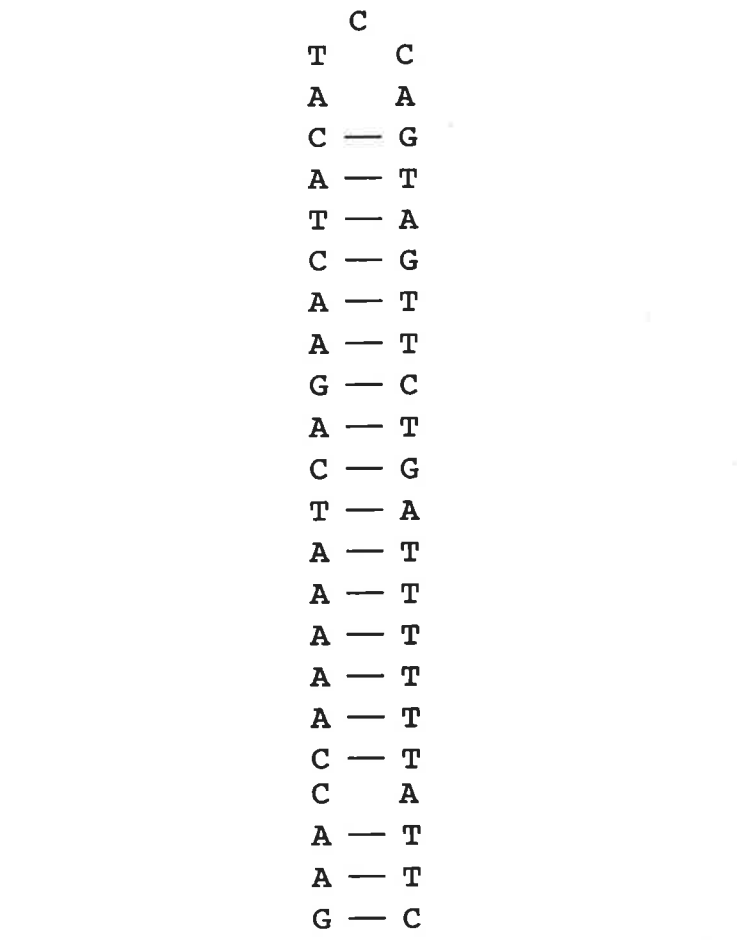
Table 5.4 Comparison of the putative promoter elements and ribosome binding site of *ctpA* with the consensus δ^{70} promoter elements (–10 and –35 regions) and ribosome binding site (RBS) from *E. coli*

Organism	Nucleotide sequence of regulatory elements for gene expression		
	–10 region	–35 region	RBS
E. coli	TATAAT	TTGACA	AAGGAGG
L. monocytogenes ctpA			**AAGGAG**A
$P1_{487}$	**TATGAA**	**AGGACG**	
$P2_{470}$	**CATAAT**	**TTCACT**	
$P3_{469}$	**CATAAT**	**TTCACT**	

E. coli consensus regulatory elements were reported by Hoopes and McClure, (1987). Nucleotides identical to *E. coli* regulatory sequences are indicated in bold typeface.

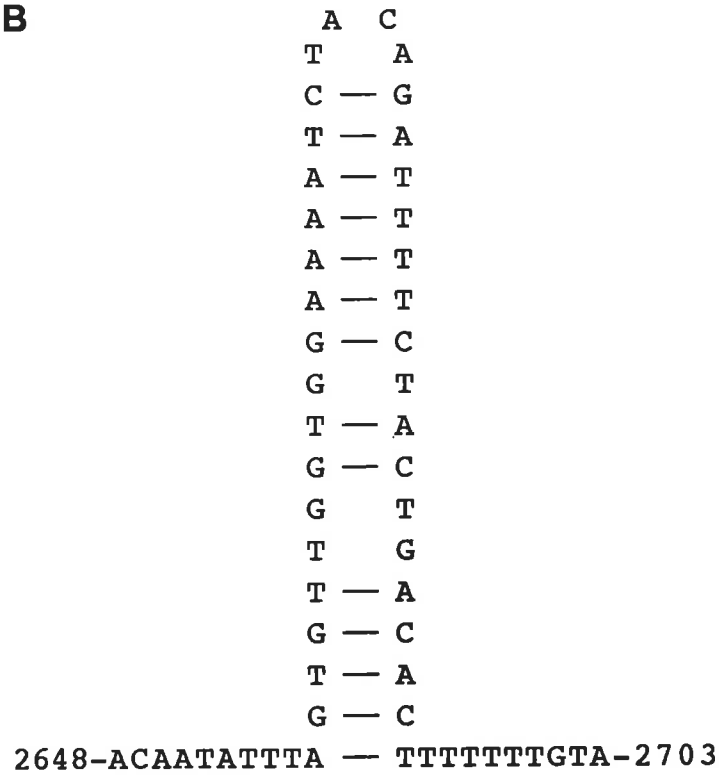
Figure 5.22: Diagrammatic representation of two inverted repeat sequences downstream of *ctpA*, identified by the University of Wisconsin GCG StemLoop algorithm, using default parameters (Devereux *et al.*, 1984). These repeat sequences are potentially involved in transcriptional termination of *ctpA*. Nucleotide positions, length of repeat sequence, and binding energy are indicated.

A



Length: 21bp
Energy: 46.0 kcal

B



Length: 17 bp
Energy: 36.0 kcal

Table 5.5 Comparison of the amino acid composition and isoelectric point of CtpA with closely related P-type ATPases

Amino acid residue	CtpA (653aa)		CadA (711aa)		PacS (747aa)		CopA (727aa)		Mcl (1500aa)	
	No.	Mol%	No.	Mol%	No.	Mol%	No.	Mol%	No.	Mol%
A (Ala)	65	9.94	70	9.85	96	12.85	76	10.45	106	7.07
C (Cys)	2	0.31	4	0.56	6	0.80	4	0.55	26	1.73
D (Asp)	35	5.35	34	4.78	28	3.75	32	4.40	66	4.40
E (Glu)	27	4.13	45	6.33	33	4.42	41	5.64	92	6.13
F (Phe)	26	3.98	29	4.08	18	2.41	29	3.99	43	2.87
G (Gly)	57	8.72	59	8.3	67	8.97	60	8.25	92	6.13
H (His)	7	1.07	11	1.55	6	0.80	16	2.20	35	2.33
I (Ile)	66	10.09	70	9.85	57	7.63	67	9.22	129	8.60
K (Lys)	35	5.35	44	6.19	13	1.74	48	6.60	88	5.87
L (Leu)	62	9.48	66	9.28	84	11.24	71	9.77	135	9.00
M (Met)	25	3.82	20	2.81	14	1.87	28	3.85	44	2.93
N (Asn)	24	3.67	25	3.52	16	2.14	24	3.30	67	4.47
P (Pro)	20	3.06	18	2.53	30	4.02	22	3.03	62	4.13
Q (Gln)	15	2.29	29	4.08	63	8.43	35	4.81	54	3.60
R (Arg)	19	2.91	19	2.67	37	4.95	13	1.79	48	3.20
S (Ser)	50	7.65	35	4.92	36	4.82	42	5.78	133	8.87
T (Thr)	41	6.27	51	7.17	51	6.83	50	6.88	100	6.67
V (Val)	61	9.33	60	8.44	66	8.84	50	6.88	141	9.4
W (Trp)	4	0.61	6	0.84	16	2.14	4	0.55	9	0.60
Y (Tyr)	12	1.83	16	2.25	10	1.34	15	2.06	30	2.00
Isoelectric point (pH)	5.71		5.42		5.33		6.09		6.26	
	% of residues with common chemical characteristics									
Acidic	9.5		11.1		8.2		10.1		10.5	
Basic	9.3		10.4		7.5		10.6		11.4	
Neutral-Polar	58.5		55.1		57.8		55.4		50.2	
Neutral-Nonpolar	22.7		23.4		26.5		23.9		27.9	

All data are derived from the sequence shown in Figure 5.14 (CtpA), and from the Genbank database. Accession numbers of the *L. monocytogenes* CadA, *Synechococcus* spp. PacS, *E. hirae* CopA, and *H. sapien* Mcl (Menkes disease) proteins are summerised in Table 5.7. Compositional analysis of an amino acid residue is determined as a percentage of the total number of amino acid residues per sequence. Acidic (D, E); Basic (H, K, R), Neutral Polar (A, F, G, I, L, M, P, V), Neutral Nonpolar (C, N, Q, S, T, W, Y).

point of all protein sequences examined was similar. These proteins are typically acidic (pH values range between 5.33 for PacS to 6.26 for the Menkes associated protein).

The pattern of codon usage, as well as the G+C of *L. monocytogenes* CtpA and CadA, is documented in Table 5.6. Interestingly, striking differences in codon usage were observed between CtpA and CadA. For example, alanine, arginine, proline, serine, threonine, tyrosine, and valine were predominantly encoded by different codons. Nevertheless, CtpA generally conformed to the codon usage established for *L. monocytogenes* based on translated sequences of genes isolated from this bacterium. Moreover, the G+C content of CtpA and CadA was consistent with the overall G+C content of *L. monocytogenes* reported previously (Seeliger and Jones, 1986; Feresu and Jones, 1988).

5.2.9 Evolutionary relatedness of CtpA

In this section, an assessment of evolutionary relatedness of CtpA among all known bacterial P-type ATPases was performed essentially as described by Fagan and Saier, (1994). The P-type ATPases associated with Menkes and Wilson disease in human's were included in this comparison. A summary of all proteins used in the analysis is given in Table 5.7. Aligned segments of each amino acid sequence were subjected to phylogenetic analysis. Segment 1 comprised the N-terminal sequence which consisted of the Metal binding, Ion transduction, and Ion channel domains of these ATPases. Segment 2 consisted of the highly conserved Aspartyl kinase, Hinge and ATP binding domains. Segment 3 included the remaining C-terminal region. The full length P-type ATPase protein sequences were also subjected to phylogenetic analysis (multiple sequence alignments are shown in Appendix A).

Essentially, analysis consisted of computation of distance measures using maximum likelihood estimates based on the Dayhoff PAM matrix (Dayhoff *et al.*, 1983). These distance matrix data was then used to estimate phylogenies under an ultrametric model assuming an evolutionary clock, the Fitch-Margoliash and other least squares criteria. A detailed explanation of the methods are described in documents accompanying the Phylip package by J. Felsenstein, University of Washington, USA, available via the WWW (http://evolution.genetics.washington.edu/phylip.html).

With the exception of Segment 3 sequences, phylogenetic analysis indicated CtpA was most closely related to a class of P-type ATPases involved in copper transport including PacS

Table 5.6 Codon usage of *L. monocytogenes* CtpA, compared to *L. monocytogenes* CadA and a collection of sequenced genes of *L. monocytogenes*. The G+C content (Mol%) of each gene is also indicated.

Amino acid	Codon	CtpA	CadA	*L. monocytogenes*[a]
Phe	UUU	69.2	79.3	63.6
	UUC	30.8	20.7	36.4
Leu	UUA	35.6	36.3	41.0
	UUG	29.0	18.2	13.9
	CUU	22.6	16.7	19.2
	CUC	0.0	3.0	5.6
	CUA	6.4	15.2	16.3
	CUG	6.4	10.6	4.0
Ile	AUU	62.1	75.7	54.4
	AUC	16.7	15.7	30.1
	AUA	21.2	8.6	15.5
Met	AUG	100.0	100.0	100.0
Val	GUU	44.2	38.3	35.1
	GUC	19.7	11.7	11.3
	GUA	27.9	21.7	35.1
	GUG	8.2	28.3	18.4
Ser	UCU	22.0	23.7	19.7
	UCC	12.0	18.4	11.7
	UCA	38.0	18.4	19.9
	UCG	6.0	18.4	9.6
	AGU	14.0	15.8	23.1
	AGC	8.0	5.3	16.0
Pro	CCU	45.0	27.8	28.4
	CCC	0.0	16.7	6.5
	CCA	55.0	44.4	52.7
	CCG	0.0	11.1	12.4
Thr	ACU	43.9	31.4	32.1
	ACC	19.5	9.8	9.0
	ACA	31.7	41.2	42.7
	ACG	4.9	17.6	16.2
Ala	GCU	41.5	22.9	35.8
	GCC	9.3	22.9	9.0
	GCA	41.5	35.7	39.1
	GCG	7.7	18.5	16.1
Tyr	UAU	66.7	87.5	70.6
	UAC	33.3	12.5	29.4
His	CAU	57.1	54.5	64.3
	CAC	42.9	45.5	35.7
Gln	CAA	80.0	82.8	85.4
	CAG	20.0	17.2	14.6

Table 5.6 continued over .....

Table 5.6 continued

Asn	AAU	66.7	66.7	65.5
	AAC	33.3	33.3	34.5
Lys	AAA	77.1	79.5	86.1
	AAG	22.9	20.5	13.9
Asp	GAU	80.0	64.7	73.1
	GAC	20.0	35.3	26.9
Glu	GAA	66.7	73.3	84.5
	GAG	33.3	26.7	15.5
Cys	UGU	100.0	75.0	62.5
	UGC	0.0	25.0	37.5
Trp	UGG	100.0	100.0	100.0
Arg	CGU	47.4	15.8	32.1
	CGC	10.5	5.3	14.2
	CGA	15.8	31.6	11.4
	CGG	10.5	5.3	8.7
	AGA	10.5	36.7	30.4
	AGG	5.3	5.3	3.2
Gly	GGU	33.3	33.8	38.6
	GGC	14.1	15.3	20.9
	GGA	36.8	35.6	29.7
	GGG	15.8	15.3	10.8
Stop	UAA	0.0	100.0	81.8
	UAG	100.0	0.0	6.8
	UGA	0.0	0.0	11.4
G+C Mol%		39.2	40.0	36-42[b]

[a] codon usage tabulated from 44 open reading frames from *L. monocytogenes* that were listed in the Genbank database (release no. 92), calculated using the CUTG program (Nakamura *et al.*, 1996)

[b] average G+C Mol% of *L. monocytogenes* reported by Seeliger and Jones, (1986) and Feresu and Jones, (1988)

Table 5.7 Summary of P-Type ATPases examined in this study.

Cation specificity	Organism	Abbreviation used	Gene	Length (amino acids)	Genbank accession number
Ca^{2+}	*Synechococcus* spp.	Syn I Ca^{2+}	*pacL*	926	D16436
Ca^{2+}	*Synechocystis* spp.	Syn Ca^{2+}	*pma1*	915	X71022
Cd^{2+}	*B. firmus*	Bfi E Cd^{2+}	*cadA*	723	M90750
Cd^{2+}	*L. monocytogenes*	Lmo E Cd^{2+}	*cadA*	711	L28104
Cd^{2+}	*S. aureus*	SauA E Cd^{2+}	*cadA*	804	L10909
Cd^{2+}	*S. aureus*	SauB E Cd^{2+}	*cadA*	727	J04551
Cu^{2+}	*E. hirae*	Ehi I Cu^{2+}	*copA*	727	L13292
Cu^{2+}	*E. hirae*	Ehi E Cu^{2+}	*copB*	745	L13292
Cu^{2+}	*H. pylori*	Hpy E Cu^{2+}	*copA*	611	L33259
Cu^{2+}	*H. sapiens*	HsaA Cu^{2+}	*mcl*	1500	L06133
Cu^{2+}	*H. sapiens*	HsaB Cu^{2+}	*wcl*	1411	U03464
Cu^{2+}	***L. monocytogenes*[a]**	**Lmo I Cu^{2+}**	***ctpA***	**653**	**U15554**
Cu^{2+}	*Synechococcus* spp.	Syn E Cu^{2+}	*pacS*	747	D16437
Cu^{2+}	*Synechococcus* spp.	Syn Cu^{2+}	*ctaA*	790	U04356
K^{+}	*E. coli*	Eco I K^{+}	*kdpB*	682	K02670
Mg^{2+}	*S. typhimurium*	StyA I Mg^{2+}	*mgtA*	902	U07843
Mg^{2+}	*S. typhimurium*	StyB I Mg^{2+}	*mgtB*	915	M57715
nk	*B. japonicumi*	Bja	*fixI*	730	X95634
nk	*E. coli*	EcoA	*hra-1*	721	U16658
nk	*E. coli*	EcoB	*hra-2*	731	U16659
nk	*E. coli*	EcoC	*orf*	732	U00039
nk	*E. coli*	EcoD	-	834	U58330
nk	*E. coli*	EcoE	*mgtA*	898	U14003
nk	*H. influenzae*	Hin	-	724	U32824
nk	*H. pylori*	Hpy	*orf*	686	L46864
nk	*M. leprae*	MleA	*ctpA*	750	Z46257
nk	*M. leprae*	MleB	*ctpB*	780	Z46257
nk	*P. mirabilis*	Pmi	-	829	U42410
nk	*R. meliloti*	Rme	*fixI*	757	M24144
nk	*Synechococcus* spp.	SynA	-	293	X05925
nk	*Synechocystis* spp.	SynB	-	945	D64005
nk	*Synechocystis* spp.	SynC	-	642	D64005
nk	*Synechocystis* spp.	SynD	-	721	D64005

[a] Evidence that CtpA functions as a copper influx transporter in *L. monocytogenes* is based on the the observation that growth of $CtpA^{-}$ mutants is significantly restricted by the Cu^{2+} chelator 8-hydroxyquinoline

I cation influx

E cation efflux

nk cation specificity not known

(Kanamaru *et al.*, 1993), CopA (Odermatt *et al.*, 1993), and an uncharacterised protein from *P. mirabilis* (Petrukhin, 1995; Genbank submission) (Figure 5.23 to 5.25). In common with other work (Silver *et al.*, 1993; Fagan and Saier, 1994; Phung *et al.*, 1994), these ATPases showed significant homology to the proteins associated with copper metabolism disorders in humans, Menkes disease (Chelly *et al.*, 1993; Mercer *et al.*, 1993; Vulpe *et al.*, 1993) and Wilson disease (Bull *et al.*, 1993). Phylogenetic analysis reliably grouped P-type ATPases of similar function. In addition to ATPases involved in copper transport, cadmium transporters and proteins more closely related to ATPases of eukaryotic origin, form distinct groups based on amino acid relatedness. However, the phenogram generated from Segment 2 amino acid sequences established sub-groups within ATPase proteins of identical cation specificity. Moreover, the distance method for representing phenograms could not be used for multiple sequence alignments of the C-terminus (Segment 3). Examination of peptide sequences from this region revealed extensive variability among a minority of proteins, although some sequence consensus among more closely related proteins was observed. Thus, the phylogenetic tree resulting from alignment of Segment 3 peptide sequences, but based on parsimony analysis (shown in Appendix B), could not reliably classify ATPases with similar cation specificity into evolutionary related groups. For example, only cadmium transporters were consistently grouped together.

5.2.10 Prediction of CtpA membrane topology

Analysis of the hydrophobicity of proteins has been routinely used to demonstrate structural relatedness (Fagan and Saier, 1994). In this study, the hydropathy profiles of CtpA and selected bacterial P-type ATPases were aligned (Figure 5.26). Using the algorithm developed by Kyte and Doolittle, (1982) to calculate hydropathy profiles, remarkable structural similarity was consistently observed. In all proteins, eight regions of high hydrophobicity were identified. However, in this alignment, CtpA was distinctive by virtue of an N-terminal truncation of a domain believed to be associated with initial cation binding.

In the past, hydrophobic measurements were used to predict α-helical transmembrane domains. This approach has now been incorporated into complex algorithms also combining evolutionary information from multiple peptide sequence alignments, statistical analysis, and neural networking designed to predict protein secondary structure. Multiple prediction algorithms have recently lead to several reports of topology models for bacterial P-type ATPase proteins which contain six to ten transmembrane domains. In view of this, the utility

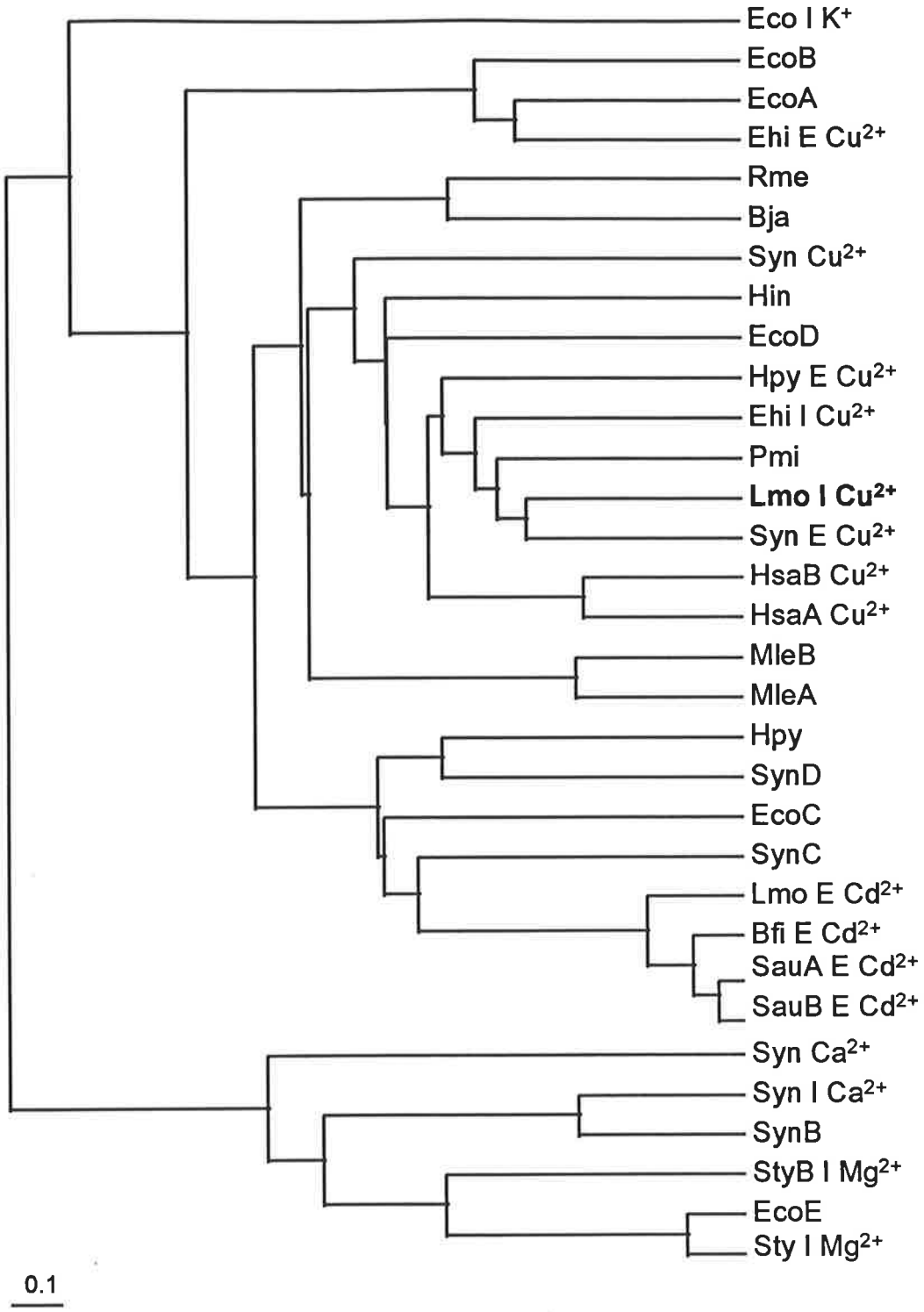


Figure 5.23: Unrooted phylogenetic tree derived from Segment 1 amino acid sequences from bacterial P-type ATPases. The putative Cu^{2+} P-type ATPases associated with Human Menkes and Wilson disease were included for comparison given their high degree of identity to CtpA over a 300 amino acid overlap (in the order of 40% identity, data not shown). Abbreviations are as indicated in Table 5.7. Scale represents arbitrary units, and the distances are considered to reflect actual evolutionary distances. The CtpA ATPase identified in this study is highlighted in bold typeface.

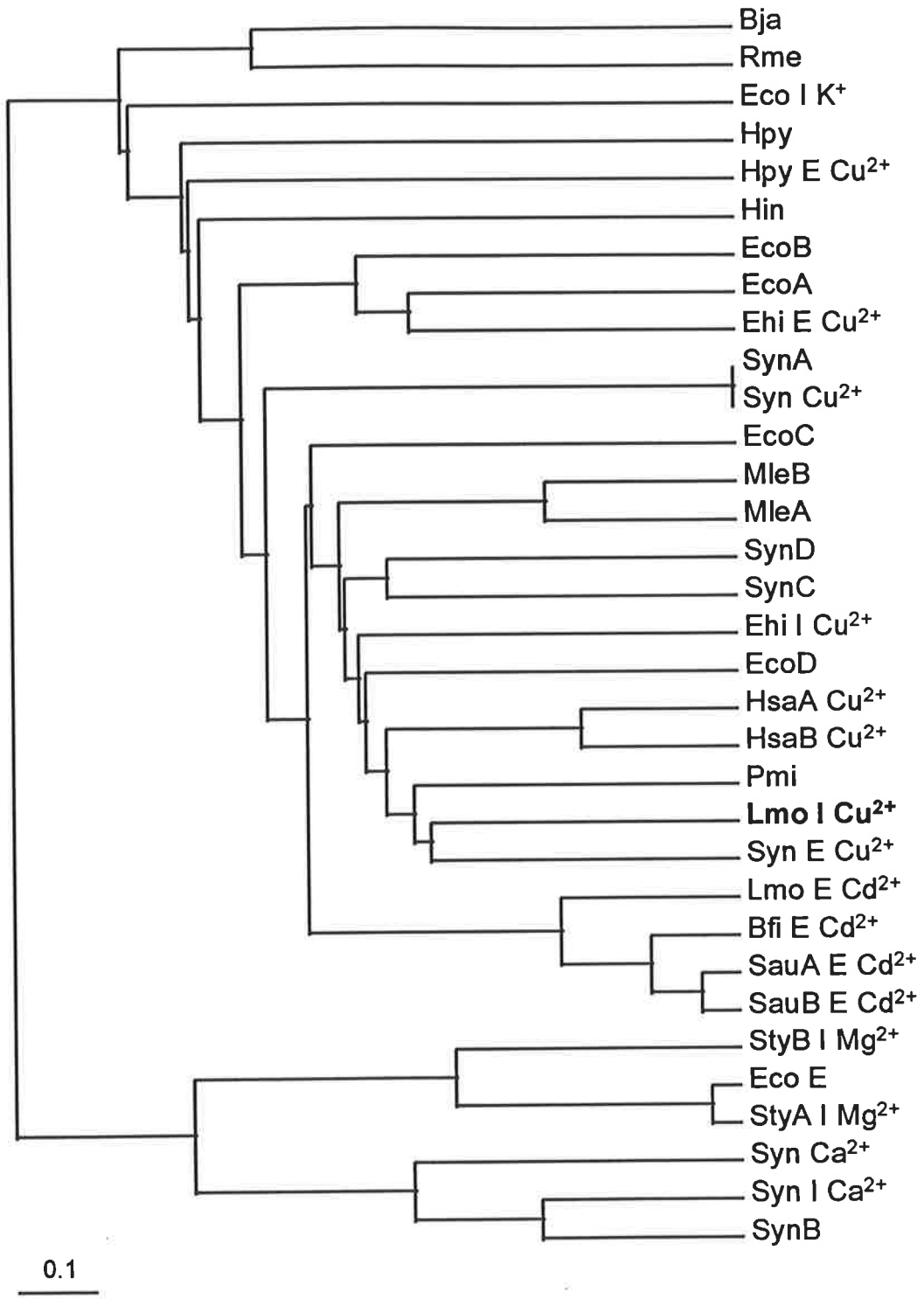


Figure 5.24: Unrooted phylogenetic tree derived from Segment 2 amino acid sequences from bacterial P-type ATPases. The putative Cu^{2+} P-type ATPases associated with Human Menkes and Wilson disease were included for comparison given their high degree of identity to CtpA over a 300 amino acid overlap (in the order of 40% identity, data not shown). Abbreviations are as indicated in Table 5.7. Scale represents arbitrary units, and the distances are considered to reflect actual evolutionary distances. The CtpA ATPase identified in this study is highlighted in bold typeface.

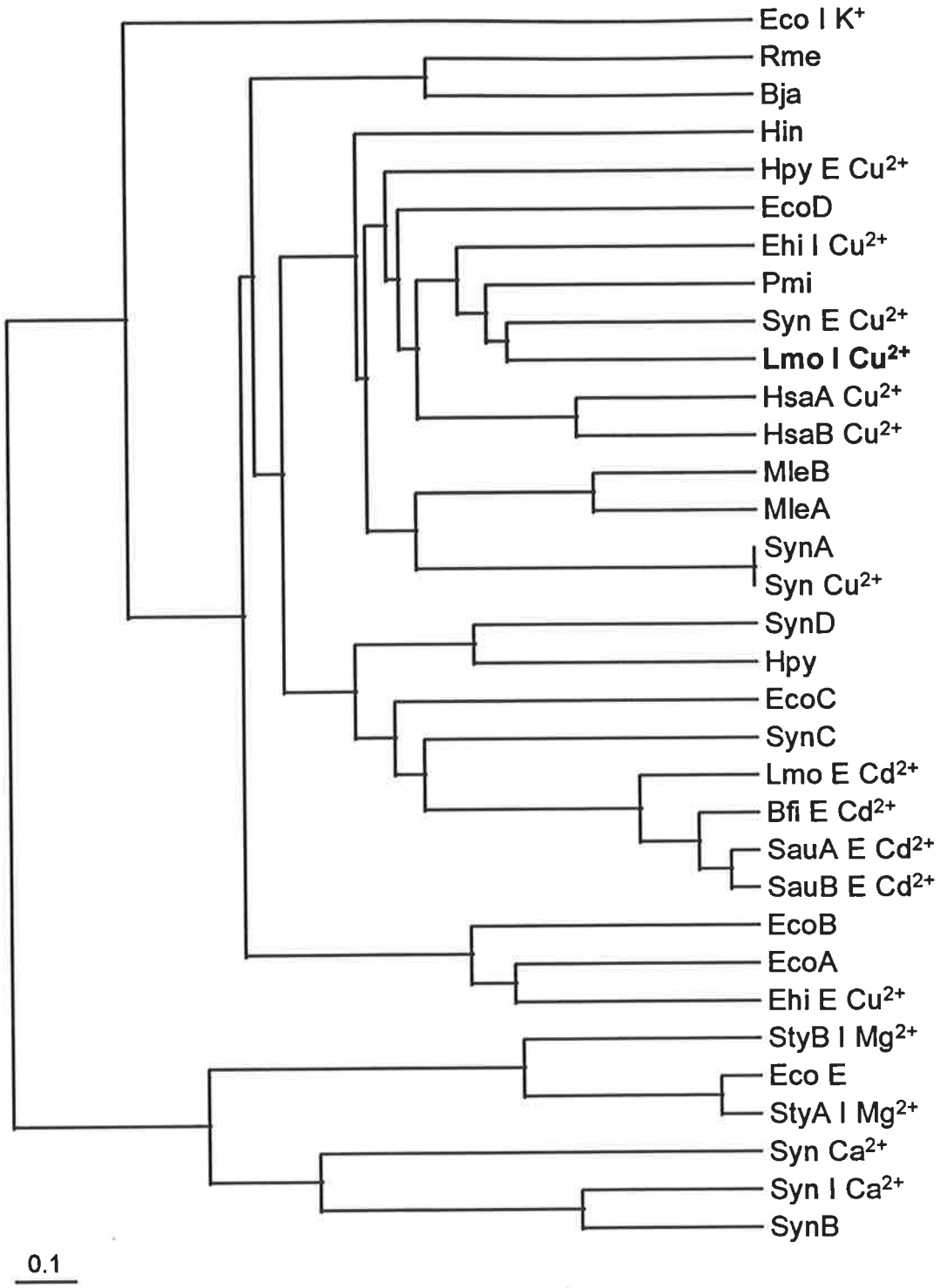


Figure 5.25: Unrooted phylogenetic tree derived from complete peptide sequences from bacterial P-type ATPases. The putative Cu^{2+} P-type ATPases associated with Human Menkes and Wilson disease were included for comparison given their high degree of identity to CtpA over a 300 amino acid overlap (in the order of 40% identity, data not shown). Abbreviations are as indicated in Table 5.7. Scale represents arbitrary units, and the distances are considered to reflect actual evolutionary distances. The CtpA ATPase identified in this study is highlighted in bold typeface.

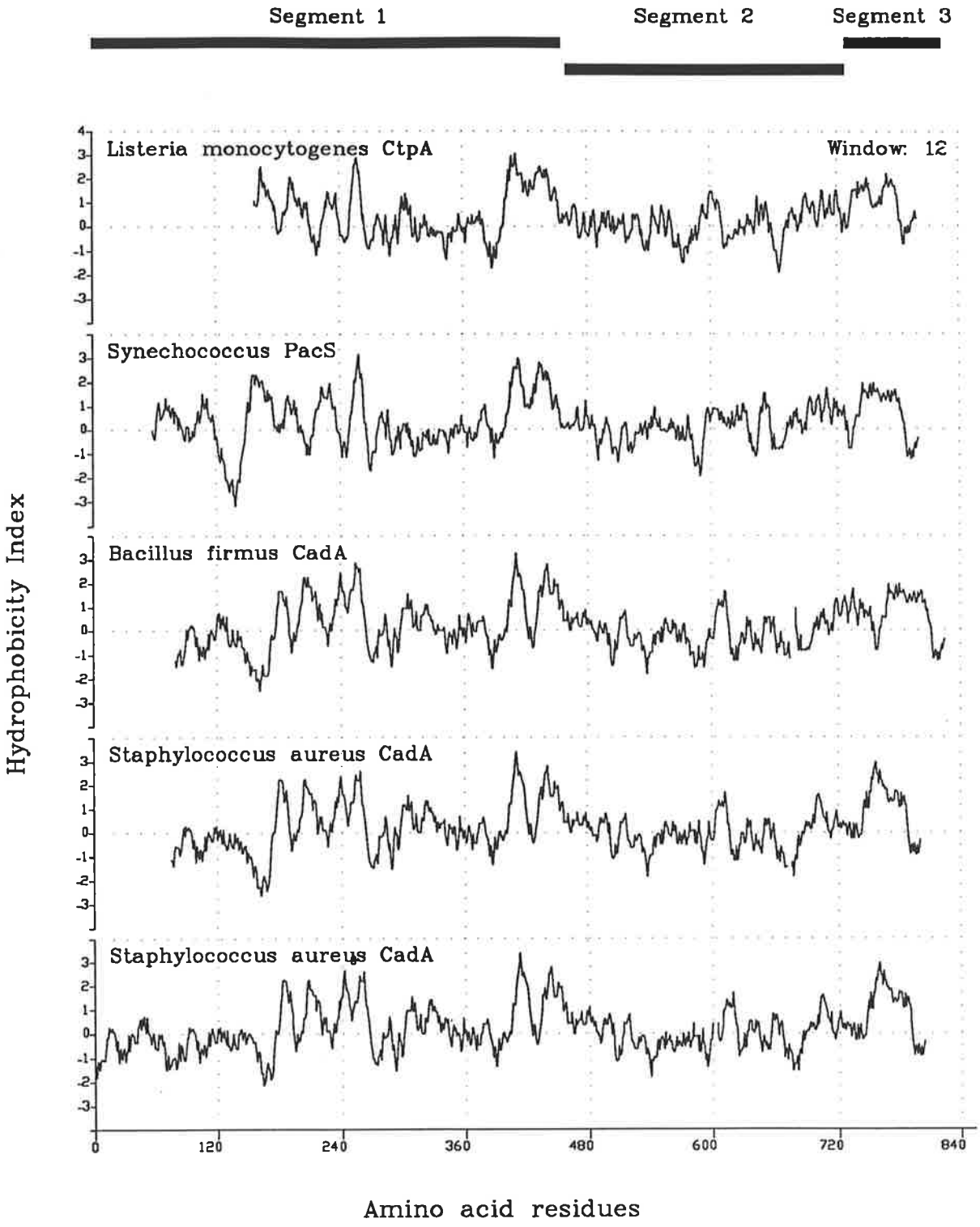


Figure 5.26: Aligned hydropathy profiles of CtpA and selected bacterial P-type ATPases calculated by the method of Kyte and Doolittle, (1982), using a window span of 12 residues. Hydrophobic domains are indicated by regions of the graph above the horizontal axis. Also shown are the amino acid segment divisions used in evolutionary relatedness studies of bacterial P-type ATPases (see Section 5.2.9).

of computer based modelling is best demonstrated in combination with protein fusion or epitope mapping data. However, this data is only available for a limited number of proteins. In most cases therefore, use of prediction algorithms is the only available option.

In an effort to establish a relevant topology model of CtpA on the basis of sequence data alone, several prediction algorithms available via the WWW were employed. Secondary structures predicted by these algorithms were evaluated using the following criteria: 1) approximately 21 amino acids are required to transverse the membrane in an α-helical structure; 2) cytoplasmic boundaries of transmembrane segments are preferentially defined by positively charged amino acid residues in the cytoplasm (Von Heijne, 1992); 3) periplasmic boundaries predominantly locate negatively charged residues near the boundary surface within the periplasmic space; 4) cytoplasmic location for the amino and carboxyl termini of P-type ATPase proteins; 5) the C-P-C amino acid stretch associated with the Ion channel is located in a membrane spanning region; and 6) conserved amino acid residues associated with Ion transduction, Aspartyl kinase, Hinge and ATP binding domains are located within two large cytoplasmic loops. These criteria have been routinely used to predict membrane topology of ATPases (Smith *et al.*, 1993; and references therein). Secondary structures predicted by the algorithms: SSP (Solovyev and Salamov, 1994), SOPMA (Geourjon and Deleage, 1994), nnPredict (Kneller *et al.*, 1990), SSPRED (Mehta *et al.*, 1995), PHDsec (Rost and Sander, 1993; Rost and Sander, 1994), and TMpred (Hofmann K. and Stoffel W., Swiss Institute for Experimental Cancer Research, Lausanne, Switzerland) (see Appendix C for α-helical predictions) were rejected for the following reasons. Firstly, the C-P-C residues in CtpA, presumably associated with the Ion channel, were not located within a transmembrane domain. Secondly, the predicted positions of the putative functional domains based on the location of corresponding consensus amino acid sequences, were rarely located within the large cytoplasmic loops. Thirdly, the N- and C-termini were rarely simultaneously located within the cytoplasm. Finally, α-helical domains predicted by individual algorithms were randomly distributed throughout the peptide sequence. As a result, no consensus putative membrane spanning domains could be assigned for CtpA.

Nevertheless, the TMAP (Persson and Argos, 1994), PHDhtm (Rost *et al.*, 1995), and PHDtopology (Protein Design Group, European Molecular Biology Laboratory, Heidelberg, Germany) algorithms predicted transmembrane domains for CtpA which satisfied most of the

criteria for transmembrane prediction outlined in this section. To investigate the reliability of transmembrane domain prediction by these algorithms, two approaches were undertaken. First, membrane spanning domains predicted for CtpA peptide sequence were compared to those predicted for the closely related PacS ATPase from *Synechococcus* spp. (Kanamaru *et al.*, 1993) and an artificially generated N-terminal truncated PacS peptide sequence (designated TrPacS). The PacS protein is reported to contain eight transmembrane domains (Kanamaru *et al.*, 1994). TrPacS resulted from the removal of 95 aa from the N-terminal region of the wild type PacS peptide sequence ($Met_1 \rightarrow Ala_{95}$), determined from amino acid sequence alignment of CtpA and PacS. Interestingly, the TMAP algorithm predicted six PTMD's for each peptide sequence (Table 5.8, Part A). Furthermore, PTMD 1 to 6 were located in analogous regions of all peptide sequences. Using the algorithm PHDhtm, six analogous PTMD's were also predicted for all peptide sequences (Table 5.8, Part B). Moreover, the C-terminal PTMD's were consistent with those predicted by the TMAP algorithm. However, additional membrane spanning domains PTMD B (Phe_{98}-Gly_{111}) and PTMD A (Ser_6-Met_{21}), were also predicted for CtpA and PacS protein sequences respectively. The PHDtopology algorithm predicted seven PTMD's for the CtpA peptide sequence (Table 5.8, Part C). Analogous PTMD's were also predicted in TrPacS and PacS peptide sequences. Interestingly, an additional transmembrane domain was predicted for PacS situated near the N-terminus (PTMD A; Gly_7-Ile_{24}), analogous to PTMD A predicted by PHDhtm. This suggests that the N-terminal truncation of CtpA and TrPacS may prevent the prediction of an additional PTMD at the N-terminus of these proteins. Generally, PHDhtm and PHDtopology predicted analogous PTMD's. The only exception being that the PHDhtm algorithm predicted PTMD B (Phe_{98}-Gly_{111}) from CtpA peptide sequence, but not from TrPacS or PacS. The moderate variation in transmembrane prediction at the N-terminus between the algorithms may suggest that this region is not sufficiently hydrophobic for reliable prediction.

A second approach used in this study involved analysis of each PTMD predicted by TMAP, PHDhtm, and PHDtopology algorithms by display of relevant amino acid segments on a helical wheel (Figure 5.27 and Figure 5.28). Helical wheels were developed using the protein analysis program ANTHEPROT V2.7e (G. Deleage, Institut de Biologie et Chimie des Protéines, Lyon, France). Generally, because these α-helices are predicted to span the hydrophobic environment of the bacterial membrane, the majority of residues are hydrophobic. However, hydrophilic residues are randomly distributed throughout each wheel. This is consistent with

Table 5.8 Prediction of transmembrane sequences of CtpA using secondary structure prediction algorithms

Representation of output data from algorithms that predict putative transmembrane domains (PTMD) for amino acid sequences and which comply with the selection criteria for membrane spanning regions of proteins, outlined in Section 5.2.10. The reliability of PTMD prediction by these algorithms was investigated by comparing output data from CtpA peptide sequence and the closely related PacS ATPase from *Synechococcus* spp. (Kanamaru *et al.*, 1994). To examine the effect of the N-terminal truncation in CtpA on PTMD prediction, CtpA and PacS peptide sequences were aligned to localise a suitable site for creation of an artificial N-terminal truncation in PacS. The first 95 aa were removed from the full length PacS protein ($Met_1 \rightarrow Ala_{95}$) to generate a PacS-derived peptide (designated TrPacS) comparable in size to CtpA. For convenience, numbering of amino acids in TrPacS and PacS began with the first amino acid after the site of truncation starting at number one (for example: Gln_{96} became Gln_1). PTMD A, B, C, and D are additional domains predicted by certain algorithms. NP indicates domains not predicted by algorithms. Numbers in parentheses indicates the length of each domain. TMAP (Persson and Argos, 1994), PHDhtm (Rost *et al.*, 1995), and PHDtopology (Protein Design Group, European Molecular Biology Laboratory, Heidelberg, Germany).

A: TMAP

PTMD	CtpA (653 aa)	TrPacS (652 aa)	PacS (747 aa)
1	Gly_{52}-Ile_{80} (29)	Ser_{52}-Phe_{80} (29)	Arg_{62}-Phe_{89} (28)
2	Thr_{96}-Met_{118} (23)	Gln_{96}-Arg_{118} (23)	Val_{102}-Arg_{123} (22)
3	Val_{245}-Ser_{273} (29)	Ala_{245}-Trp_{273} (29)	Leu_{250}-Trp_{276} (27)
4	Val_{276}-Val_{304} (29)	Trp_{276}-Pro_{304} (29)	Val_{280}-Ala_{302} (23)
5	Ile_{587}-Leu_{614} (28)	Arg_{587}-Ile_{614} (28)	Gln_{595}-Pro_{617} (23)
6	Ser_{615}-Leu_{632} (18)	Leu_{615}-Met_{632} (18)	Leu_{618}-Ser_{635} (18)

B: PHDhtm

PTMD	CtpA (653 aa)	TrPacS (652 aa)	PacS (747 aa)
A	NP	NP	Ser_{6}-Met_{21} (16)
1	Trp_{38}-Phe_{55} (18)	Leu_{38}-Phe_{53} (16)	Leu_{38}-Phe_{53} (16)
2	Gly_{75}-Met_{89} (15)	Val_{72}-Ala_{85} (14)	Val_{72}-Ala_{85} (14)
B	Phe_{98}-Gly_{111} (14)	NP	NP
3	Phe_{252}-Ser_{273} (22)	Phe_{258}-Gly_{278} (21)	Phe_{258}-Gly_{278} (21)
4	Ala_{278}-Gly_{305} (28)	Ala_{283}-Gly_{310} (28)	Ala_{283}-Gly_{310} (28)
5	Phe_{590}-Ala_{609} (20)	Phe_{599}-Pro_{617} (19)	Phe_{599}-Leu_{615} (17)
6	Thr_{613}-Leu_{632} (20)	Trp_{621}-Val_{640} (20)	Leu_{619}-Val_{640} (22)

C: PHDtoplogy

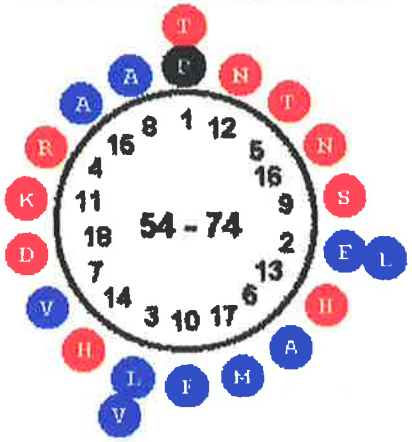
PTMD	CtpA (653 aa)	TrPacS (652 aa)	PacS (747 aa)
A	NP	NP	Gly_{7}-Ile_{24} (18)
1	Trp_{38}-Phe_{55} (18)	Gly_{37}-Phe_{54} (18)	Gly_{37}-Phe_{54} (18)
2	Val_{74}-Ala_{91} (18)	Thr_{70}-Thr_{87} (18)	Thr_{70}-Thr_{87} (18)
3	Thr_{96}-Tyr_{113} (18)	Tyr_{103}-Leu_{120} (18)	Tyr_{103}-Leu_{120} (18)
4	Val_{253}-Phe_{270} (18)	Phe_{258}-Trp_{276} (19)	Val_{259}-Trp_{276} (18)
5	Met_{279}-Ile_{296} (18)	Ala_{283}-Pro_{304} (22)	Ala_{283}-Ser_{306} (24)
6	Asn_{588}-Gly_{605} (18)	Ala_{600}-Ser_{624} (25)	Arg_{594}-Ala_{611} (18)
7	Leu_{610}-Thr_{629} (20)	Gly_{629}-Leu_{646} (19)	Tyr_{616}-Ala_{633} (18)

Figure 5.27: Segments of CtpA amino acid sequence associated with N-terminal putative transmembrane domains predicted by TMAP (Persson and Argos, 1994) (Part A) and PHDhtm (Rost *et al.*, 1995) and PHDtopology (Protein Design Group, European Molecular Biology Laboratory, Heidelberg, Germany) (Part B) algorithms, displayed on helical wheels. Helical wheels of 21 consecutive amino acids, were generated using the protein analysis program ANTHEPROT V2.7e (G. Deleage, Institut de Biologie et Chimie des Protéines, Lyon, France). The first 18 residues are displayed in one circumference with adjacent residues placed every 100 degrees, and the remaining residues are displayed in the second circumference. The position of amino acid segments is numbered in the center of each helical wheel, and wheels are labelled according to the nomenclature used in Table 5.8. The following colour code is used: blue is an amino acid with a hydrophobic side chain; red is a hydrophilic side chain; and black are all remaining amino acids.

Part A: TMAP

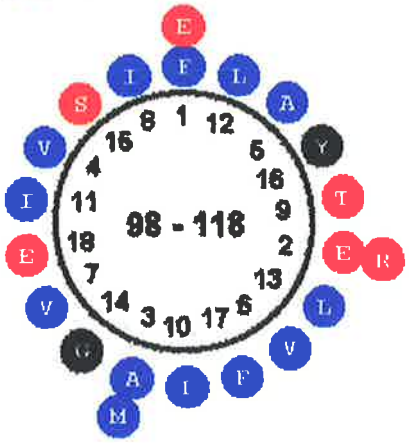
PTMD 1

PFLRTAVASFKNHHANMDTLV



PTMD 2

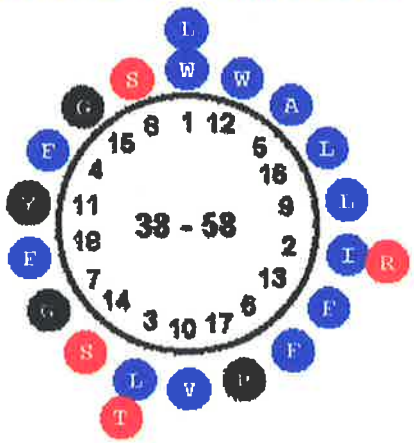
FEAVAVVITLILLGSYFEERM



Part B: PHDhtm and PHDtopology

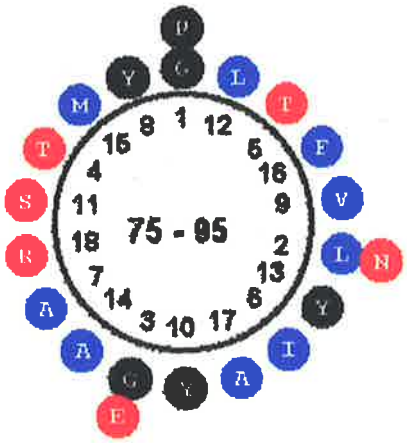
PTMD 1

WILFAFGSLVYWFSGLPFLRT



PTMD 2

GLGTTYAYVYSLYAMFARPNE



PTMD 3 (PTMD B in PHDhtm)

FEAVAVVITLILLGSYFEERM

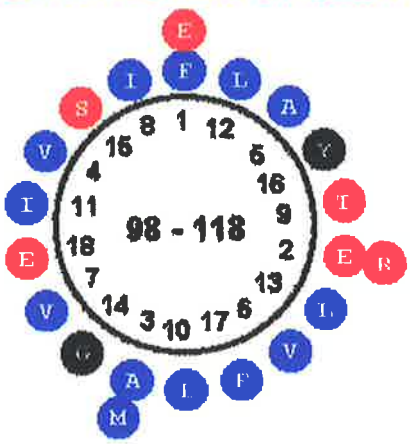
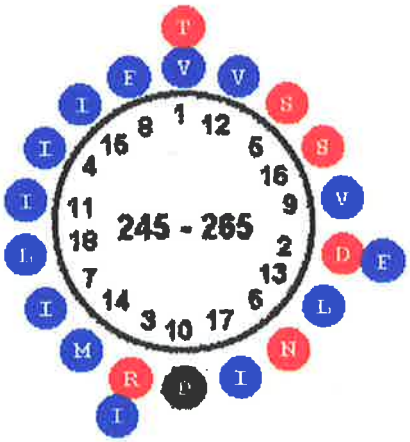


Figure 5.28: Segments of CtpA amino acid sequence associated with C-terminal putative transmembrane domains predicted by TMAP (Persson and Argos, 1994), PHDhtm (Rost *et al.*, 1995) and PHDtopology (Protein Design Group, European Molecular Biology Laboratory, Heidelberg, Germany) algorithms, displayed on helical wheels. Helical wheels of 21 consecutive amino acids, were generated using the protein analysis program ANTHEPROT V2.7e (G. Deleage, Institut de Biologie et Chimie des Protéines, Lyon, France). The first 18 residues are displayed in one circumference with adjacent residues placed every 100 degrees, and the remaining residues are displayed in the second circumference. The position of amino acid segments is numbered in the center of each helical wheel, and wheels are labelled according to the nomenclature used in Table 5.8. The following colour code is used: blue is an amino acid with a hydrophobic side chain; red is a hydrophilic side chain; and black are all remaining amino acids.

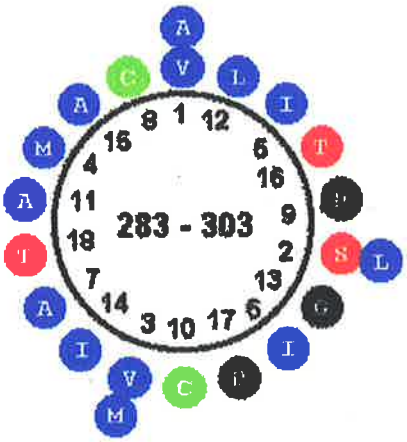
PTMD 3 (PTMD 4 in PHDtopology)

VDRISNIFVPIVLMISILTFI



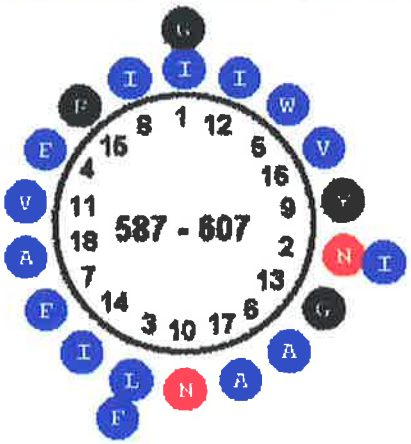
PTMD 4 (PTMD 5)

VSVMIIACPCALGIATPT



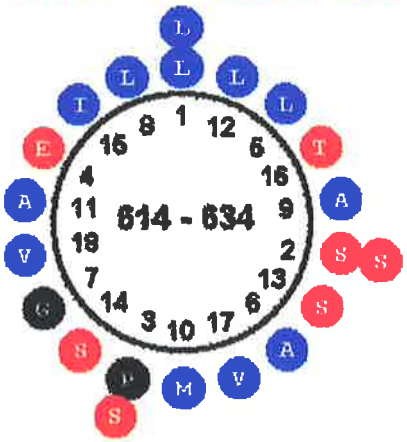
PTMD 5 (PTMD 6)

INLFWAFIYNVIGIPVAAGIF



PTMD 6 (PTMD 7)

LSPELAGLAMALSSITVVLSS



properties of integral membrane proteins which contain several transmembrane spanning domains. Hydrophilic amino acid residues within each α-helical segment can participate as inter-helical hydrogen-bond donors to enable formation of bundles of α-helices within the membrane to form a functional protein (Kyte, 1995).

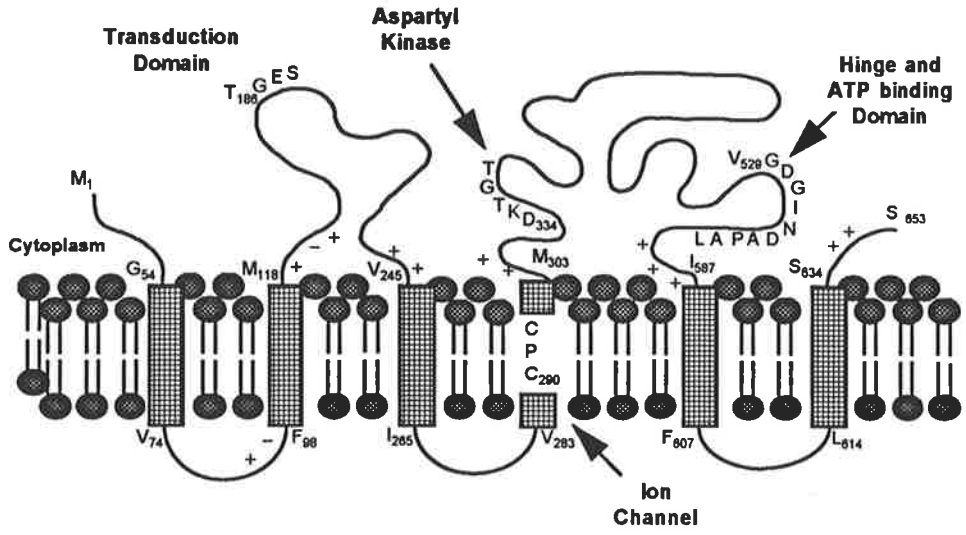
This data was combined to develop a putative CtpA topology model. The first model contained six transmembrane regions predicted by the TMAP algorithm (Figure 5.29, Model 1). An additional model (Model 2), also contained six membrane spanning regions predicted by the PHDhtm and PHDtopology algorithms, also shown in Figure 5.29. However, this model contained an additional α-helix segment (Phe_{98}-Met_{118}), not associated with the membrane to accommodate for the cytoplasmic location of the amino and carboxyl termini of P-type ATPases. Each model consists of two large cytoplasmic loops and the amino and carboxyl termini were located in the cytoplasm which conforms with previously published topology models of P-type ATPases. In these models, the putative functional domains and their respective consensus sequences, including 1) Ion transduction, 2) Aspartyl kinase, 3) ATP binding, and 4) Hinge domain, were also contained in the two large cytoplasmic loops. Furthermore, in accordance with the "positive inside rule" developed by Von Heijne, (1992), positively charged amino acid residues are located immediately following most predicted membrane spanning segments at the cytoplasmic face. However, it should be stressed that the CtpA topology shown in this section is only a model. Confirmation of these models is required using protein fusion or epitope mapping techniques.

5.2.11 Expression of the *ctpA* polypeptide in *E. coli*

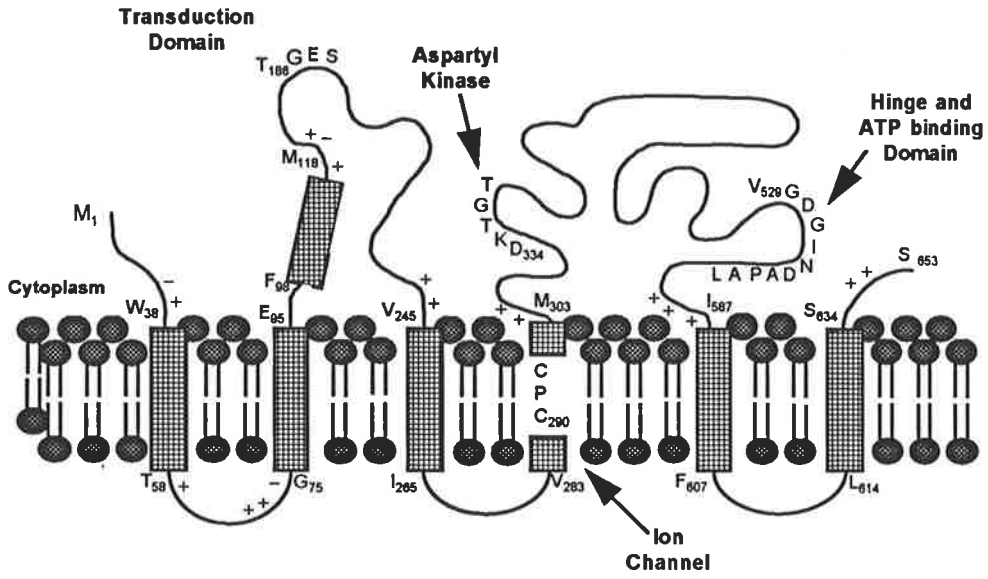
A specific antiserum raised to purified CtpA protein would be a useful tool in the functional characterisation of this protein. In particular, the antibody could be used in Western analysis to examine the electrophoretic mobility of wild type CtpA and enable confirmation of the N-terminal truncation which was predicted by the 653 amino acid protein encoded by *ctpA* (see Section 5.2.10, Figure 5.26). To facilitate the development of an anti-CtpA antiserum, purified CtpA is required. This section describes the use of two independent protein expression systems in attempts to isolate purified CtpA.

Figure 5.29: Membrane folding models of CtpA. Model 1: predicted by the TMAP algorithm developed by Persson and Argos, (1994); Model 2: predicted by the PHDhtm (Rost *et al.*, 1995) and PHDtopology (Protein Design Group, European Molecular Biology Laboratory, Heidelberg, Germany) algorithms. Six transmembrane α-helical domains were predicted which is consistent with other models for bacterial P-type ATPases. An additional α-helix in Model 2 was not associated the membrane to maintain both the amino and carboxyl termini in the cytoplasm. Conserved sequences common to all P-type ATPases are indicated: TGES is located within the Transduction/Phosphatase domain; DKTGT is the site of phosphorylation in the Aspartyl kinase domain; and VGDGINDAPAL is found within the Hinge and ATP binding domain. The conserved CPC sequence found within the fourth membrane spanning region is indicated and thought to be critical for ion channel formation. The charged residues are displayed and the numbers in subscript indicate positions of amino acid residues in CtpA.

Model 1



Model 2



5.2.11.1 Temperature inducible expression of CtpA under the control of T7 RNA polymerase

In this section, *ctpA* was over-expressed in *E. coli* under the control of the temperature inducible T7 RNA polymerase using a modification of the method described by Tabor and Richardson, (1985). Each *E. coli* host strain harboured two plasmids. The first plasmid contained the *ctpA* gene under the control of the T7 promoter contained on pGEM-7Zf(+) (pCT220 or pCT203), and the second plasmid pGP1-2, contained the T7 RNA polymerase regulated by a temperature sensitive repressor. The strains used in this study were MF002 [*E. coli*/pCT220 and pGP1-2], MF003 [*E. coli*/pCT203 and pGP1-2], and the negative control MF001 [*E. coli*/pGEM-7Zf(+) and pGP1-2]. Proteins extracted from MF001 and MF002, following induction by temperature, were electrophoresed on a 8.5% polyacrylamide gel and visualised by Coomassie stain (Figure 5.30). In comparison to proteins prepared from MF001, an abundantly expressed 84.2 kDa protein band from MF002 was identified. In contrast, the calculated M_r value for CtpA was 69.5 kDa. MF003 was not used in this experiment.

However, as the specificity of the 84.2 kDa protein for CtpA could not be confirmed, proteins expressed during the temperature induction of MF001, MF002, and MF003, were pulsed with [^{35}S]-Methionine and analysed by autoradiography of SDS-PAGE. The protein profile generated from MF003, identified a 83.5 kDa protein by [^{35}S]-Methionine incorporation, not observed in proteins produced by MF001 (Figure 5.31), yet similar in size to the 84.2 kDa protein previously identified (see Figure 5.30). Suprisingly, no protein expressed by MF002 incorporated [^{35}S]-Methionine. This result was in contradiction to the data shown in Figure 5.30, and may reflect the potential difficulty in over-expression of a large integral membrane protein of Gram-positive origin in *E. coli*. Low molecular weight protein bands in Figure 5.31 (Lane c), may have arisen from proteolytic digestion of the high molecular weight protein. Given the similar size of the proteins identified in Figure 5.30 and 5.31, they were likely to represent the product encoded by *ctpA*.

However, since neither the 83.5 kDa or 84.2 kDa proteins expressed in *E. coli* could be confirmed as CtpA specific (calculated M_r value of 69.5 kDa), purification of this protein was not undertaken. In addition, the high level of expression of native proteins by *E. coli*, even in the presence of the translation inhibitor rifampicin, would make protein purification to homogeneity difficult. In view of these limitations, a system for protein expression in *Bacillus subtilis* was employed. Expression of foreign proteins relies on the generation of a N- or

Figure 5.30: Analysis of the CtpA protein expressed in *E. coli* under T7 polymerase control by Coomassie stained SDS-PAGE of whole cell protein preparations. Lane: [a], Protein molecular weight markers [Phosphorylase b (94 kDa), Bovine Serum Albumin (67 kDa), Ovalbumin (43 kDa)]; [b], MF001; [c], MF002. The putative CtpA polypeptide (84.2 kDa) is indicated by an arrowhead.

a b c

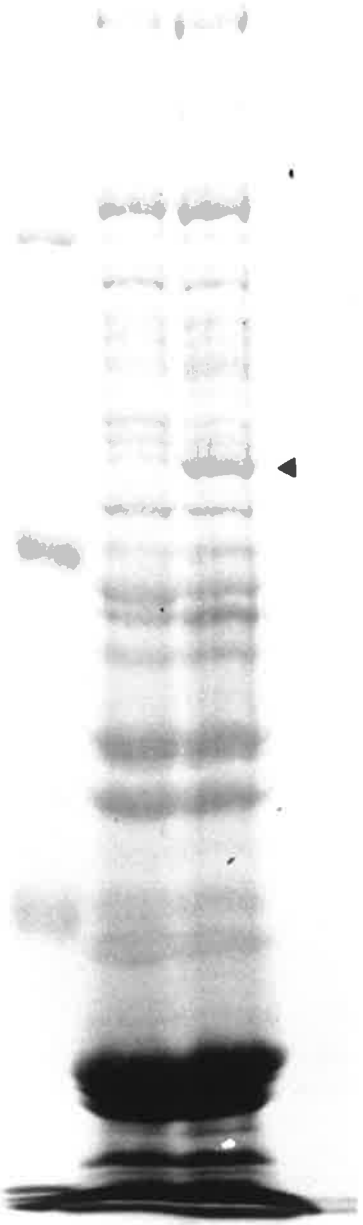
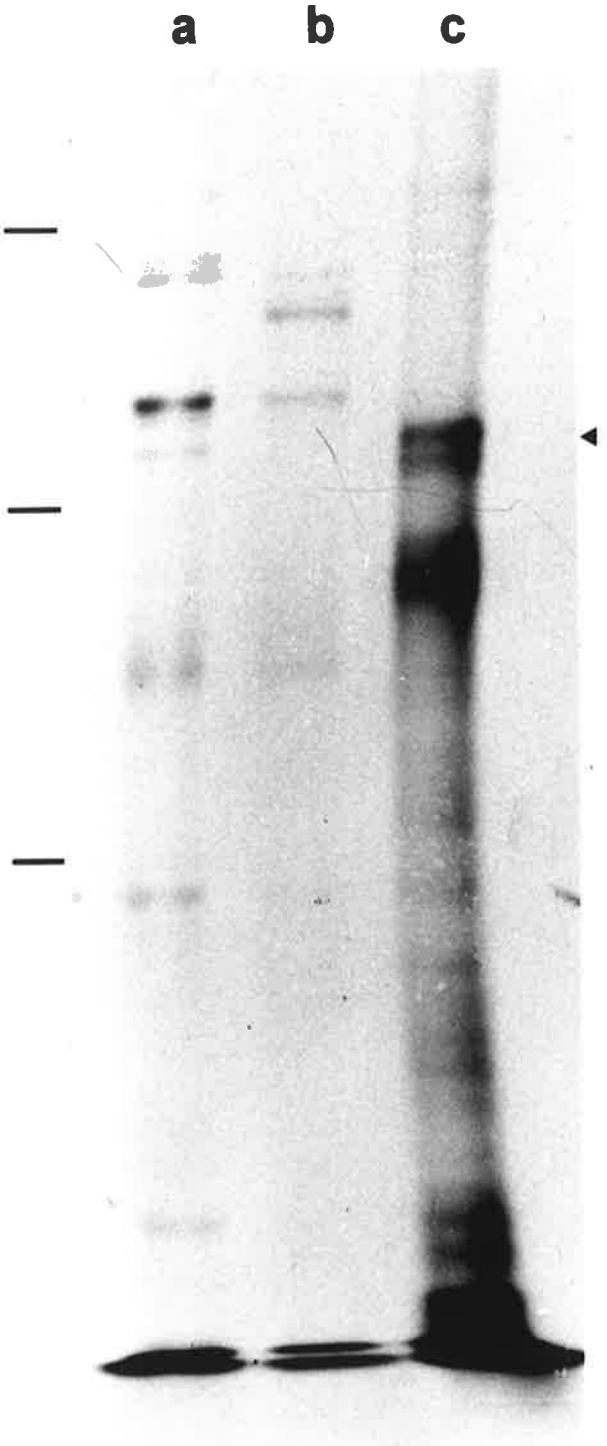


Figure 5.31: Analysis of the CtpA protein expressed in *E. coli* under T7 polymerase control by autoradiography of SDS-PAGE with [^{35}S]-Methionine labelled whole cell protein preparations. Lane: [a], MF001; [b], MF002; [c], MF003. The putative CtpA polypeptide (83.5 kDa) is indicated by an arrowhead. Protein molecular weight markers are indicated on the left hand side by solid bars; Phosphorylase b (94 kDa), Bovine Serum Albumin (67 kDa), Ovalbumin (43 kDa).


a
b
c

C-terminal translational fusion of the foreign protein with a stretch of six Histidine residues (His_6-tag) contained on the vector pUSH1 (Schön and Schumann, 1994). The over-expressed protein can then be readily purified by metal chelate affinity chromatography.

5.2.11.2 IPTG inducible expression of CtpA under the control of the T5 promoter contained on pUSH1

In this section, a cloning system which promotes inducible over-expression of foreign proteins in *B. subtilis* and purification by metal chelate affinity chromatography (Schön and Schumann, 1994), was applied to purify the CtpA polypeptide. The rationale used to generate a N-terminal His_6-tag::CtpA translational fusion contained in the vector pUSH1 is schematically outlined in Figure 5.32. PCR was used to incorporate 5′ and 3′ terminal *Bam*HI restriction sites in an amplified *ctpA* DNA fragment. The protocol for the PCR reaction using the *ctpA* specific synthetic oligonucleotides p2022 and p2023 is described in Table 2.5. Using plasmid pCT220 as the *ctpA* specific template, a 2024 bp DNA product was amplified that contained a 38 bp truncation in the 5′ end of *ctpA* (Figure 5.33). The specificity of the PCR product was confirmed by digestion with *Apa*I, *Hpa*I, and *Hin*dIII restriction enzymes internal to the *ctpA* gene identified from nucleotide sequence (see Section 5.2.4, Figure 5.14). Direct sequencing by PCR was also attempted, but this was consistently unsuccessful.

Nevertheless, *Bam*HI digested *ctpA* amplified DNA and plasmid pUSH1 were ligated, transformed into *E. coli* DH5α, and selected on solid media containing Cm. From three independent ligation reactions, plasmid DNA from at least 300 transformants were screened by *Bam*HI restriction enzyme digestion without identifying the 2024 bp *ctpA* insert (data not shown). In addition, 1000 Cm^R transformants failed to hybridise to the DIG labelled 2024 bp PCR amplified *ctpA* DNA probe in colony hybridisation experiments (data not shown). Furthermore, when either *Bam*HI digested or undigested *ctpA* DNA was ligated into pGEM-7Zf(+), or the PCR cloning vector pGEM-T, no positive clones were isolated.

5.3 Discussion

In this chapter, the cloning of *L. monocytogenes* chromosomal DNA flanking Tn*917-lacZ-cat86* insertions in genes regulated by environmental stress, such as, temperature upshift (25°C → 37°C), iron limitation, cation limitation, or carbon dioxide was described. In particular, DNA from *L. monocytogenes* C185 flanking the Tn*917-lacZ-cat86* transcriptional

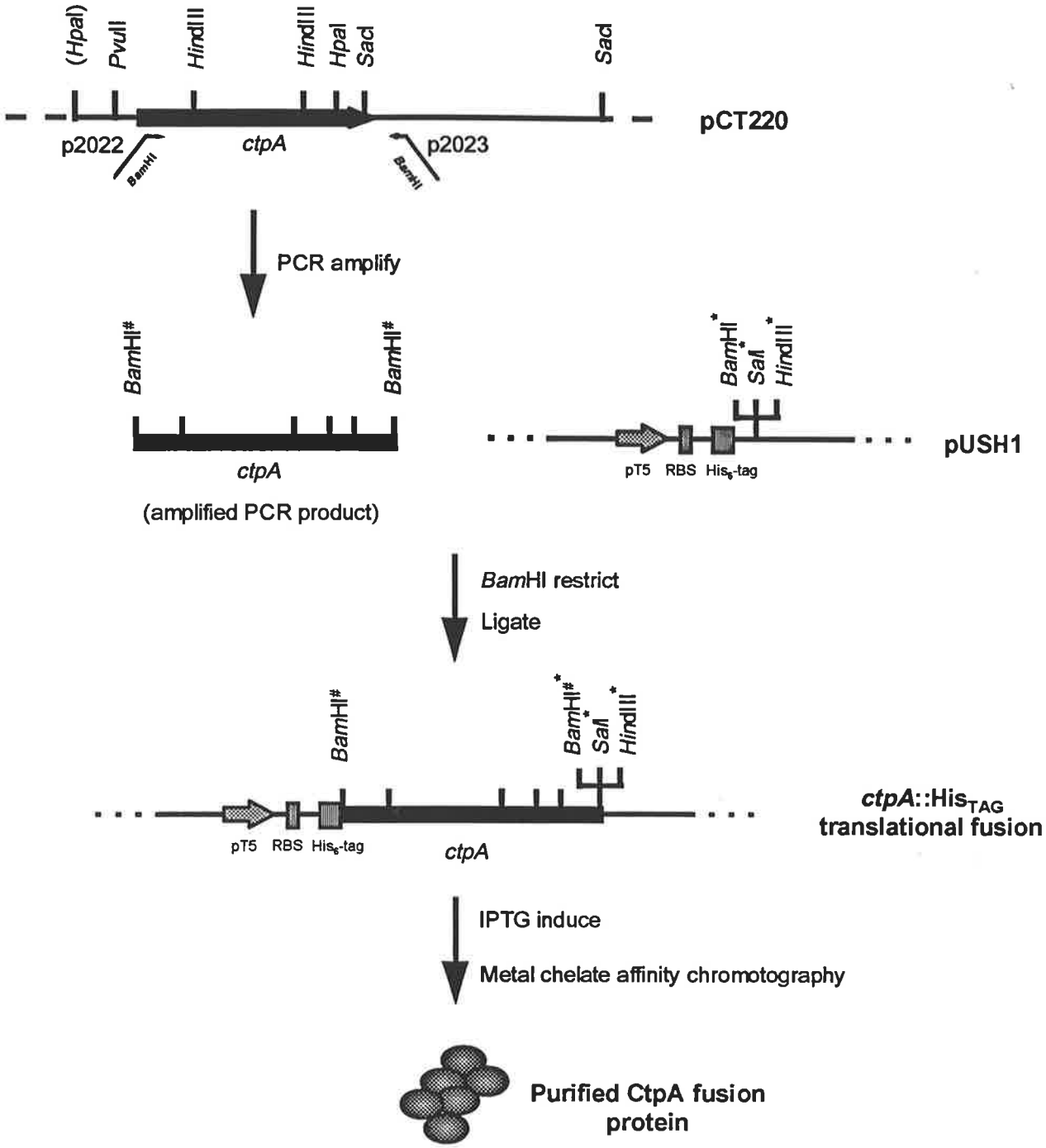
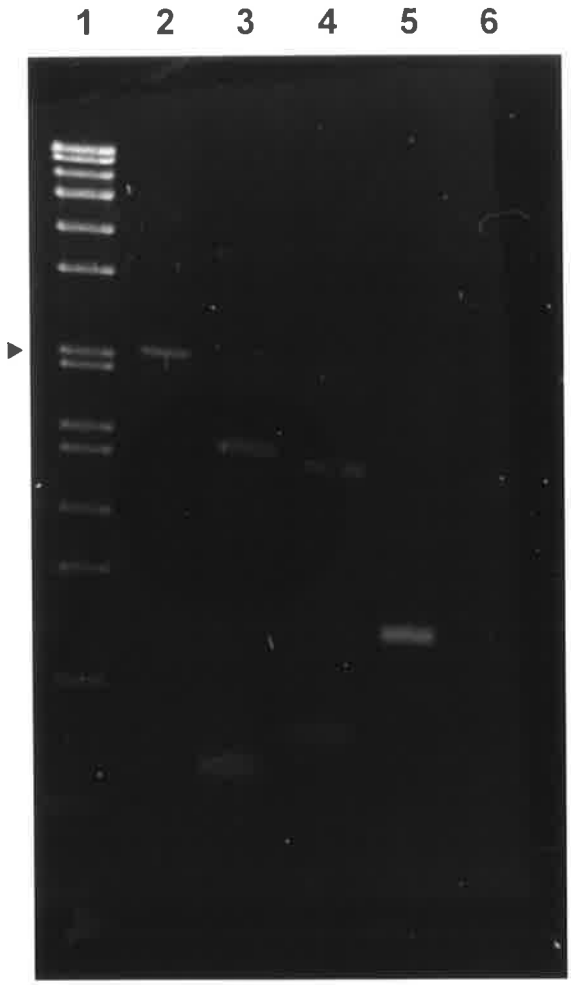


Figure 5.32. Schematic representation of the construction of an IPTG inducible *ctpA* expression vector, for over-expression and purification of a His_6-tag::CtpA translational fusion product by metal chelate affinity chromatography. The location of synthetic oligonucleotides p2022 and p2023, are shown relative to *ctpA*. PCR amplification of *ctpA* DNA using these primers would yield a 2024 bp DNA product, containing a 38 bp truncation at the 5′ end of *ctpA*. Restriction enzyme sites derived from vector, pUSH1 (asterisk), and the *Bam*HI sites incorporated into the PCR amplified *ctpA* DNA fragment (crosshatch), are indicated.

Figure 5.33: PCR amplification of a 2024 bp *ctpA* DNA product from plasmid pCT220, using the synthetic oligonucleotides p2022 and p2023. Lane: [1], *Eco*RI digested Bacteriophage SPP-1 DNA; [2], undigested PCR product; [3], *Apa*I digested PCR product; [4], *Hpa*I digested PCR product; [5], *Hin*dIII digested PCR product; [6], control PCR (no template DNA). The calculated restriction fragment sizes after digestion of the PCR product with *Apa*I were 0.54 kb and 1.5 kb; *Hpa*I (0.6 kb and 1.4 kb); *Hin*dIII (0.31 kb, 0.75 kb, and 0.85 kb). The determined fragment lengths were consistent with those predicted from nucleotide sequence (see Figure 5.14). The arrowhead indicates the 2024 bp PCR product.


1
2
3
4
5
6

fusion induced by 2.5 mM EGTA, was cloned. A series of subclones was used to facilitate nucleotide sequence analysis of DNA flanking Tn*917-lacZ-cat86* in *L. monocytogenes* C185.

Sequence analysis of cloned DNA flanking this insertion identified an open reading frame (*ctpA*) of 1962 nucleotides with the potential to encode for a protein of 653 amino acids with a predicted M_r value of 69,463. Sequence comparison of CtpA with available protein data bases revealed remarkable similarity to a family of P-type ATPase transporters found in both prokaryotes and eukaryotes. Most notable sequence identity was observed to *Synechococcus* spp. PacS (Kanamaru *et al.*, 1993) and *E. hirae* CopA (Odermatt *et al.*, 1993) ATPases involved in copper transport. Amino acid similarity was also observed to proteins associated with defective copper transport disorders in humans, Menkes (Chelly *et al.*, 1993; Mercer *et al.*, 1993; and Vulpe *et al.*, 1993) and Wilson disease (Bull *et al.*, 1993).

This finding was in good agreement with the evolutionary relatedness of all known bacterial P-type ATPase sequences. These sequences were grouped into 3 segments based on the divisions previously described (Fagan and Saier, 1994). With the exception of C-terminal peptide sequences, CtpA was reliably associated with a class of P-type ATPases involved in copper transport. Other proteins were also grouped according to their cation specificity. Similar observations, has lead to the claim that P-type ATPases should be divided into distinct classes based on ion specificity, biological occurrence, and sequence (Green, 1992). Interestingly, multiple alignment of Segment 2 amino acid sequences which contained the Aspartyl kinase and Hinge and ATP binding domains, further distinguished proteins of identical cation specificity into sub-groups based on amino acid relatedness. This definitive demonstration of evolutionary relatedness, is brought about by the highly conserved nature of the internal region in all ATPase proteins (Silver and Walderhaug, 1992). In contrast, using the distance method to illustrate phylogenies, multiple aligned amino acid sequences from Segment 3, could not establish evolutionary relatedness due to considerable sequence divergence within the C-terminal domain of some proteins. However, conserved amino acid residues were observed in this segment, particularly with proteins exhibiting identical cation specificity (see Appendix A). When Segment 1 (Metal binding, Ion transduction, and Ion channel domains) and whole protein sequences were multiply aligned and used to determine the degree of evolutionary relatedness, very consistent patterns emerged for all proteins analysed. In common with other work (Fagan and Saier, 1994), the degree of amino acid divergence within the structural domains of bacterial ATPase proteins was

Segment 2 < Segment 1 < Segment 3. Therefore, Segment 2 sequences were deemed the most suitable for assessment of evolutionary relatedness between P-type ATPase proteins.

To date, there is no current consensus regarding the membrane topology of bacterial P-type ATPases. Using prediction algorithms, models which contain six to ten transmembrane domains have been reported. The fact that several different models can be predicted for a family of proteins with a high degree of amino acid identity is interesting. Clearly, the individual parameters incorporated into each prediction algorithm has uppermost significance in analysis of a peptide sequence. For example, several different matrices are used for performing statistical analysis of multiple sequence alignments. Therefore, depending on the prediction algorithm used, each individual data set can be interpreted differently which leads to variations in output information. Similarly, the window size used to view a consecutive region of amino acid residues from a peptide sequence directly influences secondary structure prediction. Significantly, most prediction algorithms utilise a novel window size. In view of this, caution should be exercised when comparing topology models of ATPases developed by independent algorithms. These models should be used as a guide to design suitable protein fusion or epitope mapping experiments to enable a more accurate determination of protein topology. For example, the membrane topology of a P-type ATPase from *H. pylori* was investigated by *in vitro* translational fusion technology (Melchers *et al.*, 1996). This study reported the potential for at least eight transmembrane domains. In another topological study using protein fusions, the MgtB ATPase from *S. typhimurium* which closely resembles proteins of eukaryotic origin, was found to possess ten transmembrane domains (Smith *et al.*, 1993).

To establish a membrane topology model of CtpA, several prediction algorithms were used. In this analysis, most algorithms were unable to predict a model of CtpA that was consistent with those reported for other ATPases. One possible explanation for this result is that these particular algorithms may be more suited to water soluble proteins rather then lipid soluble integral membrane proteins such as P-type ATPases (Rost and Sander, 1993; Rost and Sander, 1994). However, two topology models for CtpA were presented in this study which consisted of six transmembrane regions predicted by the TMAP, and PHDhtm and PHDtopology algorithms, respectively. Amino acid segments predicted to reside within the bacterial membrane were displayed on a helix wheel. While most residues were hydrophobic, consistent with a membrane location of CtpA, hydrophilic residues were also present. These residues may be intimately associated with inter-helical hydrogen-bond formation (Kyte, 1995),

responsible for the tertiary structure of this protein. In both CtpA topology models presented in this study, the putative functional domains of this family of proteins were located within two large cytoplasmic loops, consistent with models reported for other ATPase proteins. Other common features include the cytoplasmic position of the amino and carboxyl termini of each protein, and the affiliation of an absolutely conserved proline residue with the fourth transmembrane domain. Similar topology models of other P-type ATPases have been described that contain six membrane spanning domains (Kahn *et al.*, 1989; Silver *et al.*, 1989; Saier *et al.*, 1994; Ge *et al.*, 1995), although models consisting of eight membrane spanning domains have been reported (Odermatt *et al.*, 1993; Kanamaru *et al.*, 1994; Melchers *et al.*, 1996).

However, it is impossible to suggest which model, if any, is appropriate for CtpA. Prediction of a definitive topology model of integral membrane proteins based entirely on computer modelling is difficult. For example, in this study the PacS ATPase from *Synechococcus* spp. (Kanamaru *et al.*, 1993), was found to contained either six (TMAP algorithm) or eight (PHDtopology algorithm) transmembrane domains predicted from peptide sequence. Therefore, even though PacS was previously reported to contain eight transmembrane domains (Kanamaru *et al.*, 1994), other models could be described depending on the algorithm used in each analysis, especially if individual algorithms utilise a different scale to determine hydrophobicity of individual amino acid residues. Another difficulty in predicting membrane topology by this approach, concerns the fact that channel-forming transmembrane helices of P-type ATPases probably contain charged residues facing the ion channel. Such helices would give quite different hydropathy plots (Branden and Tooze, 1991). This demonstrates the absolute requirement for biochemical data including protein fusion or epitope mapping techniques to be used in combination with prediction algorithms in order to obtain an accurate topology model of ATPase proteins. Moreover, only after electron microscopy and X-ray crystallographic data become available, will the uncertainty of membrane topology of this family of proteins be completely resolved. In the future, this information will determine if P-type ATPases do indeed differ fundamentally in membrane topology, and if these differences are related to variations in cation specificity.

An understanding of the biochemical nature of P-type ATPase function has generally been restricted to proteins of eukaryotic origin. However, given this protein family displays remarkable sequence conservation in regions associated with protein activity, functional

models developed from biochemical investigation of eukaryotic P-type ATPases have been applied to prokaryotic proteins. Critical amino acid residues associated with protein function have been identified which are absolutely conserved in all previously reported P-type ATPases. Multiple sequence alignments of CtpA with other P-type ATPases, identified these amino acid residues in the putative functional domains of CtpA. Moreover, predicted models for the membrane topology of CtpA, associated the functional domains within two large cytoplasmic loops. This was in excellent agreement with predicted topology models of other bacterial P-type ATPases (Kahn *et al.*, 1989; Silver *et al.*, 1989; Odermatt *et al.*, 1993; Smith *et al.*, 1993; Kanamaru *et al.*, 1994; Ge *et al.*, 1995; Melchers *et al.*, 1996).

The first cytoplasmic loop from the N-terminus contains the Ion transduction or phosphatase domain that is thought to be responsible for moving the cation closer to the membrane ion channel (Brandl *et al.*, 1986). Movement would occur in response to the phosphorylation state of the protein. This domain is characterised by a stretch of amino acids including T-G-E-S, which is believed to promote phosphatase activity (Serrano and Portillo, 1990). In CtpA, this consensus sequence begins at residue 186. In the fourth transmembrane region beginning at position 290, the three amino acid stretch C-P-C was observed, and is characteristic of an ion channel involved in the translocation of cations through the membrane (Brandl and Deber, 1986; Deber *et al.*, 1990). The proline residue is invariant in these proteins, whereas the neighbouring cysteine residues are typical of proteins involved in heavy metal transport (Silver and Walderhaug, 1992). An aspartate residue is situated within the Aspartyl kinase domain, found in the second cytoplasmic loop of other P-type ATPases. This aspartate residue is the site of protein phosphorylation (Hesse *et al.*, 1984; Shull *et al.*, 1985; Walderhaug *et al.*, 1985), and is part of an absolutely conserved five amino acid stretch (D-K-T-G-T) in all P-type ATPases. By analogy, residue D_{334} is likely to be the aspartate residue that undergoes phosphorylation in CtpA. The next domain functions in ATP binding probably through the invariant lysine residue (K_{514} in CtpA) (Farley and Faller, 1985; Ohta *et al.*, 1986). Lastly, the Hinge domain is involved in the conformational change between the Ion transduction and Aspartyl kinase domains (Brandl *et al.*, 1986). This domain is the most highly conserved in sequence compared to any other part of the protein including an amino acid stretch of G-D-G-I-N-D-A-P-A-L beginning at residue 529 in CtpA. On the basis of sequence comparisons, CtpA is apparently a P-type ATPase from *L. monocytogenes*. Only one other P type ATPase has been isolated from this organism, and this confers resistance to cadmium (Lebrun *et al.*, 1994).

Analysis of the CtpA amino acid sequence suggests that in common with the ATPase involved in copper transport (hpCopA) in *H. pylori* (Ge *et al.*, 1995), this putative copper transport protein lacks an N-terminal domain normally responsible for initial binding of cations. In *H. pylori*, an open reading frame (*ORF2*), which encodes a 66 amino acid protein (hpCopP), is thought to be responsible for initial cation binding. This gene lies immediately downstream of the *ORF1* gene encoding hpCopA.

To investigate if a similar gene arrangement exists in the *ctpA* region from *L. monocytogenes*, two approaches were considered. First, one way to characterise the N-terminal truncation in CtpA is to calculate the M_r value of wild type CtpA by Western blot analysis to compare this value to the predicted M_r value of 69,463 for the 653 amino acid CtpA protein. However, this approach required an anti-CtpA antibody. Preparation of purified CtpA initially involved temperature induced over-expression of *ctpA* in *E. coli* under T7 RNA polymerase control using the method of Tabor and Richardson, (1985). Analysis by SDS-PAGE of protein preparations isolated from *E. coli*, identified a comparatively more abundant 83.5 kDa (Coomassie stained) and 84.2 kDa ([^{35}S]-Methionine labelled) protein, significantly larger then the predicted M_r value of CtpA (calculated M_r value of 69.5 kDa). Interpretation of these results is difficult. However, similar variations in predicted M_r values and electrophoretic mobilities of wild type ATPase proteins have been observed (Yoon and Silver, 1991; Odermatt *et al.*, 1994), which may suggest the 83.5 kDa or 84.2 kDa proteins are the product of *ctpA*, contained on the plasmids pCT203 and pCT220. In the absence of immuno-detection techniques, one approach to confirm or otherwise, that this protein band is representative of CtpA, would be to attempt to over-express a mutagenised *ctpA* gene, such as the *ctpA*::*erm* allele maintained on the plasmid, pCT221 (see Section 6.2.1.1) in *E. coli*.

Thus, this limitation lead to the use of a system for over-expression and rapid purification of proteins in *B. subtilis* (Schön and Schumann, 1994). However, in this study at least, the PCR amplified *ctpA* DNA fragment designed to create an N-terminal translational fusion (His_6-tag::CtpA), could not be cloned into the expression vector pUSH1, or the routine cloning vectors pGEM-7Zf(+) and pGEM-T. An explanation for this result maybe related to the lethality of the *ctpA* product to *E. coli*, a factor dependent on copy number of the vector used to maintain *ctpA*. Nevertheless, this does not seem likely for the following reasons. While pGEM-7Zf(+) and pGEM-T are high copy number vectors (500 to 700 copies per cell),

the expression vector pUSH1 has a low copy number (15 to 20 copies per cell). Second, *ctpA* contained in the pGEM-7Zf(+) derived constructs pCT203 and pCT220, were stable in *E. coli*, and *ctpA* was expressed in this organism under T7 RNA polymerase control (see Section 5.2.11). Overall, data related to the over-expression of CtpA was unable to confirm the N-terminal truncation of this protein. Nevertheless, production of an CtpA antibody is critical to overcome this problem and should remain an objective for future studies.

A second approach to determine the genetic organisation of *ctpA*, involved analysis of nucleotide sequence flanking this gene for open reading frames with potential to encode proteins involved in cation binding or regulation of gene expression. However, similarity to proteins with these functions was not observed. Furthermore, location of promoter regulatory elements necessary for *ctpA* expression, immediately upstream of the putative translational start codon of CtpA (see Section 5.2.7), provides solid evidence for the N-terminal truncation of this protein. In addition, extensive amino acid residue identity to features characteristic of IS elements from Gram-positive bacteria was consistently observed upon translation of nucleotide sequence downstream of *ctpA* (see Section 5.2.5.2). Therefore, in the absence of open reading frames which flank *ctpA*, the genetic organisation of this locus is different to the *hpcopAP* locus from *H. pylori*. Nevertheless, several other Cu^{2+} transporting ATPases have been described in which no accompanying regulatory genes have been identified.

Sequence comparisons established that CtpA is apparently a P-type ATPase from *L. monocytogenes* with significant similarity to a family of proteins involved in the transport of copper in both bacteria and eukaryotes. CtpA may have a related function for *L. monocytogenes*, since the level of *ctpA* mRNA reproducibly and specifically increased by both low and high concentrations of copper in the growth medium (see Section 5.2.6). Other metal ions did not induce this affect. In this analysis, the chelating agents 8-hydroxyquinoline and EGTA were used to deplete the bacterial culture medium of copper. 8-hydroxyquinoline has been used to chelate free Cu^{2+} ions from culture media as part of studies designed to functionally characterise the copper transport systems (CopA and CopB) of *E. hirae* (Odermatt *et al.*, 1993; Odermatt *et al.*, 1994). Similarly, EGTA has been consistently used to chelate free Ca^{2+} ions (Garduno *et al.*, 1992; Van Leengoed and Dickerson, 1992; Laoudji *et al.*, 1994) from bacterial culture media, and was initially employed in this study for a similar purpose (see Section 4.2.4). However, it has been recently confirmed that EGTA has reduced specificity for Ca^{2+} ions (Youatt, 1993). Indeed, at high concentrations, EGTA preferentially

chelates other trace elements including Cu^{2+}. Moreover, the utility of EGTA and 8-hydroxyquinoline as chelators of Cu^{2+} ions was reported by Dawson *et al.*, (1986), which claimed these compounds possess a comparatively high binding coefficient for Cu^{2+}, relative to other divalent cations. These reports provide good evidence for the use of EGTA and 8-hydroxyquinoline as chelators of free Cu^{2+} ions. However, in this study, the depletion of Cu^{2+} ions in the culture medium was not confirmed by analytical methods. While CtpA is apparently involved in maintenance of intracellular copper, this function has not been demonstrated. This is the focus of experimental evidence reported in Chapter 6.

In conclusion, this chapter described the isolation and characterisation of a P-type ATPase from *L. monocytogenes* identified by Tn*917-lacZ-cat86* insertion mutagenesis. This mutant contained a transcriptional *lacZ-cat86* gene fusion identified by induced β-galactosidase production when grown in media containing the cation chelator EGTA. Nucleotide sequence analysis of DNA flanking the transposon revealed an open reading frame (designated *ctpA*), with potential to encode a 653 aa protein with a M_r value of 69, 463. Analysis of DNA upstream of *ctpA* identified putative regulatory sequences involved in expression of this gene. While active cation transport by CtpA has not been demonstrated, a role in copper transport in *L. monocytogenes* is predicted, given the significant degree of identity to P-type ATPases involved in the translocation of copper in both bacteria and eukaryotes. Consistent with this view, *ctpA* mRNA is specifically induced by high and low levels of copper. However, CtpA is distinctive from other ATPase proteins by virtue of an N-terminal truncation in the domain thought to be responsible for initial cation binding. Furthermore, the genetic arrangement of this putative copper transport system is unusual, as no open reading frame with potential to be associated with *ctpA* in copper homeostasis was identified. In particular, deduced peptide sequence from DNA flanking the 3′ end of *ctpA*, shared identity to proteins associated with IS elements of Gram-positive origin.

Chapter 6 Construction and characterisation of mutants in CtpA from *L. monocytogenes*

6.1 Introduction

In chapter 5, the isolation of a gene in *L. monocytogenes* (*ctpA*) which encodes a 653 aa polypeptide with strong identity to cation transporting P-type ATPases was described. P-type ATPase proteins are produced by diverse life forms from both *Prokaryotae* and *Eukaryotae* super kingdoms. While these proteins are responsible for ATP-driven translocation of cations (Pedersen and Carafoli, 1987; Silver *et al.*, 1989), cells encode numerous independent transport mechanisms which mediate resistance to inorganic ions. For example, multiple transporters for a single ion and ion transporters with multiple specificities have been reported (Silver, 1978). In addition, both influx and efflux transporters for the same ion have been described. Consequently, mechanisms of ion transport and homeostasis within cells are likely to be extremely complex (Silver, 1978). In view of this, functional characterisation of P-type ATPases has proven difficult, especially in the presence of a network of transport systems within a single cell.

In general, prediction of the physiological function of bacterial P-type ATPases has been restricted to analysis of peptide primary structure. Identification of absolutely conserved amino acid residues known to be essential for protein function in eukaryotic proteins, has been consistently accepted as evidence for the isolation of bacterial analogues. However, recent studies have provided insights into the specificity and regulation of P-type ATPases involved in cation transport in bacterial species. For example, the plasmid-borne cadmium resistance determinant *cadA,* (Nucifora *et al.,* 1989) from *Staphylococcus aureus,* has been well characterised. Direct biochemical analysis of CadA function, has shown this protein is directly responsible for the efflux of Cd^{2+} ions and that this process is ATP dependent (Tsai *et al.,* 1992; Tsai and Linet, 1993). Similarly, MgtB is responsible for influx of Mg^{2+} ions in *S. typhimurium* (Snavely *et al.,* 1991) and CopB for efflux of Cu^{2+} ions from *E. hirae* (Odermatt *et al.,* 1994). However, the radioisotopic analogues of Mg^{2+} and Cu^{2+} ions used to demonstrate cation transport may not have provided a true representation of the physiological role of either MgtB or CopB.

Although an understanding of the biochemical properties of P-type ATPase function is limited, a significant number of laboratories have focussed on an analysis of regulation of expression of ATPase genes. In this respect, regulation of specific mRNA transcription has been consistently correlated with concentrations of a specific cation in the culture media. For example, transcriptional gene fusions were used to show that Cd^{2+} was a strong inducer of *cadA* expression in *S. aureus* (Yoon *et al.*, 1991; Corbisier *et al.*, 1993). Similarly, Cd^{2+} significantly elevated levels of *cadA* mRNA transcription in *L. monocytogenes* (Lebrun *et al.*, 1994), and Cu^{2+} increased levels of *pacS* and *copA* and *copB* transcripts in *Synechococcus* spp. (Kanamaru *et al.*, 1994) and *E. hirae* (Odermatt *et al.*, 1994), respectively. However, increased expression of P-type ATPase genes by cations not apparently transported by these proteins has been observed (Yoon *et al.*, 1991; Corbisier *et al.*, 1993; Kanamaru *et al.*, 1994; Odermatt *et al.*, 1994). This feature highlights the complex nature of cation transport in these organisms. Not only does it appear that these proteins can transport more than one cation, but they are apparently also capable of transporting both monovalent and divalent cations across the bacterial membrane. Nevertheless, the CtpA transport system from *L. monocytogenes* is apparently an exception. Levels of *ctpA* mRNA are specifically induced by low and high concentrations of Cu^{2+} (see Section 5.2.6). This data suggests that the CtpA transporter may display a singular specificity for copper.

Expression of metal ion transporters is apparently negatively regulated by proteins which interact with promoter regions of the ATPase genes. For example, CadC is intimately involved in expression of cadmium and zinc resistance in *S. aureus* (Yoon and Silver, 1991). The role of CadC is based on three lines of evidence. First, expression of *cadC* was cadmium inducible (Yoon *et al.*, 1991). Second, an inverted repeat was located near the promoter region of *cadC*, indicative of an operator site for a *trans*-acting DNA binding regulatory protein (Yoon *et al.*, 1991). Third, gel retardation assays and DNase I footprinting experiments showed that partially purified CadC interacted with the proposed *cadA* promoter region and that this interaction was inhibited by Cd^{2+} ions (Endo and Silver, 1995). Other proteins associated with cadmium efflux systems in *L. monocytogenes* (Lebrun *et al.*, 1994) and *Bacillus firmus* (Ivey *et al.*, 1992), share extensive similarity to the CadC protein from *S. aureus*. This suggests that the *cadC* analogues may also be involved in negative regulation of *cadCA* expression in these organisms. Furthermore, the ORF3 encoded peptide associated with the hpCopAP copper resistance system from *Helicobacter pylori*, may function in regulation of this operon (Ge *et al.*, 1995). Regulatory genes associated with other bacterial P-type ATPases including

the CtpA transport system from *L. monocytogenes*, have not yet been identified. Nucleotide sequence analysis of DNA immediately flanking *ctpA* has not identified an open reading frame with similarity to known regulatory genes (see Section 5.2.5.2). DNA downstream to *ctpA* apparently has strong identity to that encoding IS elements.

While a number of P-type ATPases have been shown to play an integral role in transport of cations across biological membranes, a direct physiological function has only been assigned to the two proteins associated with the Menkes and Wilson hereditary disorders of humans. Mutations in these alleles lead to defective copper transport (Darwish *et al.*, 1983; Danks, 1989; Sarkar *et al.*, 1993). In Menkes disease, the export of copper from intestinal cells is defective, and results in severe copper deficiency. In contrast, Wilson disease results from a failure to remove copper from the liver into bile, leading to copper toxicity. Interestingly, the CtpA ATPase from *L. monocytogenes*, has significant identity to bacterial proteins involved in translocation of copper, as well as those encoding the Menkes and Wilson disease determinants (see Section 5.2.5.1). Given *L. monocytogenes* is ubiquitously distributed throughout the environment, these findings suggest an important role for the CtpA P-type ATPase in survival of this organism in its natural habitat, and may involve maintaining copper homeostasis through transport of Cu^{2+} ions.

To establish a physiological function for CtpA, a mutant was constructed by insertion of an antibiotic-resistance cartridge into the *ctpA* gene using a novel approach involving homologous recombination. The construction of this mutant and it's use in demonstrating that CtpA is a copper transport system in *L. monocytogenes* is described. In addition, the significance of CtpA for pathogenesis was examined using tissue culture monolayer internalisation assays and mouse infection models. Finally, the prevalence of the *ctpA* determinant in *Listeria* spp., including a collection of environmental, clinical, and laboratory isolates of *L. monocytogenes* was determined. The implications of these results are discussed with respect to the potential origin of *ctpA*.

6.2 Results

6.2.1 Construction of *L. monocytogenes* DSE201, a N-terminal erythromycin resistant insertion mutant in *ctpA*

The site of transposon insertion in the *ctpA*::Tn*917-lacZ-cat86* *L. monocytogenes* C185 mutant was mapped to 9 nucleotides upstream from the translational stop codon (TAG) of

ctpA (see Section 5.2.4, Figure 5.14). The possibility that C185 may still exhibit a wild type phenotype, prompted construction of a second mutant by insertional mutagenesis in the 5′ region of *ctpA*. This was achieved by allelic replacement of wild type *ctpA*, by a copy of *ctpA* containing an erythromycin resistance gene (*erm*) inserted at the 5′ end. Copies of *ctpA*::*erm* located on a Gram-negative replicon based suicide plasmid, were introduced into *L. monocytogenes* by RP4 mediated conjugation from an *E. coli* donor.

6.2.1.1 Generation of a *ctpA* specific, mobilisable mutagenesis vector, pCT223

The strategy used to construct the mutagenesis vector is shown in Figure 6.1. Plasmid pCT221 was constructed by cloning a 1.1 kb *Bam*HI fragment encoding the *erm* gene isolated from pGI21, into a *Pst*I site internal to a partial copy of *ctpA* located on plasmid pCT203[1]. The *Pst*I site is located 173 bp from the *ctpA* translational start codon. A 1.7 kb *Bam*HI fragment encoding the recognition site for mobilisation (*mob*) was then purified from pSUP201-1 and cloned into the *Bam*HI site of pCT221 to give plasmid pCT222. Conjugal transfer of pCT222 between *E. coli* strains SM10 and S17-1 was confirmed by suitable mating experiments (Sharma *et al.*, 1989). A Gram-positive chloramphenicol resistance marker external to *ctpA* was introduced into pCT222 by cloning a 1.5 kb *Eco*RI/*Hin*dIII fragment containing *catP* purified from pJIR1243, into the *Eco*RI restriction site to create pCT223.

6.2.1.2 Isolation of *L. monocytogenes* LM001, a recipient strain for DNA mobilisation from *E. coli*

A *L. monocytogenes* recipient strain was isolated that enabled selection against the donor *E. coli* strain, following conjugative transfer of pCT223. Two *L. monocytogenes* DRDC8 derivatives, that exhibited resistance to 50 μg/ml streptomycin (Sm) (LM001), or 25 μg/ml rifampicin (Rp) (LM002), were isolated. The growth rate of these Sm and Rp resistant isolates was compared to wild type DRDC8, by measuring the optical density of the bacterial cultures at 600 nm over 24 h period. The culture conditions employed were a modification of the conditions used for the analysis of *ctpA* transcription (see Section 5.2.6). Similar growth rates were observed when all strains were grown in BHI broth (Figure 6.2, Part A). However, growth of LM002 was significantly reduced in comparison to DRDC8 and LM001, when grown in media containing either 4 mM $CuSO_4$, 10 mM EGTA, or 10 μM 8-hydroxyquinoline (Figure 6.2, Part B to D). In view of these results, LM001 was selected in preference to

[1] The *ctpA* gene contains a 9 nucleotide truncation at the 3′ end, corresponding to the site of Tn*917-lacZ-cat86* insertion (see Figure 5.3 and Figure 5.5 for details on the origin and construction of pCT203).

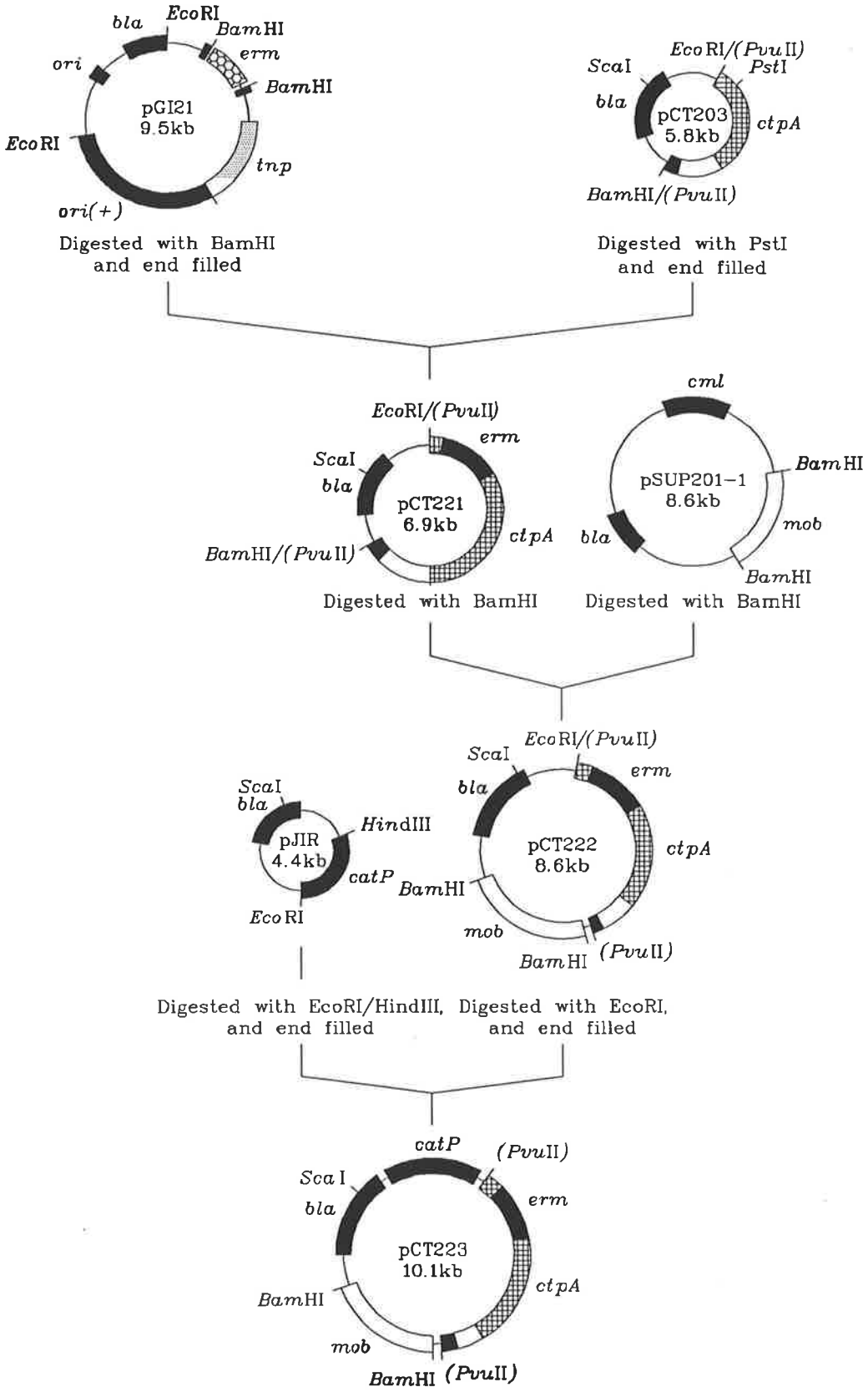


Figure 6.1: Construction of plasmid pCT223. Abbreviations: *bla*, ampicillin resistance gene; *erm*, erythromycin resistance gene; *catP*, chloramphenicol resistance gene; *ctpA*, putative Cu^{2+} transporting P-type ATPase gene from *L. monocytogenes* DRDC8; *tnp*, gene encoding for IS*10* transposase; *ori*, origin of DNA replication; *ori*(+), Gram-positive origin from the *B. thuringiensis* plasmid pHT1030; *mob*, recognition site for mobilisation. Restriction sites in parentheses have been destroyed in the cloning procedure.

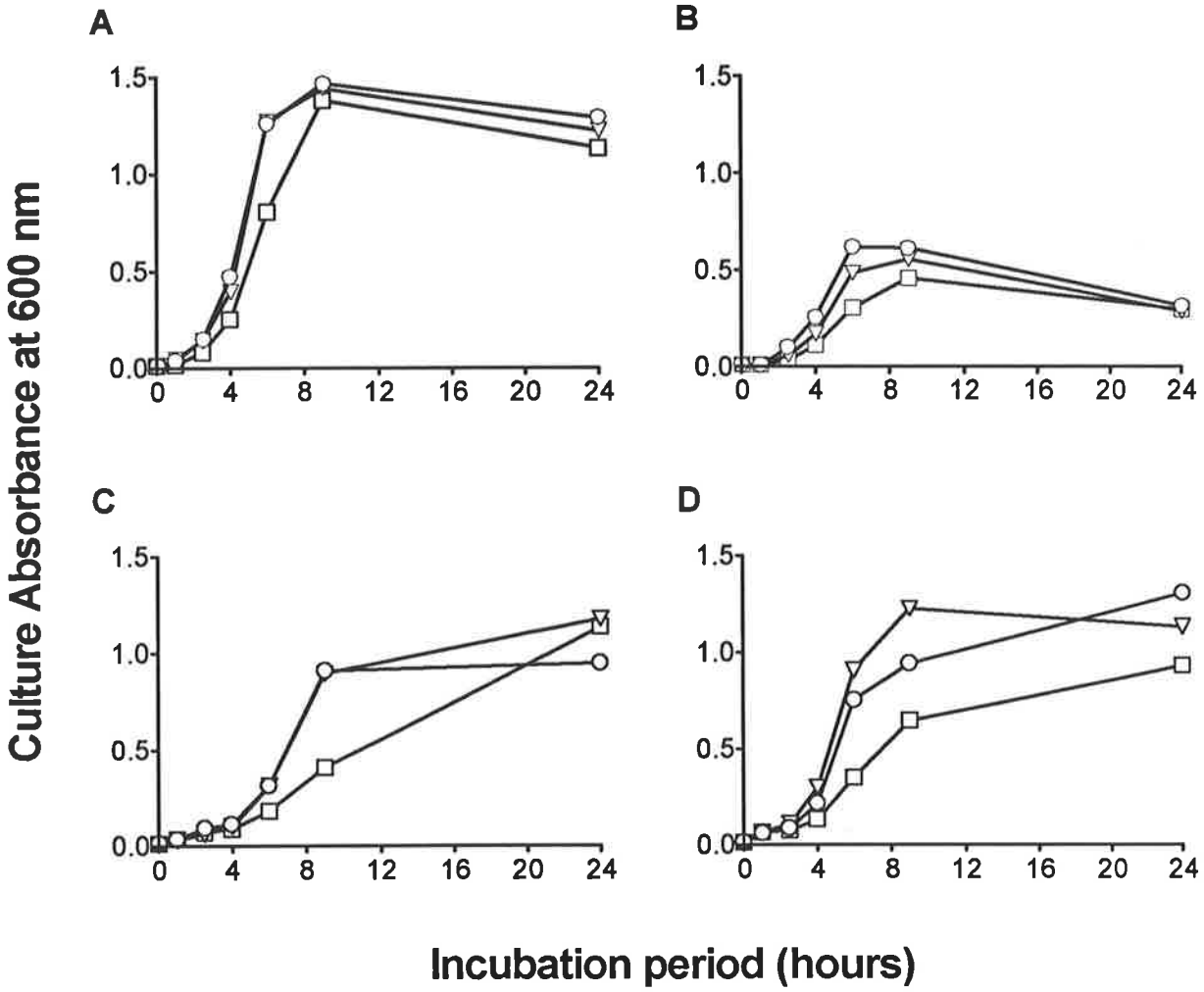


Figure 6.2: Growth of *L. monocytogenes* DRDC8 (○), LM001 (▽), and LM002 (□). Panel: [A], growth in BHI broth medium; [B], growth in BHI broth containing 4 mM $CuSO_4$; [C], growth in BHI broth containing 10 mM EGTA; [D], growth in BHI broth containing 5 μM 8-hydroxyquinoline. Each graph represents the mean of four independent experiments.

LM002, as the *L. monocytogenes* recipient strain in conjugal transfer experiments involving *E. coli* SM10/pCT223.

6.2.1.3 Introduction of *ctpA*::*erm* into *L. monocytogenes* DRDC8

The *ctpA*::*erm* allele contained on pCT223, was introduced into the *L. monocytogenes* chromosome by homologous recombination. The principle of allelic exchange (diagrammatically represented in Figure 6.3), provided a mechanism by which the chromosomal wild type *ctpA* allele was replaced by a single plasmid borne *in vitro* mutated copy (*ctpA*::*erm*). The Gram-negative replicon (*ori*) of pCT223, prevented stable maintenance of this vector in *L. monocytogenes*. Cointegration of plasmid DNA into the *L. monocytogenes* chromosome was monitored by Cm sensitivity.

Plasmid pCT223, was transferred from *E. coli* SM10 to *L. monocytogenes* LM001 by conjugation. A single cross-over event between an homologous region of plasmid derived *ctpA*::*erm* and wild type *ctpA* on the *L. monocytogenes* chromosome was selected by growth on plates containing Cm and Em. A total of 35 Cm^R, Em^R transconjugates derived from four independent filter matings were recovered after growth on appropriate media, at an average efficiency of 4.4×10^{-5} transconjugates/donor CFU. To confirm Cm^R and Em^R transconjugates were indeed *L. monocytogenes* and not resistant contaminants, a 417 bp portion of the listeriolysin O determinant *hly*, was amplified with synthetic oligonucleotides p234 and p319 (see Table 2.5 and Section 4.2.1.3, Figure 4.3), from chromosomal DNA purified from these strains using the method described by Fitter *et al.*, (1992). Figure 6.4 shows a *hly* specific 417 bp amplified DNA product obtained from a single Cm^R, Em^R *L. monocytogenes* isolate DSEC1. *L. monocytogenes* strains DRDC8 and LM001, and plasmid pLIS3, containing the *hly* gene (Cossart *et al.*, 1989) were included as controls. A total of 600 transconjugates obtained from an O/N culture of DSEC1 grown in medium containing Em, were then replica patched on solid media containing Em or Em plus Cm in order to identify Em^R, Cm^S isolates. Eleven putative *ctpA*::*erm* mutants were recovered at an efficiency of 0.18%. Four Cm^S and Em^R *L. monocytogenes* strains (DSE201, DSE221, DSE285, and DSE294), were characterised at the molecular level.

6.2.1.4 Molecular characterisation of *L. monocytogenes ctpA*::*erm* insertion mutants

Allelic replacement of wild type *ctpA* with *ctpA*::*erm* was confirmed by Southern hybridisation analysis of *Dra*I digested chromosomal DNA isolated from the Cm^S, Em^R *L. monocytogenes*

Figure 6.3: Schematic diagram describing construction of strains containing an internal insertion in *ctpA*. [A]: Chromosomal integration of pCT223 by homologous recombination between *ctpA*::*erm* allele on the plasmid, and the wild-type *ctpA* chromosomal allele. The designated cross-over points shown are arbitrary, and recombination could occur on either side of the *erm* insertion. [B]: The resulting cointegrate in the chromosome was selected by growth in the presence of Cm and Em. [C]: Upon passage of the merodiploid intermediate strain for several generations without Cm selection pressure, spontaneous excision of the integrated plasmid from the chromosome occurred. [D]: After curing of the excised plasmids by growth in the presence of Em, *L. monocytogenes* Em^R and Cm^S revertants were recovered. These resulted from excision of the integrated plasmid via homologous recombination on the opposite side of the deletion allele as shown in (C).

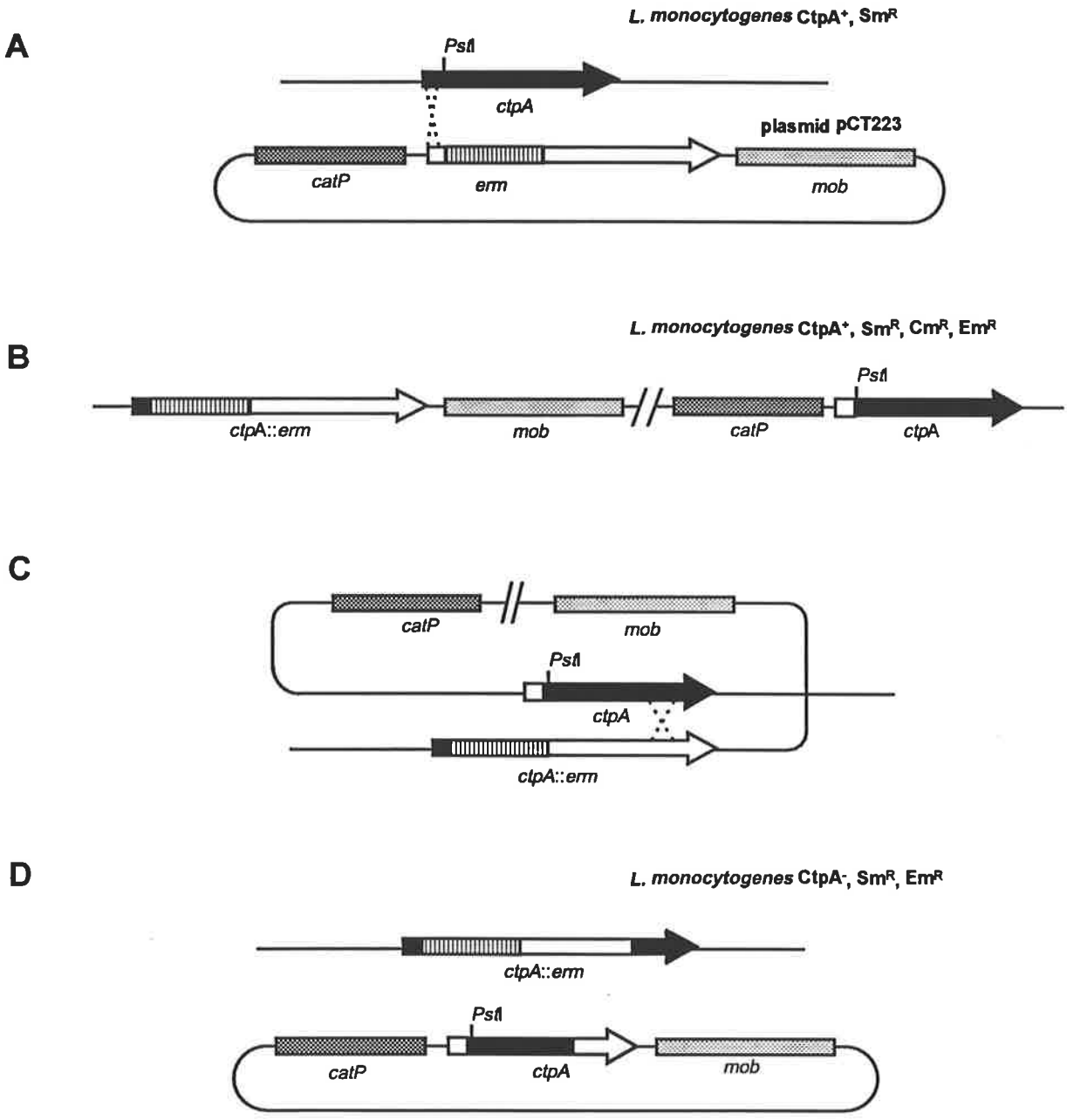
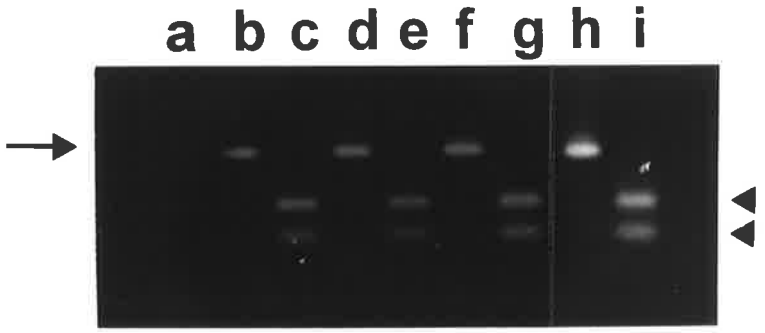

A
L. monocytogenes CtpA+, SmR
PstI
ctpA
plasmid pCT223
catP
erm
mob
B
L. monocytogenes CtpA+, SmR, CmR, EmR
PstI
ctpA::erm
mob
catP
ctpA
C
catP
mob
PstI
ctpA
ctpA::erm
D
L. monocytogenes CtpA-, SmR, EmR
ctpA::erm
PstI
catP
ctpA
mob

Figure 6.4: PCR analysis of chromosomal DNA isolated from *L. monocytogenes* wild type and Cm^R and Em^R strains using the *L. monocytogenes hly* specific synthetic oligonucleotides p234 and p319, described by Fitter *et al.*, (1992). Lane: [a], non-pathogenic *L. innocua*; [b and c], DRDC8; [d and e], DSEC1 (a Cm^R and Em^R strain of *L. monocytogenes* DRDC8 containing the plasmid, pCT223, integrated in the chromosome); [f and g], LM001; [h and i], 10 ng plasmid pLIS3 [a 4 kb *Bam*HI *hly* clone (Cossart *et al.*, 1989)]. The 0.42 kb PCR amplified DNA products are indicated by an arrow. Lanes c, e, g, and i are *Hin*dIII restricted PCR product, which generates a 162 bp and 255 bp fragment as predicted from the nucleotide sequence of the *hly* gene (Mengaud *et al.*, 1988).


a b c d e f g h i

isolates (Figure 6.5). A DIG labelled 1.73 kb *Pst*I/*Sac*I DNA fragment internal to *ctpA*, hybridised to a 3.51 kb *Dra*I chromosomal DNA fragment from DSE201, DSE221, DSE285, and DSE294. As expected, this probe also hybridised to a 3.35 kb *Dra*I chromosomal DNA fragment from DRDC8 and LM001. Plasmid pCT203 was included as the positive control. The difference in fragment sizes (~160 bp) is consistent with insertion of a 1.1 kb *erm* gene cartridge in *ctpA*, because a single *Dra*I site located at nucleotide position 190 in *erm* (Martin *et al.*, 1987), would account for the different sizes of the *Dra*I DNA fragments. This result indicates the *erm* gene must have inserted in the same orientation as *ctpA* (diagrammatically illustrated in Figure 6.6).

A combination of PCR and Southern hybridisation analysis was used to confirm this data. Using oligonucleotide primers which flanked the *Pst*I site used for insertion of the *erm* gene cartridge (Figure 6.7), PCR was used to amplify *ctpA* specific DNA. A 0.52 kb product was amplified from DRDC8 derived strains and pCT203 DNA, whereas a 1.62 kb fragment was amplified from Cm^S and Em^R isolates and pCT223 DNA (Figure 6.8, Plate A). No products were obtained for the non-pathogenic *L. innocua* (Figure 6.8, Plate A). The difference in product sizes is consistent with insertion of a 1.1 kb *erm* gene cartridge in *ctpA*. This was confirmed by hybridisation analysis of the PCR products using *ctpA* and *erm* specific DNA probes. The *ctpA* probe hybridised to the 0.52 kb and the 1.62 kb products (Figure 6.8, Plate B), whereas the *erm* specific probe hybridised to only the 1.62 kb products (Figure 6.8, Plate C). A schematic comparison of the *L. monocytogenes* C185 and DSE201 *ctpA* insertion mutants is presented in Figure 6.9.

6.2.2 *In vitro* growth comparison of *ctpA* insertion mutants and wild type *L. monocytogenes* isolates in response to heavy metal divalent ions and cation chelators

On the basis that CtpA shares sequence identity with Cu^{2+} transport associated P-type ATPases (see Section 5.2.5.1) and that *ctpA* mRNA levels respond to copper in the culture medium (see Section 5.2.6), a role for the CtpA protein in copper transport in *L. monocytogenes* is predicted. In view of this, the *ctpA*::Tn*917-lacZ-cat86* insertion mutant, C185 and the *ctpA*::*erm* insertion mutant, DSE201, were used to demonstrate that mutations in CtpA have a direct effect on sensitivity of *L. monocytogenes* to copper. In this analysis, the chelating agents EGTA and 8-hydroxyquinoline, were used to deplete the bacterial culture medium of copper.

Figure 6.5: Southern hybridisation analysis of *Dra*I digested chromosomal DNA prepared from wild type *L. monocytogenes* and *ctpA*::*erm* mutant strains. DNA was probed with a digoxigenin labelled 1.73 kb *Pst*I/*Sac*I DNA fragment purified from plasmid, pCT203 containing *ctpA* (see Figure 5.6). Lane: [a], *Eco*RI digested Bacteriophage SPP-1 DNA; [b], DRDC8; [c], LM001; [d], DSE201; [e], DSE221; [f], DSE285; [g], DSE294; [h], non-pathogenic *L. innocua*; [i], *Eco*RI/*Bam*HI digested pCT203. The arrows indicate a 3.35 kb *Dra*I DNA fragment from parent strains, and a 3.51 kb *Dra*I DNA fragment from mutant strains, that hybridised to probe DNA. The arrowhead indicates a 2.8 kb *Eco*RI/*Bam*HI *ctpA* DNA fragment from plasmid, pCT203.

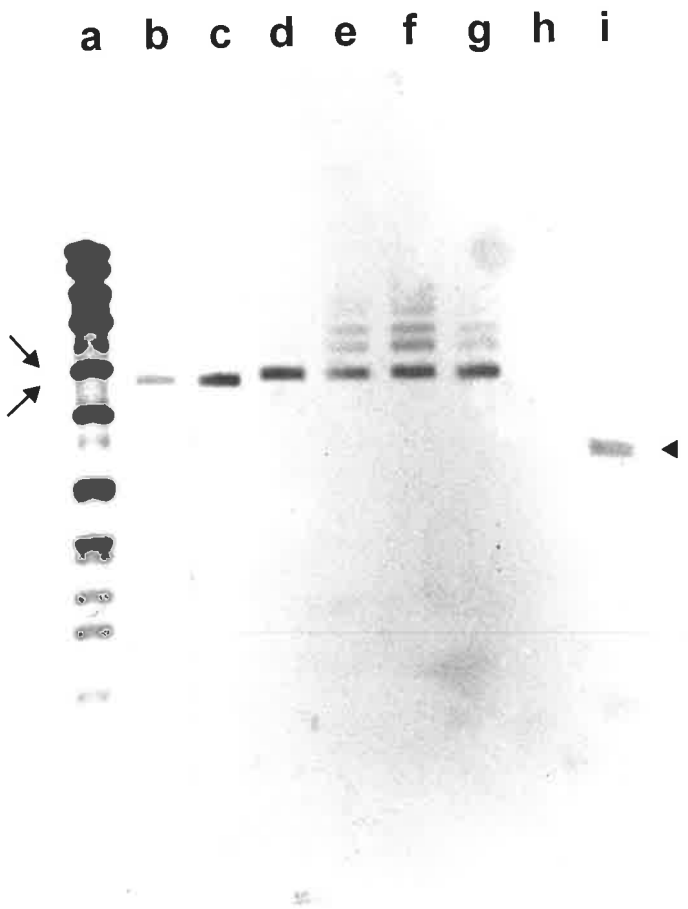
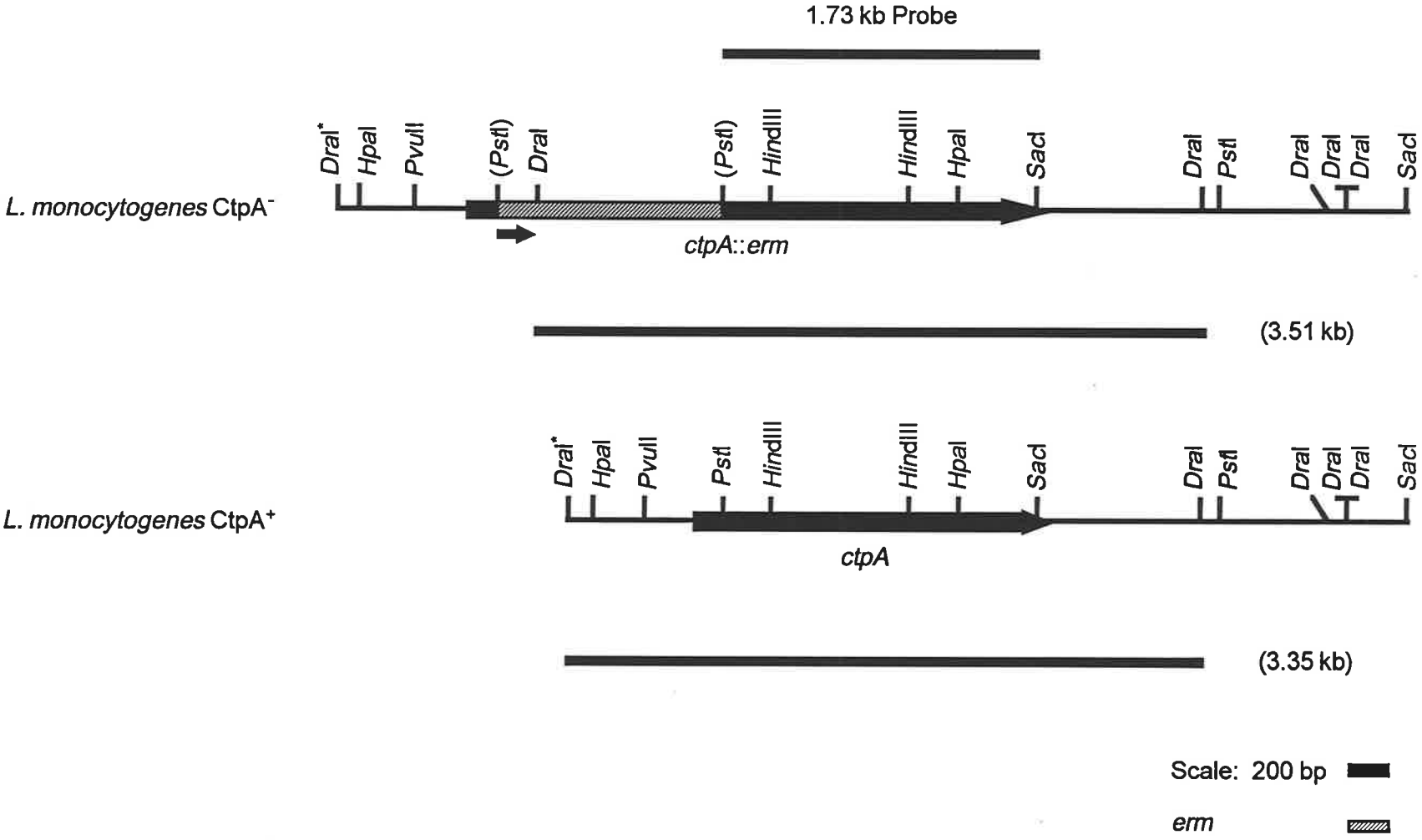

a b c d e f g h i

Figure 6.6: Schematic diagram of the *ctpA*::*erm* mutant allele in *L. monocytogenes* Cm^S and Em^R isolates (DSE201, DSE221, DSE285, and DSE294). The location of the 1.73 kb *Pst*I/*Sac*I DNA fragment purified from plasmid, pCT203, containing *ctpA* (see Figure 5.6), is indicated and the *Dra*I chromosomal DNA fragments from $CtpA^-$ and wild type strains which hybridise to probe DNA are represented by a heavy line. The putative orientation of *erm* relative to *ctpA* is shown by an arrow. Restriction sites in parentheses have been destroyed by the cloning procedure and those indicated by an asterisk have not been confirmed by nucleotide sequence. The location of these sites were determined from Southern hybridisation of *Dra*I restricted DNA (see Figure 6.5).


1.73 kb Probe
L. monocytogenes CtpA⁻
DraI*
HpaI
PvuII
(PstI)
DraI
(PstI)
HindIII
HindIII
HpaI
SacI
DraI
PstI
DraI
DraI
DraI
SacI
ctpA::erm
(3.51 kb)
L. monocytogenes CtpA⁺
DraI*
HpaI
PvuII
PstI
HindIII
HindIII
HpaI
SacI
DraI
PstI
DraI
DraI
DraI
SacI
ctpA
(3.35 kb)
Scale: 200 bp
erm

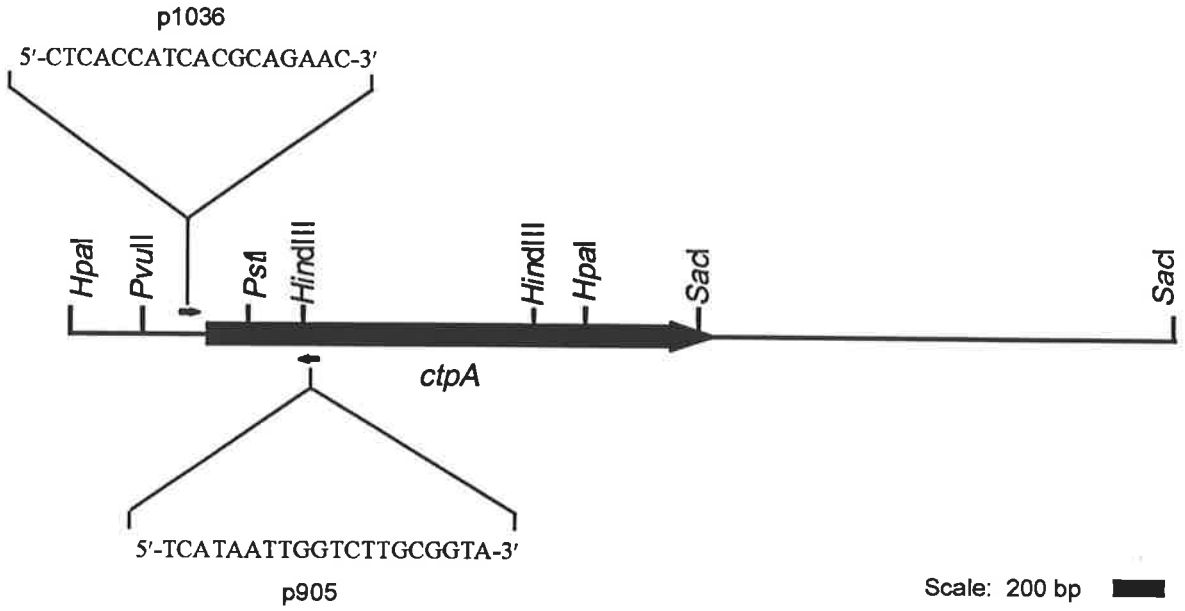
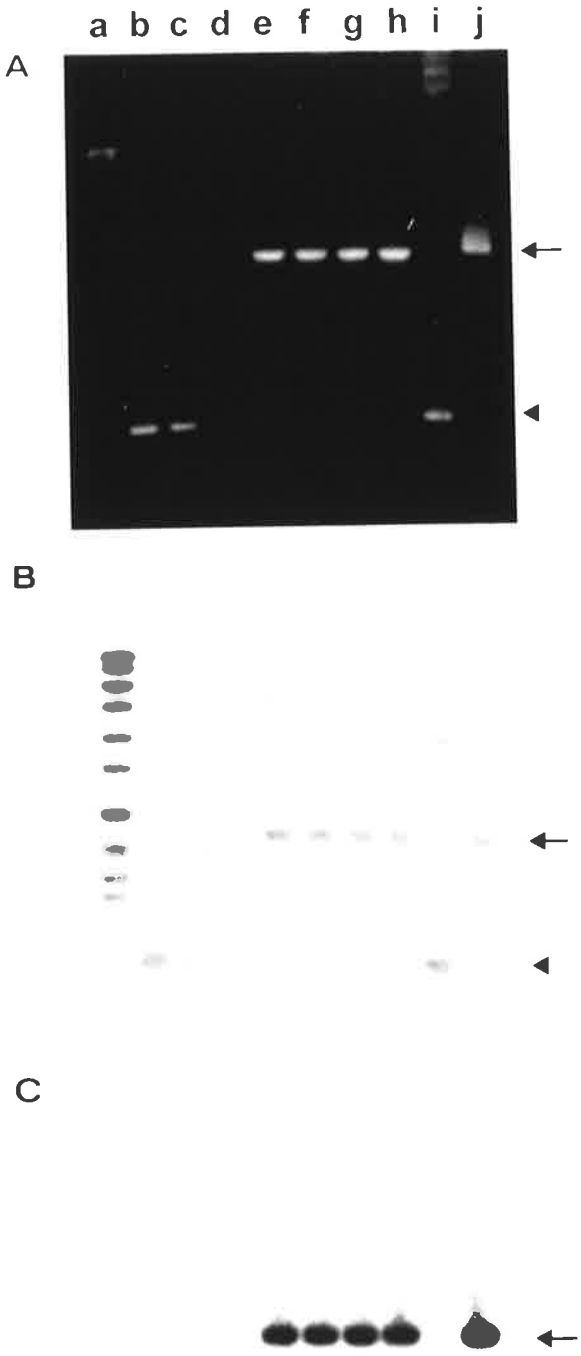


Figure 6.7: Location of the synthetic oligonucleotides p905 and p1036, used in the molecular characterisation of the *ctpA*::*erm* mutant. The oligonucleotides are complementary to nucleotide sequence flanking the *Pst*I site in *ctpA*. This *Pst*I site was used to disrupt *ctpA* by insertion of a erythromycin resistance cartridge isolated from plasmid pGI21 on a *Bam*HI fragment (see Figure 6.1). The thermal cycling profile is described in Table 2.5.

Figure 6.8: Molecular characterisation of *ctpA*::*erm*, *L. monocytogenes* DSE201. Plate A: PCR analysis of chromosomal DNA isolated from *L. monocytogenes* wild type and *ctpA* mutant strains using the synthetic oligonucleotides p905 and p1036. Lane: [a], *Eco*RI digested Bacteriophage SPP-1 DNA; [b], DRDC8; [c], LM001; [d], non-pathogenic *L. innocua*; [e], DSE201; [f], DSE221; [g], DSE285; [h], DSE294; [i], plasmid pCT203; [j], plasmid pCT223. The 1.625 kb and 0.525 kb PCR amplified DNA products are indicated by an arrow and arrowhead, respectively. Amplified DNA was subjected to Southern hybridisation analysis using the digoxigenin labelled 1.73 kb *Pst*I/*Sac*I DNA fragment internal to *ctpA* isolated from pCT203 (Plate B), or the 1.1 kb *Bam*HI *erm* cartridge from pGI21 (Plate C) as the probe.


a b c d e f g h i j
A
B
C

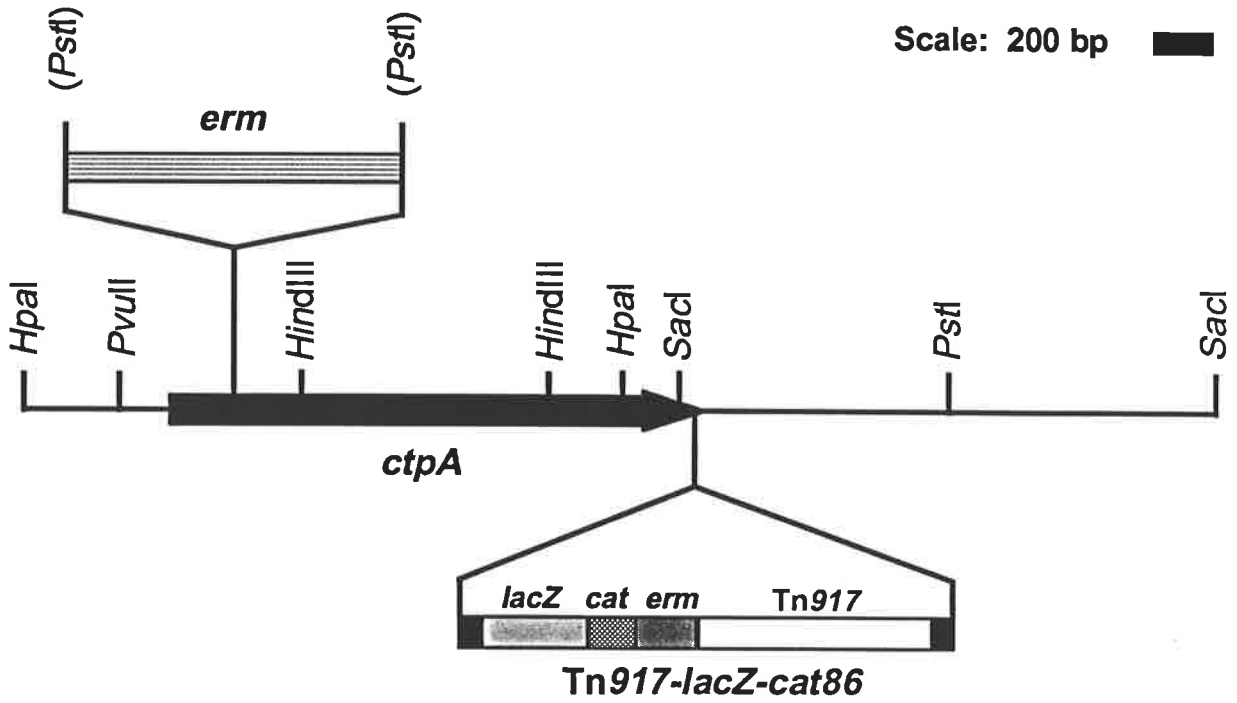


Figure 6.9: Diagrammatic representation of the *ctpA* gene. The site of insertion of Tn*917-lacZ-cat86* and *erm* in the chromosome of *L. monocytogenes* C185 and DSE201, respectively, are indicated. Tn*917-lacZ-cat86* is not drawn to scale. Restriction sites in parentheses have been destroyed in the cloning procedure.

6.2.2.1 Sensitivity of *L. monocytogenes* mutants to several heavy metal ions

Minimal inhibitory concentrations (MIC) of several heavy metal divalent ions and the chelating agents, EGTA and 8-hydroxyquinoline for the insertion mutants and parental strains, were determined by the method of Sahm and Washington, (1991). *L. monocytogenes* DRDC8, C185, LM001, and DSE201, were compared for their ability to grow on solid media containing increasing concentrations of the cations Ag^{2+}, Ba^{2+}, Ca^{2+}, Cd^{2+}, Co^{2+}, Cr^{2+}, Cu^{2+}, Hg^{2+}, Mg^{2+}, Mn^{2+}, Ni^{2+}, and Zn^{2+}, and the chelating agents EGTA and 8-hydroxyquinoline (Table 6.1). Although all strains were equally susceptible to all divalent cations tested and to EGTA, the MIC was dependent on the growth media used[2]. Nevertheless, when C185 was grown on agar media (Difco) containing the heavy metal cation chelator, 8-hydroxyquinoline, growth was inhibited at a final concentration of 10 μM, whereas the concentration required for inhibition of DRDC8 was 20 μM. Susceptibility of DSE201 grown on media supplemented with 8-hydroxyquinoline was not determined. Even though the growth inhibition of the *ctpA*::Tn*917-lacZ-cat86* insertion mutant by 8-hydroxyquinoline could be due to direct toxicity of this agent towards the mutant bacterium, it is more likely that addition of 8-hydroxyquinoline to the culture medium, leads to a depletion of the essential trace element, copper. However, the depletion of Cu^{2+} ions in the culture medium was not confirmed by analytical methods.

6.2.2.2 Effect of Cu^{2+} concentration on rate of growth of *ctpA* insertion mutants grown in broth culture

The sensitivity of the growth of both *ctpA* insertion mutants DSE201 and C185 to copper was tested in liquid media. Growth of both strains in BHI broth containing 4 mM $CuSO_4$, was comparable to the parental strains DRDC8 and LM001 respectively (Figure 6.10, Plate B), but significantly less than growth of all strains in standard culture conditions (Figure 6.10, Plate A). Growth of DSE201 and LM001 were similar in BHI broth containing 10 mM EGTA, but surprisingly, growth of C185 was reduced compared to the parental strain (Figure 6.10, Plate C). This result is contradictory to other experiments which have reliably shown that both C185 and DSE201 and the parental strains, are equally susceptible to EGTA (see Section 6.2.2.1). Nevertheless, C185 and DSE201 showed a significantly reduced capacity for growth in BHI broth containing 5 μM 8-hydroxyquinoline compared with the

[2] During the course of this study, changes to quarantine regulations mean that Difco and Oxoid BHI powder are no longer available to Australian consumers. This prevented the calculation of MIC for some agents to *L. monocytogenes* grown on media purchased from these companies.

Table 6.1 Minimal inhibitory concentration of heavy metal divalent cations and cation chelating agents for *L. monocytogenes*[a]

Growth condition[b]	Minimal inhibitory concentration (MIC)[c]											
	DRDC8			C185			LM001			DSE201		
	A	B	C	A	B	C	A	B	C	A	B	C
Ag^{+}	1.50	> 0.50	> 0.40	1.50	> 0.50	> 0.40	nt	> 0.50	> 0.40	nt	> 0.50	> 0.40
Ba^{2+}	< 16.77	nt	nt	< 16.77	nt	nt	nt	nt	nt	nt	nt	nt
Ca^{2+}	< 27.86	nt	nt	< 27.86	nt	nt	nt	nt	nt	nt	nt	nt
Cd^{2+}	0.67	< 1.60	< 2.00	0.67	< 1.60	< 2.00	nt	< 1.60	< 2.00	nt	< 1.60	< 2.00
Co^{2+}	2.15	4.00	2.00	2.15	4.00	2.00	nt	4.00	2.00	nt	4.00	2.00
Cr^{2+}	15.40	20.00	15.00	15.40	20.00	15.00	nt	20.00	15.00	nt	20.00	15.00
Cu^{2+}	12.80	8.00	10.00	12.80	8.00	10.00	nt	8.00	10.00	nt	8.00	10.00
Hg^{2+}	0.059	0.05	0.01	0.059	0.05	0.01	nt	0.05	0.01	nt	0.05	0.01
Mg^{2+}	< 20.15	nt	nt	< 20.15	nt	nt	nt	nt	nt	nt	nt	nt
Mn^{2+}	< 27.13	nt	nt	< 27.13	nt	nt	nt	nt	nt	nt	nt	nt
Ni^{2+}	17.20	18.00	< 9.00	17.20	18.00	< 9.00	nt	18.00	< 9.00	nt	18.00	< 9.00
Zn^{2+}	14.20	12.00	< 10.00	14.20	12.00	< 10.00	nt	12.00	< 10.00	nt	12.00	< 10.00
EGTA	10.00	27.50	< 30.00	10.00	27.50	< 30.00	nt	27.50	< 30.00	nt	27.50	< 30.00
8-Hq	0.02	nt	> 0.003	0.01	nt	> 0.003	nt	nt	> 0.003	nt	nt	> 0.003

[a] During the course of this study, changes to quarantine regulations mean that Difco and Oxoid BHI powder are no longer available to Australian consumers. This prevented the calculation of MIC for some agents to *L. monocytogenes* grown on media purchased from these companies.

[b] Concentration of cations and cation chelators in mM.

[c] MIC is defined as the lowest concentration that inhibits growth (Sahm and Washington, 1991), determined after a 72 h incubation of plates at 37°C. The source of Brain Heart Infusion media was: A, Difco Laboratories; B, Oxoid Ltd; and C, BBL Microbiology Systems.

nt strains not tested

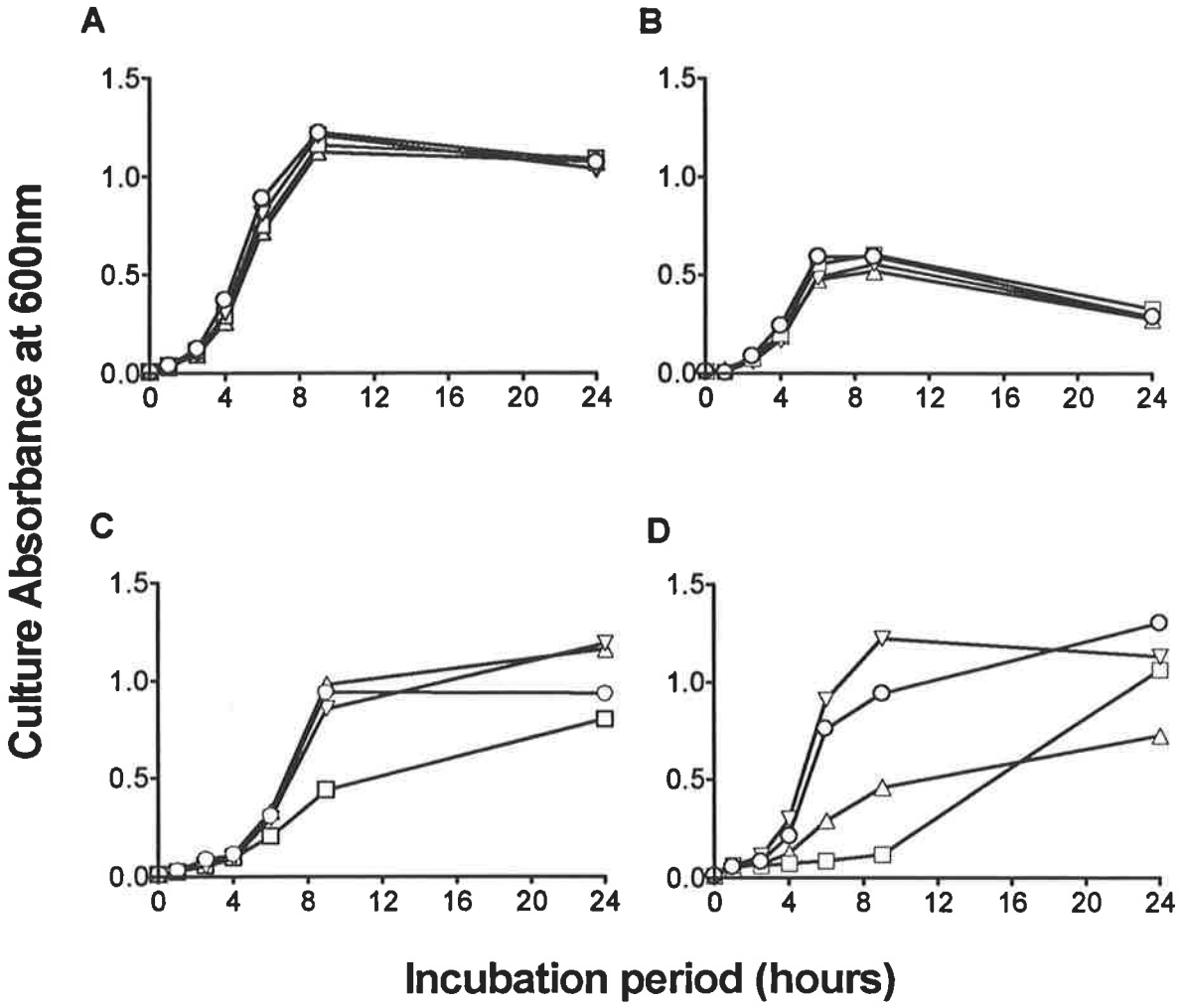


Figure 6.10: Growth of *L. monocytogenes* DRDC8 (O), LM001 (∇), C185 (□), and DSE201 (Δ). Panel: [A], growth in BHI broth medium (control); [B], growth in BHI broth containing 4 mM $CuSO_4$; [C], growth in BHI broth containing 10 mM EGTA; [D], growth in BHI broth containing 5 μM 8-hydroxyquinoline. Each graph represents the mean of at least three independent experiments.

parental strains, DRDC8 and LM001 (Figure 6.10, Plate D). Overall, these data are consistent with the effects of 8-hydroxyquinoline on CopA, a copper influx system in *E. hirae* (Odermatt *et al.*, 1993; Odermatt *et al.*, 1994), and suggests that the CtpA ATPase is involved in copper influx in *L. monocytogenes*.

6.2.3 *In vitro* pathogenicity of *ctpA* insertion mutants

Before examining the *in vivo* behaviour of the *ctpA* insertion mutants, DSE201 and C185 were characterised *in vitro* with respect to properties which might affect pathogenic potential.

6.2.3.1 Haemolytic activity of *L. monocytogenes ctpA* mutants and wild type strains

Haemolytic activity is an essential determinant in *L. monocytogenes* pathogenicity (Gaillard *et al.*, 1986; Kathariou *et al.*, 1987a; Cossart *et al.*, 1989; Michel *et al.*, 1990). DSE201, C185 and parental strains exhibited comparable levels of β-haemolysis when cultured on CHBA media. The *hly*::Tn*917-lacZ-cat86* insertion mutant, *L. monocytogenes* LLO17, and a non haemolytic, non pathogenic *L. innocua* strain used as controls, showed no haemolytic activity when cultured on identical media.

6.2.3.2 Growth competition between *L. monocytogenes ctpA* mutants and wild type strains

The ability of *ctpA* mutants to grow in the presence of the parent strain was determined by *in vitro* competition experiments in Difco BHI broth (Table 6.2). None of the *ctpA*::*erm* *L. monocytogenes* isolates were disadvantaged for growth in competition experiments with LM001, with wild type parent:mutant ratios of *ca.* 2.5:1 consistently recorded following sampling after 0 h, 4 h, and 8 h incubations. In contrast, the *ctpA*::Tn*917-lacZ-cat86* mutant was significantly disadvantaged when grown in the presence of DRDC8, achieving an increase in the wild type parent:mutant ratio from *ca.* 4.5:1 to *ca.* 51:1 after 8 h incubation. Nevertheless, similar growth rates were observed when all strains were grown independently (see Section 6.2.2.2, Figure 6.10). Control competition experiments between DRDC8:LLO17 and DRDC8:LM001, consistently resulted in wild type parent:mutant ratios of *ca.* 2:1 at all sampling times.

6.2.3.3 Intracellular growth and cell to cell spread of *ctpA* insertion mutants in tissue culture cell monolayers

Given the facultatively intracellular properties of *L. monocytogenes*, the behaviour of the CtpA insertion mutants and wild type strains in infected tissue culture monolayers were compared.

Table 6.2 Growth of *L. monocytogenes* wild type and *ctpA* mutant strain combinations in BHI broth[a]

L. monocytogenes strain combination	0 h			4 h			8 h		
	Parent	Mutant	Ratio	Parent	Mutant	Ratio	Parent	Mutant	Ratio
DRDC8 / C185	7.03×10^2	1.55×10^2	4.54:1	2.74×10^4	3.20×10^3	8.56:1	2.86×10^6	5.60×10^4	51.07:1
DRDC8 / LLO17	1.06×10^3	5.30×10^2	2.00:1	4.55×10^4	2.10×10^4	2.17:1	4.37×10^6	1.20×10^6	3.64:1
DRDC8 / LM001	1.14×10^3	5.88×10^2	1.94:1	4.03×10^4	2.41×10^4	1.67:1	3.36×10^6	1.78×10^6	1.89:1
LM001 / DSE201	1.13×10^3	5.48×10^2	2.06:1	3.96×10^4	1.62×10^4	2.44:1	3.09×10^6	1.40×10^6	2.21:1
LM001 / DSE221	1.16×10^3	5.40×10^2	2.15:1	4.73×10^4	1.71×10^4	2.77:1	3.75×10^6	1.30×10^6	2.88:1
LM001 / DSE285	1.15×10^3	4.25×10^2	2.71:1	4.98×10^4	1.76×10^4	2.83:1	3.72×10^6	1.30×10^6	2.86:1
LM001 / DSE294	1.21×10^3	4.15×10^2	2.92:1	4.86×10^4	1.93×10^4	2.52:1	3.36×10^6	1.52×10^6	2.21:1

[a] Represents the mean viable bacterial counts at 0 h, 4 h, and 8 h post inoculation, from at least two independent experiments.

Cell to cell spread during bacterial infection was confirmed using immunofluorescent techniques. HeLa cells infected at an MOI of 1:1 with DRDC8, LM001, C185, and DSE201, demonstrated classical features indicative of bacterial induced actin polymerisation and cell to cell spread (Figure 6.11). As expected, the non-haemolytic *L. monocytogenes* strain, LLO17, was unable to polymerise actin or engage in cell to cell spread. Control strains containing the erythromycin gene or Tn*917-lacZ-cat86* in unrelated loci were not used in this study. Rates of growth of each bacterial strain in the cytoplasm of cell lines was not significantly different (Figure 6.12). The intracellular doubling times of all strains in the J774 and HeLa cell lines was ~130 min and ~70 min, respectively. *L. monocytogenes* non-haemolytic mutant (LLO17), did not undergo extensive intracellular multiplication in either cell line. The increase in numbers of DRDC8 and LM001 per well compared to the mutant strains, may reflect marginal differences in the original inoculum sizes used to infect cell monolayers.

6.2.4 *In vivo* comparison of persistence of *L. monocytogenes ctpA* insertion mutants with wild type strains in a murine model for virulence

6.2.4.1 Analysis of growth curves of *L. monocytogenes* strains in organs of infected mice

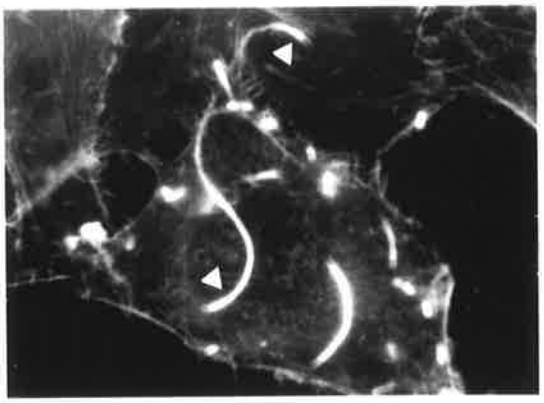
The effect of disrupting the *ctpA* gene on virulence of *L. monocytogenes* was assessed, using the mouse infection model, by determining the rate of clearance of these organisms inoculated intravenously from infected mouse tissue. *L. monocytogenes* DSE201 and C185 were cleared more rapidly from livers of infected mice, than the wild type strains (Figure 6.13). This effect was most notable 3 days after infection. However, no significant difference was observed in the rate of clearance from spleens of infected mice (Figure 6.13). Non-haemolytic *L. monocytogenes* LLO17 used as a negative control, was rapidly cleared from both livers and spleens of infected mice.

6.2.4.2 *In vivo* competition growth experiments

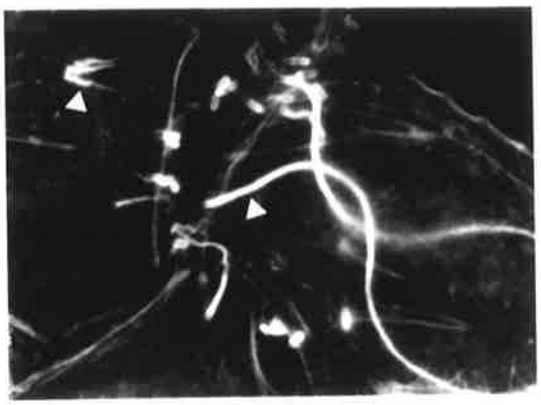
The capacity of parent and mutant strains to persist in the organs of infected mice were directly compared in a competition experiment. A mixed inoculum was used to infect a group of mice by the intravenous route, and the ratio of bacteria present in the inoculum compared with that recovered from the spleen or liver 1, 3 and 6 days later. These results were consistent with data obtained for mice infected with individual strains of *L. monocytogenes* (see Section 6.2.4.1).

Figure 6.11: Immunofluorescent photomicrographs (1000×) of HeLa cells after infection with *L. monocytogenes* isolates at a multiplicity of infection of 1 bacteria to 1 cell. Monolayers were stained with phalloidin rhodamine conjugate prior to examination. Panel: [A], DRDC8; [B], C185; [C], LM001; [D], DSE201; [E], non-haemolytic *hly*::Tn*917-lacZ-cat86* insertion mutant, LLO17; [F], uninfected control. Arrowheads indicate bacterial induced actin polymerisation and pseudopod-like structures indicative of cell to cell spread of *L. monocytogenes*.

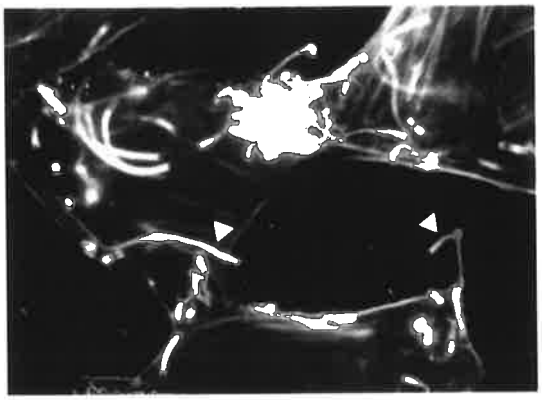
A



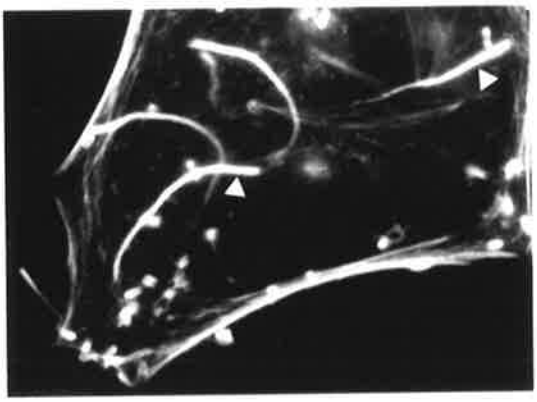
B



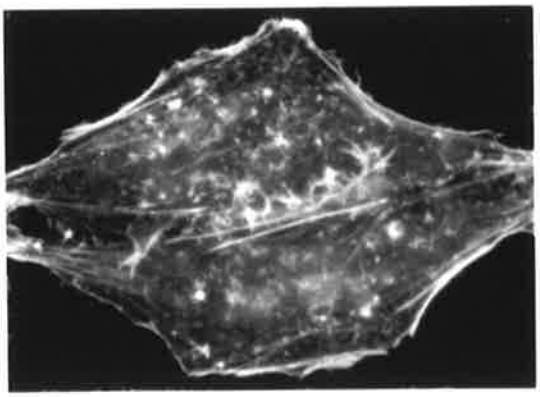
C



D



E



F

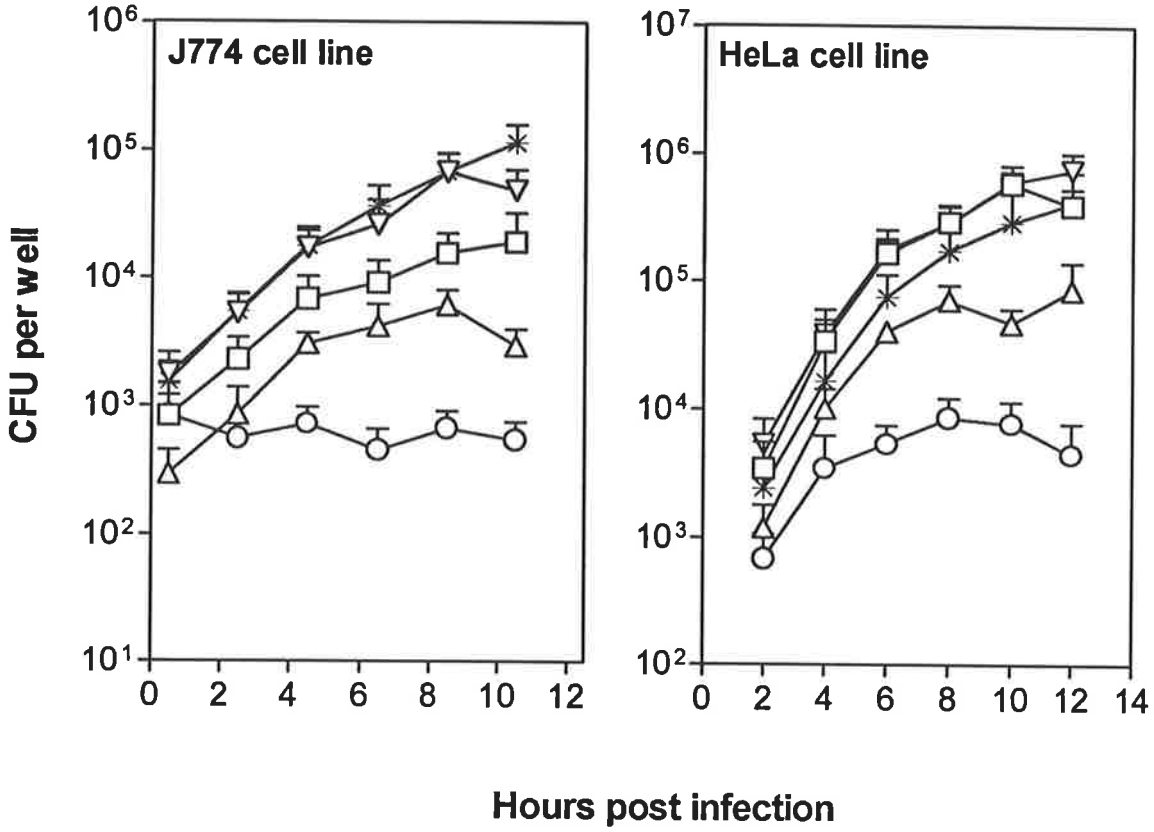


Figure 6.12: Intracellular growth of *L. monocytogenes* strains in tissue culture cell lines. J774 cell monolayers were overlayed with 7.03×10^3 (DRDC8), 4.23×10^3 (C185), 6.12×10^3 (LM001), 4.35×10^3 (DSE201), and 4.18×10^3 (LLO17) bacteria per well. HeLa cell monolayers were overlayed with 2.95×10^4 (DRDC8), 1.18×10^4 (C185), 2.3×10^4 (LM001), 1.85×10^4 (DSE201), and 2.38×10^4 (LLO17) bacteria per well. [□], wild type DRDC8; [Δ], C185; [▽], LM001; [✳], DSE201; [○], LLO17. Numbers are viable bacterial counts (CFU) per well. Each graph represents the mean ± SEM ($\log_{10}$) of three independent experiments.

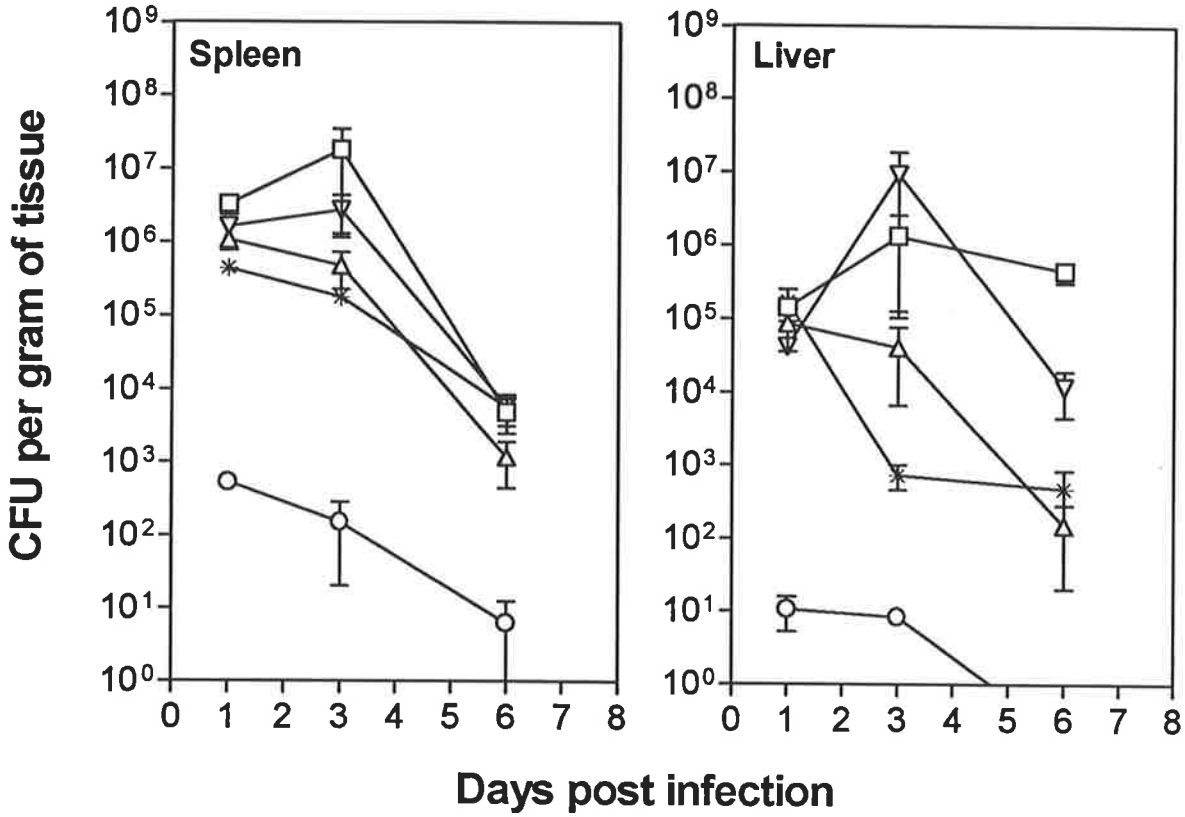


Figure 6.13: Course of infection by wild type and CtpA$^-$ *L. monocytogenes.* Mice were inoculated intravenously with 9.5× 10^3, 1.19× 10^4, 6.5× 10^3, 1.08× 10^4, 1.07× 10^4 bacteria of the wild type DRDC8 [□], C185 [Δ], LM001 [▽], DSE201 [✳], and Hly$^-$ *L. monocytogenes* LLO17 [○] strains, respectively. The numbers are viable bacterial counts (CFU) per gram of tissue, 1, 3, and 6 days after infection. Each number represents the mean ± SEM (log_{10}) from five animals.

Persistence of mutant DSE201 in liver was dramatically impaired in this respect, with median recovery ratio's of wild type parent:mutant ranging from *ca.* 1:1 after day 1, to *ca.* 100:1, 6 days post infection (Figure 6.14, Plate B). Persistence of mutant DSE201 in spleen was also significantly reduced, with median recovery ratio's ranging from *ca.* 5:1 after day 1, to *ca.* 500:1 6 days post infection (Figure 6.14, Plate B). Similar results were observed with DRDC8:C185 infections. Median recovery ratio's of wild type parent:mutant recovered from liver ranged from *ca.* 50:1 to *ca.* 1000:1 after six days infection (Figure 6.14, Plate A). However, persistence of mutant C185 was not notably reduced in spleen, with ratio's of wild type parent:mutant recovered ranging from *ca.* 20:1 after day 1, to *ca.* 70:1 6 days post infection (Figure 6.14, Plate A). The non-haemolytic mutant control LLO17, was rapidly cleared from both liver and spleen of infected mice as early as one day after infection and median recovery ratio's of wild type parent:mutant bacteria were *ca.* 30000:1 and *ca.* 900000:1, respectively (Figure 6.14, Plate C).

6.2.5 Distribution of *ctpA* in *Listeria* spp., and environmental, clinical and laboratory isolates of *L. monocytogenes*

L. monocytogenes DRDC8, is an Australian environmental isolate and may be genotypically distinct from strains isolated from other countries and serotyped *L. monocytogenes* laboratory strains routinely used in studies of *Listeria* infection. To test the hypothesis that *ctpA* is widely distributed in *L. monocytogenes,* Southern hybridisation analysis and PCR were used to identify *ctpA* homologues in a range of environmental, clinical and laboratory isolates.

Southern hybridisation analysis of *Dra*I digested chromosomal DNA isolated from a collection of *Listeria* spp. including *L. ivanovii, L. seeligeri, L. welshimeri, L. grayi,* and *L. innocua,* and *L. monocytogenes* strains obtained from the Special Listeria Culture Collection (SLCC), was probed with a 1.73 kb *Pst*I/*Sac*I DNA fragment internal to *ctpA* (Figure 6.15). A 3.35 kb DNA fragment from DRDC8 hybridised to *ctpA* probe DNA. However, no DNA isolated from other *Listeria* spp. analysed, was found to hybridise probe DNA under the stringency conditions used. Nevertheless, a 1.73 kb *Bam*HI/*Eco*RI internal *ctpA* DNA fragment from control plasmid pCT210, hybridised to probe DNA.

One explanation for this result may be related to the inability of Southern hybridisation analysis to detect DNA homologous to *ctpA* using the stringency conditions outlined in Figure 6.15. To overcome this, two independent PCR protocols (see Table 2.5), were used as alternative

Figure 6.14: *In vivo* persistence of *ctpA*::*erm* and *ctpA*::Tn*917-lacZ-cat86* mutants compared to wild type parental strains in the liver and spleen of mice. Plate: [A], DRDC8:C185; [B], LM001:DSE201; [C], DRDC8:LLO17. Each dot represents the ratio of parent:mutant bacteria recovered from the spleen or liver of an individual mouse; horizontal lines show median values. Note the different scales on the vertical axes. Inoculum strain ratios were as follows: DRDC8:C185, 6.38:1; LM001:DSE201, 2.16:1; DRDC8:LLO17, 3.83:1.

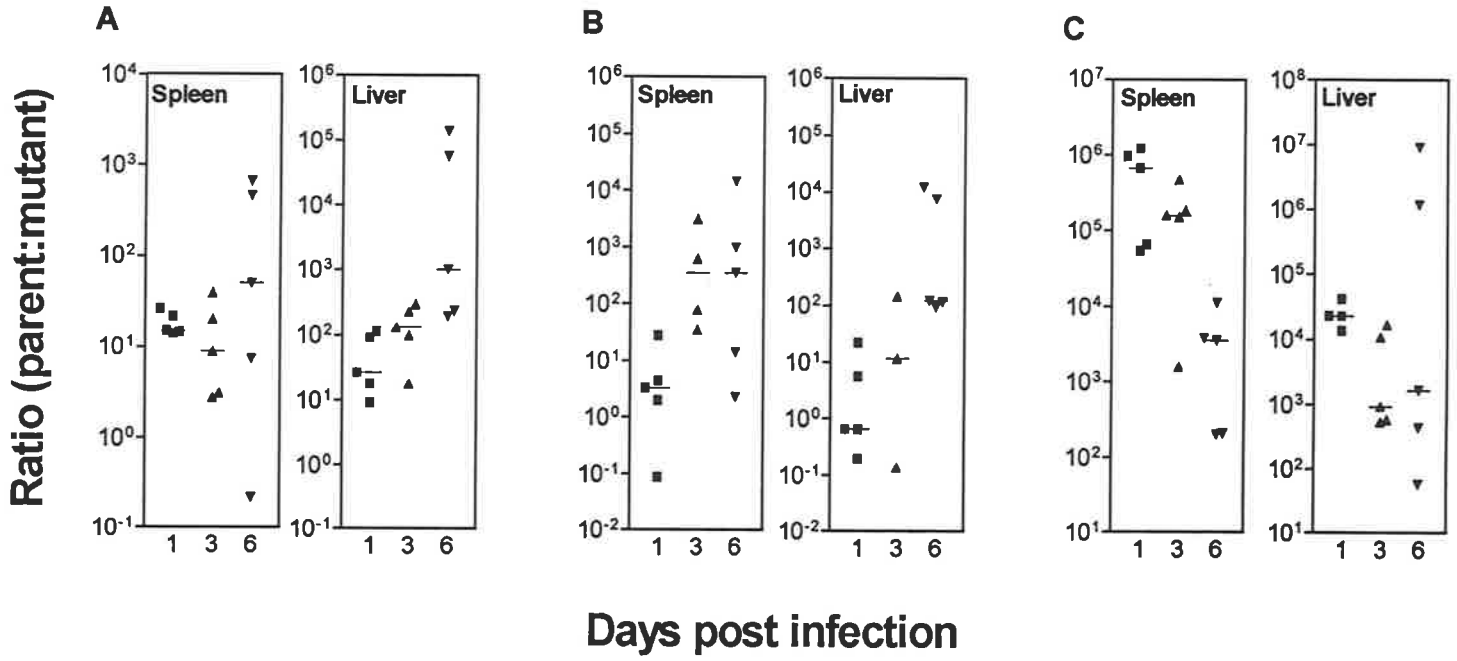
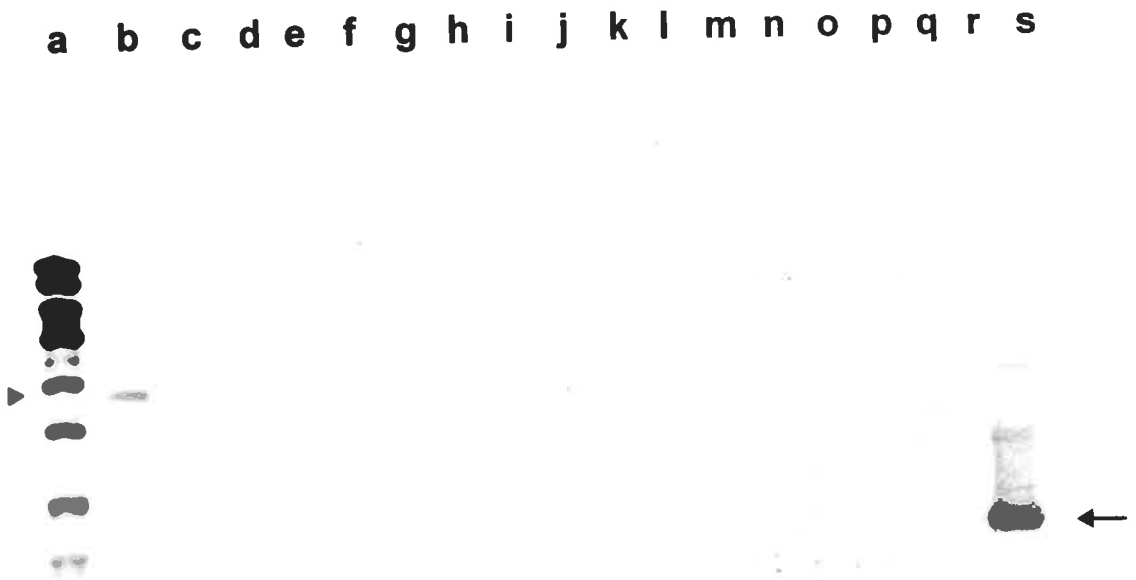

A
B
C
Spleen
Liver
Ratio (parent:mutant)
Days post infection
1 3 6

Figure 6.15: Southern hybridisation analysis of *Dra*I digested chromosomal DNA prepared from *Listeria* spp., probed with a digoxigenin labelled 1.73 kb *Pst*I/*Sac*I DNA fragment purified from plasmid, pCT203 containing *ctpA* (see Figure 5.6). Lane: [a], *Eco*RI digested Bacteriophage SPP-1 DNA; [b], DRDC8; [c], SLCC 2371; [d], SLCC 2372; [e], SLCC 2373; [f], SLCC 2374; [g], SLCC 2377; [h], SLCC 2378; [i], SLCC 2540; [j], SLCC 2755; [k], SLCC 5764; [l], EGD (Bubert strain); [m], EGD (Kaufmann strain); [n], *L. ivanovii*; [o], *L. seeligeri*; [p], *L. welshimeri*; [q], *L. grayi*; [r], *L. innocua*; [s], *Eco*RI/*Bam*HI digested plasmid pCT210. The arrowhead indicates a 3.35 kb *Dra*I DNA fragment from DRDC8, that hybridised to probe DNA. The arrow indicates the 1.73 kb *Eco*RI/*Bam*HI DNA fragment from the control plasmid, pCT210 (see Figure 5.6), equivalent to the DNA used as the probe. After hybridisation, unbound probe was removed from the filter by washing twice in 2 x SSC, 0.1% (w/v) SDS at RT for 5 min, followed by two high stringency washes in 0.1 x SSC, 0.1% (w/v) SDS at 68°C for 20 min each.


a b c d e f g h i j k l m n o p q r s

detection methods. This assay utilised oligonucleotide primer pairs specific for 1) the 5′ region of *ctpA* (p905/p1036), and 2) the 3′ region of *ctpA* including downstream flanking DNA (p945/pS32) (Figure 6.16). Thirty seven environmental and clinical *L. monocytogenes* isolates, which belonged to either serogroup 1 or serogroup 4 were used in this study. Moreover, these serogroups were further subdivided into five groups based on *Hin*dIII restriction fragment length polymorphisms (RFLP) within the *hly* virulence operon (Thomas, 1995). Results of the PCR analysis using p905/p1036 and p945/pS32 oligonucleotides are summarised in Table 6.3. Independent of serogroup, 43.2% (16/37) of all environmental/clinical isolates analysed were positive for a 0.52 kb amplified product consisting of the 5′ portion of *ctpA*. Interestingly, 63.2% (12/19) of these 'positive' isolates belonged to either RFLP group B or B1. However, when the p945/pS32 oligonucleotide pair was used to amplify the 3′ end of *ctpA* and downstream flanking DNA, a 1.45 kb amplified DNA product was identified in only 8.1% (3/37) of all isolates. Moreover, these isolates were restricted to serogroup 4 (RFLP group B), of which *L. monocytogenes* DRDC8 is the prototype member. The reduced incidence of amplified DNA products using the p945/pS32 oligonucleotide pair compared to p905/p1036 is interesting. The fact that DNA downstream of *ctpA* has significant identity to that which encodes for IS elements, suggests that the location of oligonucleotide pS32 is not suitable for this analysis. Amplification of DNA using the p945/pS32 oligonucleotide pair depends on *ctpA* being consistently flanked by the IS element in all *L. monocytogenes* strains. Given the unlikelihood of this association, it is not surprising that DNA was amplified in only a few *L. monocytogenes* isolates. Significantly, PCR analysis of all routine *L. monocytogenes* laboratory strains obtained from SLCC and other *Listeria* spp., did not result in the amplification of a DNA product, regardless of the PCR protocol used, which is in agreement with the data from Southern hybridisation analysis (see Figure 6.15).

6.3 Discussion

In seeking to evaluate the functional significance of CtpA for *L. monocytogenes,* a *ctpA*::*erm* N-terminal mutant was constructed by allelic displacement. This method involved introduction of an *in vitro* induced *ctpA*::*erm* mutation into *L. monocytogenes* replacing wild type *ctpA* by homologous recombination. The principle of allelic displacement has been routinely applied in studies of *L. monocytogenes* pathogenesis. For example, the utility of this technique was first described in *L. monocytogenes* using the listeriolysin O determinant, *hly* (Michel *et al.*, 1990; Wuenscher *et al.*, 1991). Subsequent studies involving this pathogen, have since used this

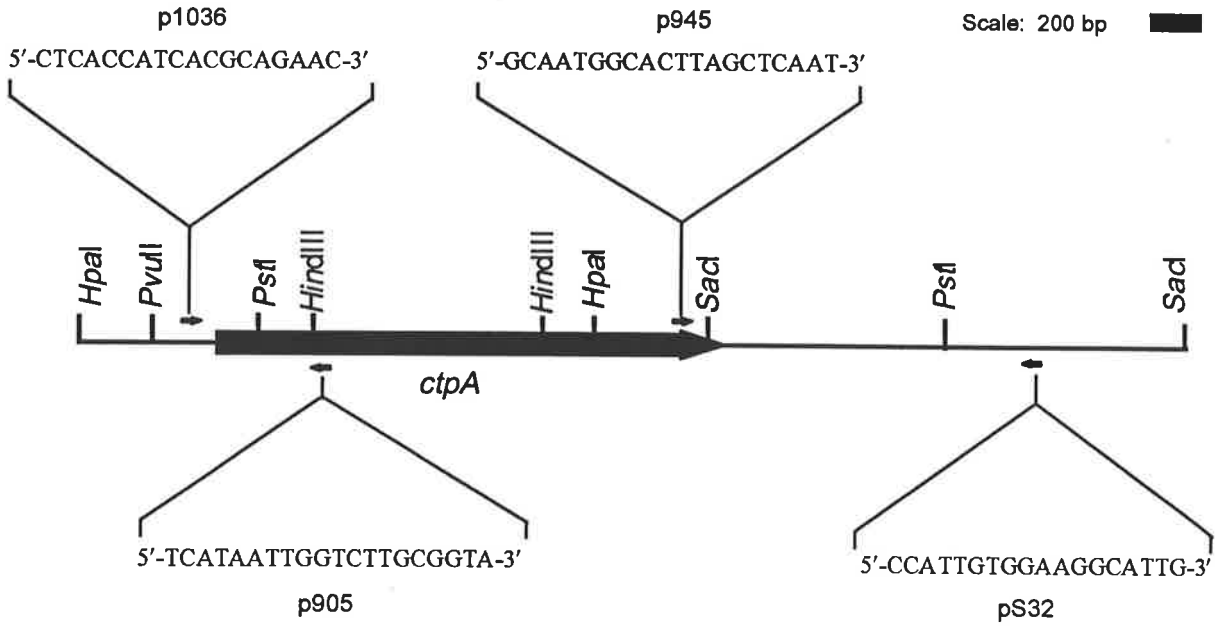


Figure 6.16: Location of the synthetic oligonucleotide pairs p905/p1036 and p945/pS32, relative to *ctpA* and flanking DNA. These oligonucleotide pairs were used in PCR analysis to determine the presence of DNA homologous to *ctpA*, in laboratory, clinical and environmental isolates of *L. monocytogenes*, and other *Listeria* spp. (see Table 6.3). The thermal cycling profile for each oligonucleotide pair is described in Table 2.5.

Table 6.3 PCR amplification of *ctpA* specific DNA from environmental and clinical isolates of *L. monocytogenes* using two oligonucleotide primer pairs

Listeria spp.[a]	RFLP group[b]	Serotype	Number of strains	PCR amplification using oligonucleotide pair[c]	
				p905/p1036	p945/pS32
L. monocytogenes					
environmental and clinical isolates	A	1	11	9.1	0
	A1	1	6	50.0	0
	B	1	9	44.4	0
	B	4	7	71.4	42.9
	B1	1	2	100.0	0
	B1	4	1	100.0	0
	C	1	1	0	0
serotyped laboratory isolates	A	1/2a	1	0	0
	nd	1/2a	3	0	0
	nd	1/2b	1	0	0
	nd	1/2c	1	0	0
	A	3a	1	0	0
	nd	3b	1	0	0
	nd	4a	1	0	0
	nd	4d	1	0	0
	nd	4e	1	0	0
L. ivanovii	na	nd	1	0	0
L. seeligeri	na	nd	1	0	0
L. grayi	na	nd	1	0	0
L. welshimeri	na	nd	1	0	0
L. innocua	na	nd	1	0	0

[a] Serotyped laboratory isolates belonged to the Special Listeria Culture Collection (SLCC)

[b] Five *Hin*dIII Restriction Fragment Length Polymorphisms surrounding the *hly* gene indicated by the nomenclature A, A1, B, B1, and C (Thomas, 1995).

[c] Percent of isolates positive for a PCR amplified DNA product.

nd not determined

na not applicable

approach to demonstrate the role in pathogenesis of other recently isolated determinants including invasion associated protein (*iap*: Wuenscher *et al.*, 1993), actin polymerisation (*actA*: Domann *et al.*, 1992; Kocks *et al.*, 1992), internalin locus (*inlAB*: Dramsi *et al.*, 1995), phospholipase (*plcA*: Mengaud *et al.*, 1991b; Camilli *et al.*, 1993), and positive regulatory factor (*prfA*: Chakraborty *et al.*, 1992; Freitag and Portnoy, 1994).

On the basis of sequence comparisons, CtpA is predicted to be a P-type ATPase from *L. monocytogenes*. Most similarity was to a family of proteins involved in the transport of copper in both bacteria and eukaryotes, suggesting a related function for CtpA (see Section 5.2.5.1). In support of this claim, the intracellular level of *ctpA* mRNA was reproducibly increased by both low and high concentrations of copper in the growth medium (see Section 5.2.6). In view of this, the *ctpA*::Tn*917-lacZ-cat86* insertion mutant, C185 (see chapter 5), and the *ctpA*::*erm* insertion mutant, DSE201 (this Chapter), were used to demonstrate that CtpA corresponds to an active copper transport system. While the MIC of all cations used and EGTA, were similar for both mutants and parental strains, the *ctpA*::Tn*917-lacZ-cat86* insertion mutant was more sensitive to 8-hydroxyquinoline. This result prompted examination of copper sensitivity of the mutant strains in liquid media. Growth of DSE201 and LM001 were similar in BHI broth containing 10 mM EGTA, but surprisingly, growth of C185 was reduced compared to the parental strain. This result is contradictory to other experiments which have reliably shown that both C185 and DSE201 and the parental strains, are equally susceptible to EGTA (see Section 6.2.2.1). Since C185 is competitively inhibited by growth in the presence of the parent strain DRDC8, while growth of DSE201 is comparable to LM001 when cultured together (see Section 6.2.3.2), this suggests that insertion of Tn*917-lacZ-cat86* in C185, conferred an additional growth defect on this organism, not associated with CtpA. Nevertheless, C185 and DSE201 show a significantly reduced capacity for growth in BHI broth containing the chelating agent 5 μM 8-hydroxyquinoline compared with the parental strains, DRDC8 and LM001. Overall, these data are consistent with the effect of 8-hydroxyquinoline on the CopA copper transport system in *E. hirae* (Odermatt *et al.*, 1993; Odermatt *et al.*, 1994). In these reports, $CopA^-$ mutants were unable to grow in Cu^{2+} limiting medium, and suggested that CopA was necessary for Cu^{2+} influx in *E. hirae*. A similar role for the CtpA ATPase is involved in copper influx in *L. monocytogenes* appears likely. Even though growth inhibition of the *ctpA* insertion mutants by 8-hydroxyquinoline could be due to direct toxicity of this agent towards the mutant bacterium, it is likely that addition of 8-hydroxyquinoline to the culture medium, leads to a

depletion of the essential trace element, copper. However, the depletion of Cu^{2+} ions in the culture medium was not confirmed by analytical methods.

Copper is an essential nutrient cation, which is required at low levels for cell viability in all life forms as a cofactor of many enzymes involved in redox reactions (Mertz, 1981; Brown *et al.*, 1992). However, high concentrations of copper ions are also deleterious. Copper catalyses the generation of toxic hydroxyl radicals which in turn promote direct oxidation of proteins, lipids, nucleic acids and polysaccharides (Odermatt *et al.*, 1994). It is likely, therefore, that bacteria have essential Cu^{2+} transport systems responsible for maintaining Cu^{2+} ions at a relatively constant low level (Brown *et al.*, 1994). Furthermore, copper homeostasis may be important for survival of pathogenic bacteria in their host environment. For example, the Cu^{2+} transport P-type ATPases from *H. pylori* have been implicated in the pathogenesis of this human gastric pathogen, facilitating colonisation and survival in the human stomach (Ge *et al.*, 1995; Melchers *et al.*, 1996). CtpA may therefore play a critical role in the selective adaptation of *L. monocytogenes* to varying environments including the intracellular location of an infected host. For survival in this environment, *L. monocytogenes* may have acquired the mechanism to sequester copper from infected cells by the function of CtpA.

In order to determine the significance of CtpA for virulence, mutant and parental strains were compared for ability to establish infection in tissue culture monolayer internalisation assays and a murine model for virulence. All strains were able to invade and multiply intracellularly within the cytoplasm of J774 mouse macrophage like cells and human epithelial HeLa cells. Intracellular generation times were comparable to previous reports (Portnoy *et al.*, 1988; Camilli *et al.*, 1989; Sun *et al.*, 1990; Freitag and Portnoy, 1994). Nevertheless, each of the mutant strains showed significantly restricted growth in liver and to a lesser extent, the spleen, of infected mice when compared to wild type parental strains. This strongly suggests that strains with defective copies of *ctpA* are more rapidly destroyed inside infected liver and spleens cells and/or resident macrophages. To further investigate the *in vivo* consequences of the *ctpA*::*erm* and *ctpA*::Tn*917-lacZ-cat86* mutations, competition experiments comparing the persistence of wild type and mutant strains were assessed. Both *ctpA* mutant strains (C185 and DSE201), were significantly disadvantaged in terms of persistence in spleens and livers of infected mice. However, the ratios of wild type parent:mutant organisms recovered were lower than those seen with the DRDC8:LLO17 pair included for control purposes. This is expected since LLO17 is unable to produce listeriolysin O necessary for intracellular survival in

macrophages and other non-professional phagocytic cells (Gaillard *et al.*, 1986; Kathariou *et al.*, 1987; Cossart *et al.*, 1989; Michel *et al.*, 1990).

The fact that *ctpA* mutants were not defective for intracellular multiplication in tissue culture monolayers, but dramatically attenuated *in vivo,* is interesting. J774 cells, like bone marrow derived monocytes, are highly permissive for many bacteria (Portnoy *et al.*, 1988) and J774 cells elicit only weak microbiocidal activity compared with bone marrow derived monocytes, or Kupffer cells (which are the main target in the liver) for example. Nevertheless, this study and others (Portnoy *et al.*, 1988) have shown *L. monocytogenes* strains exhibit comparable growth rates in a variety of macrophage and epithelial cell lines. Consequently, differences in behaviour of *ctpA* mutants in tissue culture and *in vivo* experiments is unlikely to reflect the choice of cell lines. Furthermore, while splenic dendritic cells (Guzman *et al.*, 1996), Kupffer cells (Mackaness, 1962; North, 1974), and hepatocytes (Rogers *et al.*, 1996) are the primary defence against *L. monocytogenes* infection *in vivo*, this effect would not be reproduced *in vitro* unless the cell lines employed were able to maintain the same function in tissue culture.

This phenomenon may be related to the static nature of *in vitro* cultured cells, as distinct from cells *in vivo,* which are influenced by the surrounding cells and by soluble factors (Miller, 1995). Soluble factors, including the trace elements iron (Fe), copper (Cu), and zinc (Zn), are essential for cell viability and routine culture of cell lines provides constant optimal concentrations of these factors in culture media. However, in infected humans and laboratory animals, concentrations of trace elements in the serum can alter significantly in response to systemic inflammation (Beisel, 1977). For example, the Cu^{2+} ion concentration in livers of *Trypanosoma cruzi* or *T. lewisi* infected rats, is significantly reduced compared to normal uninfected animals (Crocker *et al.*, 1992; Matousek de Abel de la Cruz *et al.*, 1993), whereas in the spleen, Cu^{2+} ion concentration is increased (Matousek de Abel de la Cruz *et al.*, 1993). As a result of these changes in liver Cu^{2+} concentration, infected animals become more susceptible to infection. This phenomenon is not likely to occur in the controlled environment of tissue culture monolayer invasion assays. In view of these studies, it is likely that the *ctpA* mutants were not defective for growth *in vitro* in tissue culture cells because the assay does not mimic the physiological conditions *in vivo,* in response to infection. Furthermore, it is conceivable that the *ctpA* mutants were more rapidly cleared from the liver of infected mice, because *L. monocytogenes* infection induced low Cu^{2+} concentrations in the liver. Presumably, CtpA mutants exhibit attenuated virulence, in response to low Cu^{2+} availability. This data is in

good correlation to the hypersensitivity of *ctpA* mutants to low concentrations of Cu^{2+} ions *in vitro* (see Section 6.2.2.2). Taken together, these results suggest the CtpA protein is necessary to establish a tropism of *L. monocytogenes* for the liver of an infected host.

A precedent for this concept has been previously reported. The *Legionella pneumophila hel* locus, which encodes for an intracellularly induced homologue of heavy-metal ion transporters of *Alcaligenes* spp., has been implicated in the pathogenesis of *Legionella* (Arroyo *et al.*, 1994; McClain *et al.*, 1996). Disruption of this locus results in reduced host cell cytopathogenicity. Consequently, these results taken together with those of the present study, represent the first reports of heavy-metal ion transporters involved in virulence.

The concept that the CtpA ATPase is important for virulence is interesting and should remain the focus of future studies. In particular, construction of an in-frame deletion in *ctpA* is necessary to eliminate potential polar affects on downstream genes, and should confirm an involvement of CtpA in virulence when used in the mouse infection model. Another interesting aspect, concerns a recent report that *L. monocytogenes* can induce apoptosis of dendritic cells (Guzman *et al.*, 1996) and hepatocytes (Rogers *et al.*, 1996). These are significant findings as dendritic cells and hepatocytes are a primary defence against *L. monocytogenes* infection of spleen and liver respectively. Since *hly* mutants are unable to trigger apoptosis (Guzman *et al.*, 1996), the ability of *L. monocytogenes* to induce apoptosis is apparently dependent on the organisms ability to multiply within the cytoplasm of cell populations. Given apoptosis is dependent on intracellular multiplication and that *ctpA* insertion mutants are unable to persist in liver and spleen of infected mice, it would be of interest to examine the role of *ctpA* in infection of dendritic cells and hepatocytes. When examining the effect of infection, particular attention should be given to the level of intracellular multiplication and evidence for bacterial induced apoptosis of infected cells.

Although optimised methods for the transfer of plasmids into *Listeria* spp. by electrotransformation (Alexander *et al.*, 1990; Park and Stewart, 1990), or protoplast transformation (Wuenscher *et al.*, 1991), has been described, these techniques are significantly compromised when attempting to transform plasmids of unwieldy size. Furthermore, they are not often reproducible or successfully applied to other closely related strains. This limitation has been overcome by use of methods for the efficient conjugal transfer of vector DNA from *E. coli* to *L. monocytogenes* (Mengaud *et al.*, 1991b; Trieu-Cuot *et al.*, 1991; Trieu-Cuot

et al., 1993). In this study, a similar approach was employed which enabled the generation of a specific *ctpA*::*erm* insertion mutant. By utilising the IncP-type specific recognition site for mobilisation (*mob*) of RP4, the mutagenesis vector pCT223, was transferred by conjugation from *E. coli* into *L. monocytogenes*. In fact, this vector was mobilised into the recipient strain, with an efficiency equal to that reported for an optimised method for conjugal transfer of plasmid DNA from *E. coli* to *L. monocytogenes* (Trieu-Cuot *et al.*, 1993). A further advantage of this vector system is that the Gram-negative replicon used, facilitates suicide on entry into Gram-positive hosts. Thus strains in which the wild type *ctpA* gene was replaced by the mutated allele, were easily detected by antibiotic selection (Cm^S and Em^R). Another important advantage is that the Gram-negative replicon allows all DNA manipulations to be performed in *E. coli* prior to mobilisation into the recipient strain.

One disadvantage of using polar insertion mutations such as *ctpA*::*erm*, concerns the possible disruption of expression of a downstream gene(s), particularly where target genes are part of an operon. In these circumstances, interpretation of the phenotypic effects of polar mutations can be difficult and is usually overcome by construction of in-frame deletions. Nevertheless, from the present study, reduced persistence in organs of infected mice is either directly attributable to the *ctpA* defect, or is a result of polar effects on an adjacent downstream gene(s). The latter effect is unlikely for the following reasons. Nucleotide sequence analysis of *ctpA* has identified a strong transcriptional terminator immediately downstream of the open reading frame (see Section 5.2.7, Figure 5.22). In addition, a region of significant similarity to a transposable element (see Section 5.2.5.2), with no probable function in growth or virulence of *L. monocytogenes* has been identified downstream of *ctpA*. While this is strong supporting evidence for the reduced impact of these polar mutations on downstream genes, the construction of an in-frame *ctpA* deletion mutant is necessary to confirm the functional significance of CtpA.

Given the apparent significance of CtpA for virulence of DRDC8, the distribution of *ctpA* within routine laboratory, clinical and environmental strains of *L. monocytogenes* was determined. Interestingly, PCR analysis indicated *ctpA* homologous DNA was restricted to Australian isolates of *L. monocytogenes* which predominantly belonged to RFLP group B and B1. At this stage, the significance of this geographical association is unknown, but suggests that the Cu^{2+} transporting CtpA ATPase is functionally important for survival of *L. monocytogenes* isolates from Australia. RFLP group B and C strains are primarily

associated with dairy and clinical isolates (Thomas, 1995). It is possible that the increased incidence of *ctpA* in RFLP group B and B1 strains contributes to their association with clinical infections. In contrast, the occurrence of *ctpA* was less frequent in poultry isolates (RFLP groups A and A1), and may explain why these strains are not associated with clinical cases. However, the limited sample size of this study makes analysis difficult.

This data suggests that *ctpA* is not widely distributed among *L. monocytogenes* isolates, yet this gene is apparently significant for virulence. One explanation for this result may be related to the choice of sequence used for the oligonucleotide primers. Thus, strains with variations in nucleotide sequence may not be detected using PCR. For example, two oligonucleotides used in this study (p1036 and pS32, see Figure 6.16) are located external to *ctpA*. Given the likelihood that sequence in these non-coding regions flanking *ctpA* would be less conserved among *L. monocytogenes* isolates, some strains may not be detected. Furthermore, the p945/pS32 oligonucleotide pair amplified DNA in DRDC8 which is primarily external to *ctpA*, and has sequence identity to DNA that encodes IS elements. This mobile genetic element may not be represented in all *L. monocytogenes* isolates and if present, would be rarely associated with *ctpA*. Thus, pS32 DNA sequence is not an ideal choice for assessing distribution of *ctpA*, and would explain why few strains were detected with this oligonucleotide pair (see Section 6.2.5). Therefore, in future work it is necessary to assess the distribution of *ctpA* among Australian isolates with a oligonucleotide pair which is located internal to the *ctpA* gene. Moreover, studies which involve PCR should be performed in combination with Southern hybridisation analysis of chromosomal DNA probed with an internal fragment of *ctpA* using an appropriate hybridisation stringency.

The origin of this allele is not understood. A number of heavy metal resistance determinants have been acquired by transposon insertion (Nucifora *et al.*, 1989; Lebrun *et al.*, 1994). Interestingly, sequence flanking the downstream region of *ctpA* in DRDC8, shares similarity to IS elements of Gram-positive origin. Putative imperfect inverted repeats were located at nucleotide positions 2635 and 4146, which border a potential transposase gene, although the open reading frame has not been confirmed. However, it is unlikely the IS element is associated with the *ctpA* copper resistance locus for the following reasons. Firstly, the IS element is not sufficiently large enough to contain both the *ctpA* copper resistance determinant and the transposase gene. Indeed, by definition, IS elements contain only genes necessary for transposition (Campbell *et al.*, 1979). Finally, DNA amplification of *ctpA* and

the flanking IS element using the p945/pS32 oligonucleotide pair was limited to a few isolates, in comparison to the prevalence of *ctpA* in *L. monocytogenes* (see Section 6.2.5). While it is unknown if the IS element is represented in all Australian isolates, collectively, this data suggests that the IS element is rarely associated with *ctpA*.

Furthermore, while the codon usage of CtpA is significantly different compared to *L. monocytogenes* CadA, a second ATPase protein from *L. monocytogenes* (Lebrun *et al.*, 1994), it does correspond to the average codon usage determined from all available *L. monocytogenes* sequences (Nakamura *et al.*, 1996) (see Section 5.2.8, Table 5.6). This is not surprising for the CadA resistance determinant, at least, as the locus resides on a transposable element most likely acquired from a source other than *L. monocytogenes*. However, a similar origin for the CtpA copper resistance determinant could not be suggested from this data.

In conclusion, this chapter has described construction of a *erm* insertion mutant in the CtpA ATPase and demonstrated that this protein is actively involved in Cu^{2+} transport in *L. monocytogenes*. Furthermore, this study provides the first example of a P-type ATPase associated with pathogenicity. CtpA mutants were attenuated in virulence in *in vivo* studies. In particular, these strains were more readily cleared from the liver of infected mice. The differential pattern associated with the physiological changes in trace element concentration in serum and tissues of infected hosts are a significant host defence mechanism. Therefore, the CtpA ATPase from *L. monocytogenes* appears to assist in the survival of this organism, particularly in resisting the toxic environment of the liver found in infected hosts. These results may provide some understanding into the cellular tropism of *L. monocytogenes*. The implications of this finding were discussed with regard to the distribution of *ctpA* in *L. monocytogenes* isolates.

Chapter 7 General discussion

7.1 Introduction

L. monocytogenes is a Gram-positive organism, frequently found in the environment and is responsible for serious foodborne diseases such as perinatal infections, septicaemia and meningoencephalitis in humans and animals (Gellin and Broome, 1989). Even though *L. monocytogenes* is an uncommon human pathogen, it's significance as a pathogen lies in a high mortality rate, often in the order of 20% to 50%, and the potential for producing epidemic infections (Bille and Doyle, 1991). In addition, listerial infections are likely to rise with increased use of immuno-suppression therapy in organ transplantation surgery and increased incidence of immuno-compromised individuals induced by clinical infections including AIDS.

Virulence factors known to play a role in pathogenicity of *L. monocytogenes* infections have been recently reviewed (Portnoy *et al.*, 1992a; Sheehan *et al.*, 1994). However, additional factors which affect cell viability may also play a role in allowing the organism to selectively establish an infection. For example, a significant number of *L. monocytogenes* strains are resistant to heavy metal cations including cadmium (Lebrun *et al.*, 1992). In this case, resistance is conferred by the CadA determinant, a P-type ATPase protein involved in cadmium transport (Lebrun *et al.*, 1994). Proteins of similar function, but specificity for different ions, may be required by *L. monocytogenes* to adapt to an intracellular environment in an infected host. Monitoring metal ion concentration is an ideal way for a pathogen to sense a particular environment because ions are crucial for cell viability, but often extremely toxic when present in excess. Consequently, all microbes and other life forms possess mechanisms to regulate the intracellular ion concentration (Silver and Walderhaug, 1992).

As pathogenic bacteria attempt to establish host infection, they encounter a dramatically different environment to their normal niche. Bacteria utilise these variations in environmental conditions as cues for controlling expression of virulence genes. This is a practical approach which bacteria employ, since virulence determinants need only be expressed during a time of microbe-host interaction. For example, environmental stimuli such as iron, temperature, calcium, osmolarity and stress (heat shock, pH and starvation) regulate virulence gene expression in a range of important Gram-negative pathogenic bacteria (Miller *et al.*, 1989; Mekalanos, 1992; Gross, 1993, and references therein). The situation for *L. monocytogenes* is

probably no different. Transcription of essential virulence determinants in this organism, are also influenced by temperature (Leimeister-Wächter *et al.*, 1992; Dramsi *et al.*, 1993b), iron limitation (Cowart and Foster 1981; Geoffroy *et al.*, 1987), osmolarity (Park *et al.*, 1992; Myers *et al.*, 1993), and heat shock (Sokolovic and Goebel, 1989).

The primary aim of this project was to isolate novel *L. monocytogenes* determinants regulated by environmental cues, and investigate their role in pathogenesis by this organism using a tissue culture internalisation assay and the murine model of infection. The approach taken involved generating a transcriptional fusion library in *L. monocytogenes* using Tn*917-lacZ-cat86* contained on the plasmid pTV53 (Youngman *et al.*, 1985a). Environmental conditions including cation stress, iron stress, carbon dioxide, and temperature upshift were initially used to establish a library of promoter::*lacZ* transcriptional fusion mutants. One of these, was selected for further study.

7.2 Optimisation of a tissue culture internalisation assay

Tissue culture internalisation assays are a convenient method for assessing the pathogenicity of bacteria. To assist in the identification of fusion mutants with reduced pathogenic potential, a tissue culture internalisation assays was optimised in this study, using the human cell lines HeLa and Caco-2. It was noted that problems associated with other internalisation assays such as extensive monolayer disruption are overcome by inoculating cell monolayers with low multiplicities of infection (1:1; bacteria:cell) and restricting the infection period to 4 h. These conditions resulted in maximum recovery of bacteria from infected cell monolayers Both cell lines reliably distinguished between pathogenic and non-pathogenic *Listeria* isolates. Thus, this internalisation assay provides a useful method for routine assessment of *L. monocytogenes* pathogenicity, able to identify bacteria which are either defective for internalisation or cell to cell spread in cell monolayers. As such, this assay was successfully applied to the functional characterisation of CtpA, a P-type ATPase involved in Cu^{2+} transport in *L. monocytogenes* (see Section 6.2.3.3).

7.3 Isolation and characterisation of *ctpA* encoding a P-type ATPase involved in copper transport

A transcriptional promoter::*lacZ* gene fusion was isolated from *L. monocytogenes* DRDC8 in response to increased expression of β-galactosidase under cation stress induced by the chelator EGTA (2.5 mM). Analysis of this fusion, lead to the isolation of *ctpA*, which encodes a

protein of 653 aa with a predicted M_r value of 69,463 (see Section 5.2.4). The deduced protein sequence displayed a high degree of similarity to bacterial P-type ATPases involved in copper homeostasis from *Synechococcus* spp. (PacS) (Kanamaru *et al.*, 1993), and *E. hirae* (CopA) (Odermatt *et al.*, 1993). Significant similarity was also observed to proteins associated with Menkes (Chelly *et al.*, 1993; Mercer *et al.*, 1993; and Vulpe *et al.*, 1993) and Wilson (Bull *et al.*, 1993) copper metabolism disorders in humans. Amino acid residues absolutely conserved in functional domains of ATPase proteins from eukaryotes and prokaryotes were identified in CtpA. Levels of *ctpA* mRNA in *L. monocytogenes* were specifically increased in the presence of copper and the chelating agents EGTA and 8-hydroxyquinoline, and this indicated that CtpA is an ATPase involved in copper transport. This determinant was distinct from the CadA Cd^{2+} transporter previously isolated from *L. monocytogenes* (Lebrun *et al.*, 1994).

7.4 CtpA is an active Cu^{2+} transport protein and is required for *L. monocytogenes* survival in organs of infected mice

To further confirm the physiological function of *ctpA*, a mutant was constructed by insertion of an erythromycin cartridge (*erm*) into the *Pst*I restriction site at the 5′ termini of this gene. When compared to parental strains, insertion mutants were hypersensitive to low concentrations of Cu^{2+} (BHI + 10 mM EGTA and BHI + 5 μM 8-hydroxyquinoline) (see Section 6.2.2.2). No difference in the growth of all strains in media containing high Cu^{2+} (BHI + 4 mM $CuSO_4$) concentrations was observed. This implied that CtpA is an active copper transporter responsible for the influx of free Cu^{2+} ions in *L. monocytogenes*. However, this result should be treated with caution for the following reasons. First, the concentration of free copper in the culture medium was not determined quantitatively. Measurements of this kind would be highly desirable to assist in future studies. Second, an insertion mutant may induce a polar effect on downstream genes making the mutant phenotype difficult to interpret (see discussion below). Nevertheless, this result is comparable to studies involving CopA, a Cu^{2+} influx transporter from *E. hirae* (Odermatt *et al.*, 1993).

A focus of future studies should include a direct demonstration that CtpA is involved in Cu^{2+} transport. Other researchers have routinely used cation binding or accumulation assays to demonstrate Cu^{2+} transport in *E. hirae* (Odermatt *et al.*, 1994), Cd^{2+} transport in *S. aureus* (Nucifora *et al.*, 1989; Yoon *et al.*, 1991), and Mg^{2+} transport in *S. typhimurium* (Snavely *et al.*, 1989; 1991). This approach essentially involves incubation of a known concentration of

radioisotopic cation with bacterial culture suspensions. Bacteria able to engage in cation transport will ultimately change the amount of remaining free cation radioisotope in the culture supernatant. This can be measured by harvesting free cation radioisotope from culture supernatants onto pre-washed filters, which can be assayed in a β-scintillation counter to quantitate the level of radioactivity. Results can be used to illustrate cation accumulation or extrusion by cells. Similar experiments performed with $CtpA^+$ and $CtpA^-$ *L. monocytogenes* strains, would confirm this Cu^{2+} transport system. Furthermore, a sophisticated study has shown CadA from *S. aureus* was directly responsible for the translocation of Cd^{2+} ions out of the bacterial cell, by a process mediated by hydrolysis of ATP (Tsai *et al.*, 1992; Tsai and Linet, 1993). This was a significant finding, in light of the complicated network of ATP-dependent pathways active in bacteria. Nevertheless, this assay would be extremely difficult to reproduce for analysis of other transport systems. Stringent optimisation of all conditions for each individual system would be required to distinguish between ATP binding ability of the P-type ATPase of interest and other ATP binding proteins within the cell.

Copper is an essential nutritional requirement for living cells, yet it is very toxic when present in excess. Cells possess transport mechanisms to maintain Cu^{2+} homeostasis, which is an absolute requirement for survival. During infection, eukaryotic host cells are exposed to dramatic changes in trace element concentrations in the serum (Beisel, 1977). For example, the Cu^{2+} ion concentration in livers of rats is significantly reduced during parasitic infection (Crocker *et al.*, 1992; Matousek de Abel de la Cruz *et al.*, 1993). With this in mind, the potential for an important role of CtpA in the virulence of *L. monocytogenes* became apparent, although no difference in the intracellular growth of $CtpA^-$ mutants and wild type strains in HeLa or J774 monolayers was observed. However, when mice were infected by intravenous inoculation with a combination of wild type and mutant strains and organ tissue was harvested from infected mice during the course of the six day infection, mutant bacteria were unable to persist in livers of infected mice (see Section 6.2.4). In addition, wild type organisms were able to persist in organs of infected mice for the duration of the experiment.

One possible explanation for this observation is that Cu^{2+} concentrations in the liver are significantly reduced in response to a *L. monocytogenes* infection. The reduced availability of Cu^{2+} prevents growth and persistence of $CtpA^-$ mutants in this environment. This is the first reported involvement of a P-type ATPase in virulence. Even though the virulence gene cluster

of *L. monocytogenes* has been established, this data clearly suggests this organism requires other determinants which complement the pathogenic process. Hypersensitivity of CtpA defective strains to low concentrations of Cu^{2+} (BHI + 10 mM EGTA and BHI + 5 μM 8-hydroxyquinoline) was also established *in vitro* (see Section 6.2.2.2). The discrepancy between the *in vitro* growth of $CtpA^-$ mutants in cell monolayers and their *in vivo* behaviour most likely reflects the static nature of tissue culture models (Miller, 1995). Conditions *in vitro* may not mimic the physiological changes *in vivo* in response to infection.

However, the insertion *ctpA* mutant could conceivably lead to polar mutations on a downstream gene(s). Therefore, to confirm the attenuation of virulence was due to a disruption in *ctpA*, an in-frame deletion in this gene should be constructed and compared to the wild type strain in a repeat of the virulence assays used in this study. In addition, analysis of virulence of the deletion mutant complemented with a wild type copy of *ctpA* contained on a self-replicating plasmid would also be desirable. Nevertheless, it is my opinion that the phenotype of insertion mutants constructed in this study is consistent with an involvement of CtpA in survival of *L. monocytogenes* in organ tissue of infected mice. Any contributing effect of polar mutations on this phenotype are unlikely. A potential open reading frame was identified downstream of *ctpA*. However, this region of DNA has significant identity to insertion elements from Gram-positive bacteria, and mutants in this region are not likely to be involved in growth or virulence of *L. monocytogenes*.

7.5 Regulation of the *ctpA* Cu^{2+} resistance determinant

A preliminary investigation into the regulation of *ctpA* was performed involving direct analysis of transcription. Levels of *ctpA* mRNA was induced when grown in low Cu^{2+} (BHI + 10 mM EGTA and BHI + 5 μM 8-hydroxyquinoline) or high Cu^{2+} (BHI + 4 mM $CuSO_4$) concentrations. Expression of *ctpA* was not detected in the presence of other heavy metals (Cd^{2+}, Hg^{2+}, Ni^{2+}, and Zn^{2+}) or in uninduced cultures (BHI broth alone) (see Section 5.2.6). To further confirm the Cu^{2+} dependent regulation of *ctpA*, it would be useful to use the approach reported by Yoon *et al.*, (1991). In this report, expression of the *cadA* cadmium resistant determinant from *S. aureus* was followed by Northern hybridisation analysis of RNA isolated at regular intervals post induction with Cd^{2+}. This technique has the advantage of allowing the size of the mRNA transcript to be determined. Furthermore, gene regulation can also be demonstrated using gene fusion technology. The approach has been extensively

applied to the study of the P-type ATPase proteins including CadA in *S. aureus* (Yoon *et al.*, 1991; Corbisier *et al.*, 1993), MgtB in *S. typhimurium* (Snavely *et al.*, 1991), and KdpB in *E. coli* (Sugiura *et al.*, 1992). Thus, the combination of *ctpA* fusions and direct measurements of transcription would provide concise information of *ctpA* regulation.

Negative regulator proteins have been identified in Cd^{2+} transport systems from *S. aureus* (Yoon and Silver, 1991), *L. monocytogenes* (Lebrun *et al.*, 1994), and *B. firmus* (Ivey *et al.*, 1992). In particular, the *cadC* determinant from *S. aureus* has been extensively characterised (Yoon and Silver, 1991, Endo and Silver, 1995). However, a *cadC*-like determinant was not identified in DNA flanking *ctpA* in *L. monocytogenes*. This is not unusual, as a similar gene has not been observed in any Cu^{2+} transporting ATPases reported to date. The mechanism of regulation in these proteins has not been determined.

7.6 N-terminal truncation of CtpA

Structural similarity between CtpA and bacterial P-type ATPases was confirmed by comparison of amino acid sequence and hydropathy profiles (see Section 5.2.10). In all proteins examined, eight regions of high hydrophobicity were identified. However, CtpA and the hpCopA Cu^{2+} transporter from *H. pylori* (Ge *et al.*, 1995), are distinctive in that they have N-terminal truncations in the putative ion binding domain characteristic of all P-type ATPases. Not suprisingly, this feature must have direct implications in the way CtpA can interact with Cu^{2+} ions. Consequently, a immediate goal for further study should involve confirmation of the existence of the N-terminal truncation. The situation in the Cu^{2+} transport system in *H. pylori* is interesting. Based on peptide sequence similarity, the ion binding motif was predicted to be associated with a second protein in the Cu^{2+} transport operon, hpCopP. In view of this report, the potential for an analogous protein involved in the CtpA transport system was considered. However, sequence analysis of DNA flanking *ctpA*, did not identify an open reading frame pertaining to metal binding function. Furthermore, analysis of the deduced peptide sequence from 5′ DNA flanking *ctpA* in all six reading frames failed to identify the C-X-X-C consensus sequence characteristic of metal binding domains (Bull and Cox, 1994). Given this information, it is unlikely a sequencing error resulting in a premature stop codon at the 5′ terminus has lead to the truncation.

It will be necessary to confirm the truncation by N-terminal amino acid sequence analysis of wild type CtpA. Purification of this protein first requires a specific antibody to identify CtpA on a SDS-PAGE prior to purification. Investigation into antibody production was initiated in this study and involved an analysis of temperature induced *ctpA* expression in *E. coli.* These experiments will need further development in the near future, so that the steps required to generate N-terminal amino acid sequence of CtpA can be readily achieved. These results will have a significant impact on attempts to understand the mechanism of Cu^{2+} transport in *L. monocytogenes.*

7.7 Membrane topology of CtpA

Most membrane topology models of bacterial P-type ATPases consist of either six or eight membrane domains. At this stage, there is no consensus model for these proteins and the type of model depends on the prediction algorithm used. For example, nine algorithms were used to predict a topology model of CtpA. However, only models predicted by three algorithms could be supported based on the guidelines used to establish topology models reported for other ATPase proteins. Models consisting of six membrane spanning domains were predicted (see Section 5.2.10). While computer based models are limited by their reliability, they serve as a guide especially in the absence of other biochemical data. For example, these models provide a suitable basis for the experimental design of protein fusion or epitope mapping strategies to develop a more accurate model of CtpA membrane topology.

Generating translation fusions between CtpA and reporter proteins including BlaM, PhoA or LacZ, would be useful to define more precisely the number and location of CtpA transmembrane segments. For example, a positive BlaM fusion ($BlaM^+$) detected by Ap^R, would indicate a fusion located in the periplasmic half of the membrane or in the periplasmic space. Similarly, $PhoA^+$ (periplasmic face) and $LacZ^+$ (cytoplasmic face) fusions are detected by selective ability to cleave chromogenic substrates. However, construction of CtpA fusions with a small peptide encoding an epitope recognised by a specific monoclonal antibody, may be more advantageous avoiding potential problems associated with bulky fusion proteins that could potentially effect secretion or transport of CtpA into the membrane. One system designed for this purpose is the commercially available (IBI FLAG™ Epitope, International Biotechnologies Inc.). Moreover, an alternative epitope mapping procedure could prove useful in the analysis of CtpA orientation. Antibodies raised against synthetic peptides of CtpA

could be used to locate the position of epitopes relative to the membrane by immunogold electron microscopy of CtpA-membrane preparations. The utility of this procedure is dependent on the ability of antibodies to recognise the tertiary structure of CtpA within target cell membranes. However, the significant disadvantage of this method is the preparative costs involved in synthesising peptides and generating antibodies. Nevertheless, electron microscopy and X-ray crystallographic studies share most potential in solving the membrane topology puzzle. Until this data becomes available for multiple P-type ATPase proteins, structure prediction will necessarily rely on interpretation of protein fusion or epitope mapping information coupled with analysis of prediction algorithms.

Establishing an accurate topology model for individual P-type ATPase proteins will substantially contribute to understanding their function. For example, while the primary sequence is highly conserved and parsimony would dictate a single membrane structure of these proteins, cation specificity is different and may suggest the requirement for each protein to have a unique structure as a prerequisite for cation-enzyme interaction. Moreover, two proteins exhibiting identical cation specificity can transport the cation in opposite directions. If the present topology hypothesis is realistic, a limited amount of the polypeptide sequence is exposed at the extracellular face of the membrane. Indeed, all functional domains, including sites of cation interaction and translocation are located in the cytoplasm. Therefore, proteins facilitating the influx of cations into the cell must possess a unique mechanism of cation interaction in the extracellular environment. One mechanism has been proposed for the MgtB Mg^{2+} transport locus of *S. typhimurium.* An accessory "docking" protein MgtC, was considered necessary for Mg^{2+} influx by binding extracellular free Mg^{2+} ions and delivery of cations to the enzyme subunit at the outer membrane face (Snavely *et al.*, 1991). A similar function has been proposed for the KdpA protein associated with K^{+} ion transport by the KdpB ATPase from *E. coli* (Polarek *et al.*, 1988). Preliminary characterisation of $CtpA^{-}$ mutants showed these strains were hypersensitive to low concentrations of copper in the growth media. This suggested that CtpA may be responsible for Cu^{2+} influx in *L. monocytogenes.* However, limited nucleotide sequence of DNA flanking *ctpA* failed to identify a gene which may function as a docking protein.

In addition, contradictory reports concerning eukaryotic ATPases, surround the number of ATPase polypeptides required for active transport. Some studies suggest that monomers were active when used to reconstitute artificial membranes, while detergent solubilisation and

genetic studies predict that oligomeric structures are required for biological activity (cited in Serrano, 1988). No data is available for bacterial ATPase proteins. It may be possible to determine the extent of CtpA oligomerisation or otherwise, by treatment of protein preparations with the cross-linking agent formaldehyde, prior to 2D PAGE and Western analysis (Phizicky and Fields, 1995).

Another aspect deserving investigation concerns the concept of generating a fusion between functional domains of independent ATPase proteins. Potentially, a fusion protein could be engineered having an altered cation specificity or direction of cation translocation. Furthermore, the membrane topology may be modified in response to variations in primary peptide sequence. Consequently, this may lead to valuable information in regard to the fundamental understanding of P-type ATPase function.

7.8 Distribution of *ctpA* among *L. monocytogenes* isolates

CtpA is necessary for *L. monocytogenes* to persist in organ tissue of infected mice. In view of the significance of CtpA in bacterial infections, the distribution of the *ctpA* Cu^{2+} transport system among *L. monocytogenes* isolates was investigated. Using PCR to identify homologous DNA in a range of isolates, 43.2% (16/37) of Australian isolates tested contained the *ctpA* determinant. However, DNA homologous to *ctpA* was not detected in laboratory strains from the SLCC, which are routinely used in the molecular analysis of *L. monocytogenes* virulence, the animal pathogen *L. ivanovii,* or in non-pathogenic *Listeria* spp. The simplest explanation for this result may be that the *ctpA* Cu^{2+} transport system is unique to Australian isolates. However, this explanation is based on the following assumptions. First, the sample size used in this analysis is large enough to provide a significant result. It is important this analysis be repeated using an increased sample population. Second, false negative or false positive results would not arise from PCR. However, this is unlikely because the technique relies on the oligonucleotide primer pair being able to anneal to conserved complementary DNA regions on the chromosome. Some isolates may contain nucleotide sequence variations which prevent primer binding, leading to unsuccessful PCR amplification with potential to give rise to false negative results. To overcome this limitation, PCR analysis must be used in combination with Southern hybridisation using an internal *ctpA* specific DNA fragment(s) as a probe.

7.9 Detection of a novel insertion element in *L. monocytogenes*

Insertion (IS) elements are mobile genetic elements that contain only genes related to transposition (Campbell *et al.*, 1979), present in multiple copies within a single genome (Galas and Chandler, 1989). Preliminary nucleotide sequence immediately downstream of *ctpA* has identified a putative IS element. Although an open reading frame was not located, the deduced protein sequence from reading frames +2 and +3 showed significant similarity to transposases from IS elements from Gram-positive origins (see Section 5.2.5.2). However, regions of DNA have only been sequenced in one direction and will need completion in future work in an effort to locate the open reading frame. Potential inverted repeats were detected flanking this region, however, direct repeats indicating the fusion junction between the IS element and *L. monocytogenes* DNA were not detected. The formation of small direct repeats upon integration is a common property of IS elements (Galas and Chandler, 1989). To precisely determine the ends of the IS element, nucleotide sequence across the fusion junction of identical IS elements cloned from different regions of the *L. monocytogenes* chromosome or from plasmid DNA is essential. This would confirm the IS element inverted repeats and identify the direct repeats that form in host DNA by duplication at the site of integration.

Another aspect of this work, which should be the focus of future studies, involves the demonstration of transposition by this IS element from plasmid to plasmid and from plasmid to chromosome. Success of these experiments would be dependent on the number of target sites in host DNA for IS element integration. Presumably, target sites would be more prevalent in the *L. monocytogenes* chromosome than in plasmid DNA. Plasmid to plasmid transposition can be demonstrated in the same *E. coli* cell, which contains a donor construct consisting of the cloned IS element and a linked antibiotic resistance marker and a recipient plasmid that contains a different resistance marker. Transposition could then be detected by isolating the plasmid DNA and re-transforming into *E. coli* selecting on media containing the appropriate antibiotics. To demonstrate transposition into the *L. monocytogenes* chromosome, a donor construct with a Gram-negative origin of replication is needed that consists of the cloned IS element and a linked antibiotic resistance marker. This construct should be unable to replicate in *L. monocytogenes* such that growth on media containing the appropriate antibiotic will enable selection of cells in which a transposition event has occurred. These experiments should also enable the efficiency of transposition to be determined.

The distribution of this IS element may have potential for use in molecular epidemiological studies of *L. monocytogenes* infections caused by foodborne transmission. Determination of IS element distribution could be achieved by Southern hybridisation analysis of chromosomal DNA from a variety of *Listeria* spp. probed with a DNA fragment internal to the IS element. Furthermore, if the DNA is digested with a restriction enzyme that does not recognise DNA from the IS element, the number of DNA fragments which hybridise to probe DNA should be representative of the multiplicity of the IS element in the genome of each *Listeria* isolate. This principle has previously been used for strain identification (Johansen and Kibenich, 1992; Derbise *et al.*, 1994). Depending on the copy number and location of the insertion sequence, a useful fingerprint for the identification of specific strains is obtained.

Appendix A

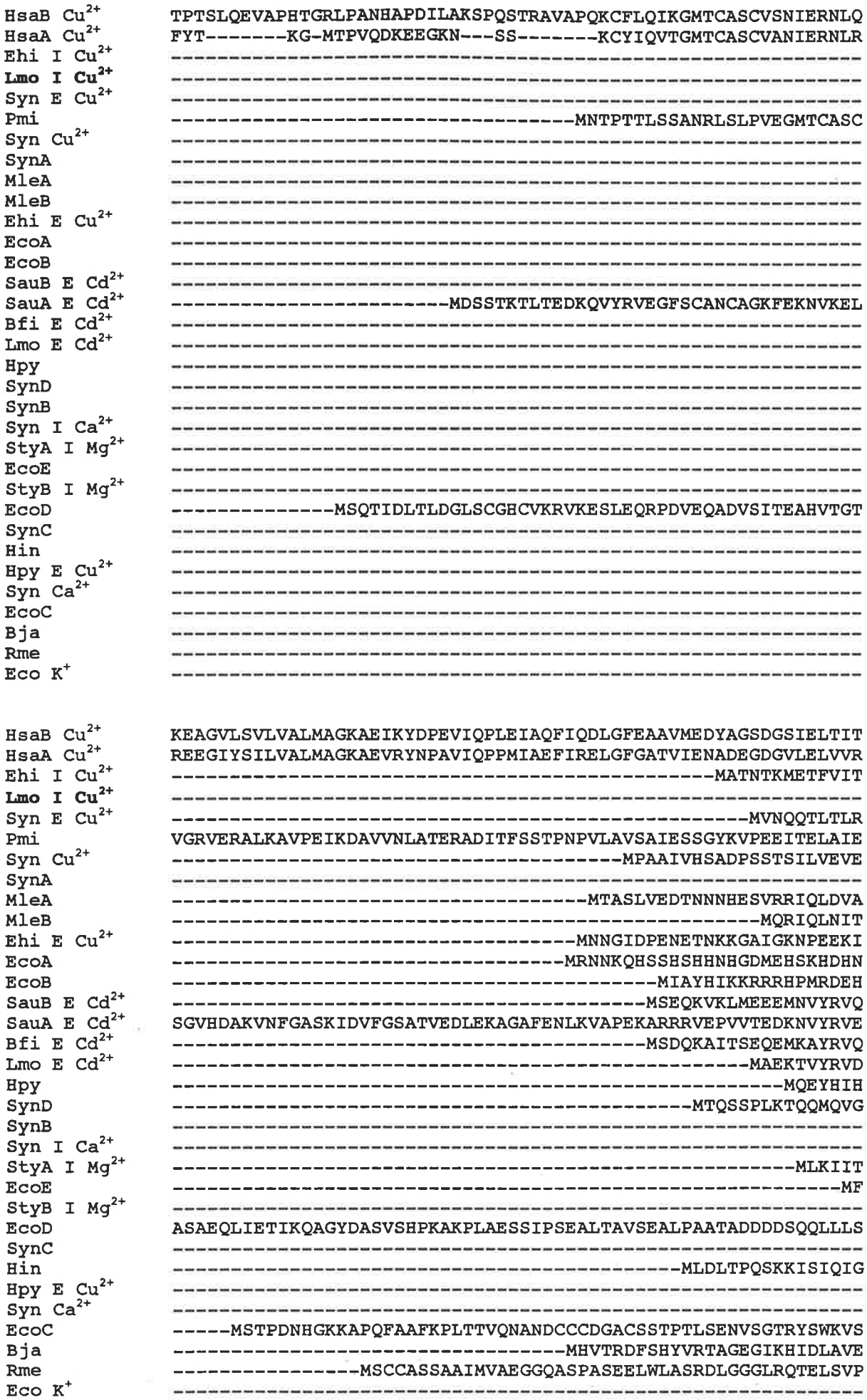
Multiple alignment of whole peptide sequences from bacterial P-type ATPases.

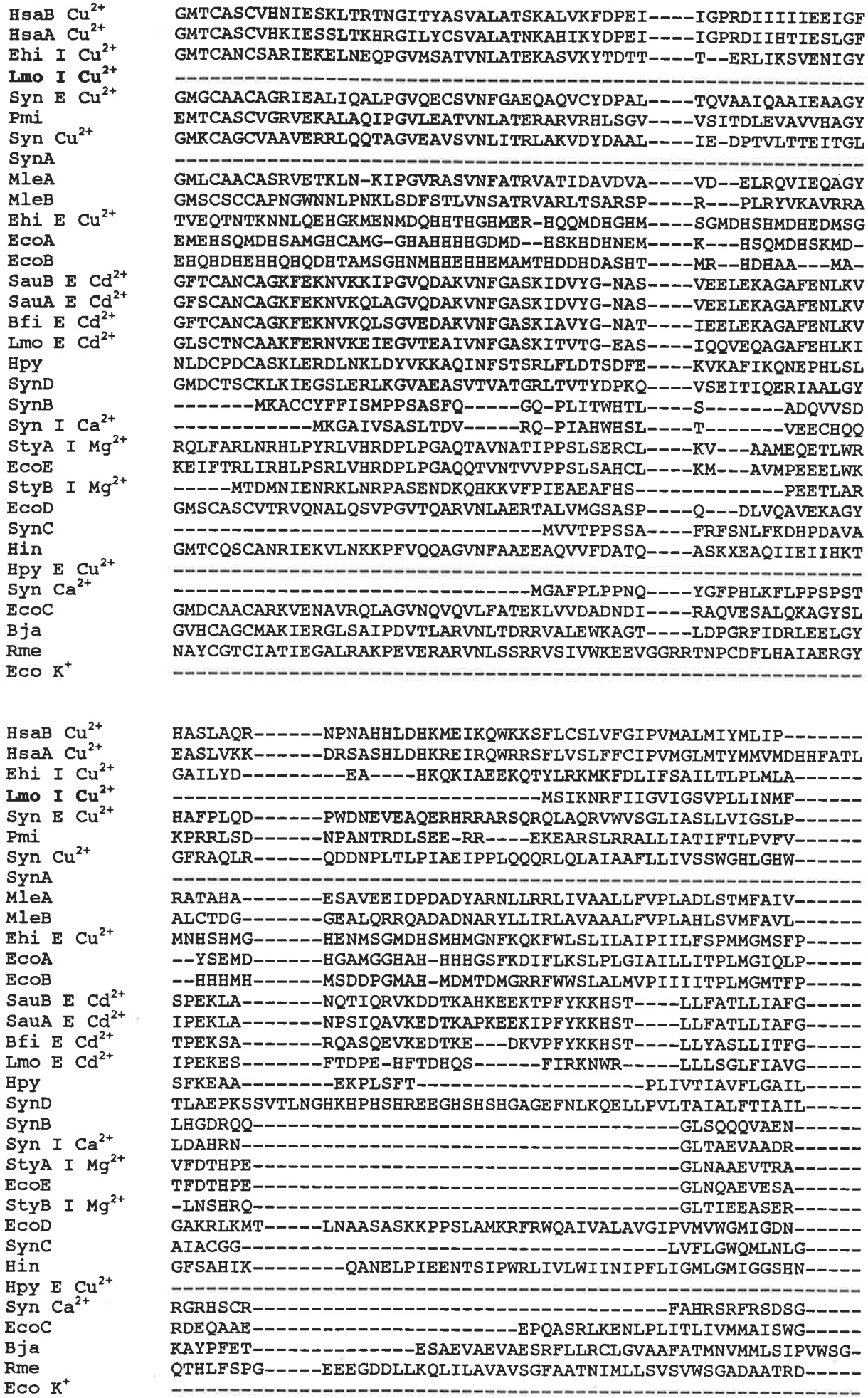
```
HsaB Cu2+       ------------------------------------------------------------M
HsaA Cu2+       MDPSMGVNSVTISVEGMTCNSCVWTIEQQIGKVNGVHHIKVSLEEKNATIIYDPKLQTPK
Ehi I Cu2+      ------------------------------------------------------------
Lmo I Cu2+      ------------------------------------------------------------
Syn E Cu2+      ------------------------------------------------------------
Pmi             ------------------------------------------------------------
Syn Cu2+        ------------------------------------------------------------
SynA            ------------------------------------------------------------
MleA            ------------------------------------------------------------
MleB            ------------------------------------------------------------
Ehi E Cu2+      ------------------------------------------------------------
EcoA            ------------------------------------------------------------
EcoB            ------------------------------------------------------------
SauB E Cd2+     ------------------------------------------------------------
SauA E Cd2+     ------------------------------------------------------------
Bfi E Cd2+      ------------------------------------------------------------
Lmo E Cd2+      ------------------------------------------------------------
Hpy             ------------------------------------------------------------
SynD            ------------------------------------------------------------
SynB            ------------------------------------------------------------
Syn I Ca2+      ------------------------------------------------------------
StyA I Mg2+     ------------------------------------------------------------
EcoE            ------------------------------------------------------------
StyB I Mg2+     ------------------------------------------------------------
EcoD            ------------------------------------------------------------
SynC            ------------------------------------------------------------
Hin             ------------------------------------------------------------
Hpy E Cu2+      ------------------------------------------------------------
Syn Ca2+        ------------------------------------------------------------
EcoC            ------------------------------------------------------------
Bja             ------------------------------------------------------------
Rme             ------------------------------------------------------------
Eco K+          ------------------------------------------------------------

HsaB Cu2+       KKSFAFDNVGYEGGLDGLGPSSQVATSTVRILGMTCQSCVKSIEDRISNLKGIISMKVSL
HsaA Cu2+       TLQEAIDDMGFDAVIHNPDPLPVLTDTLFLTVTASLTLPWDHIQSTLLKTKGVTDIKIYP
Ehi I Cu2+      ------------------------------------------------------------
Lmo I Cu2+      ------------------------------------------------------------
Syn E Cu2+      ------------------------------------------------------------
Pmi             ------------------------------------------------------------
Syn Cu2+        ------------------------------------------------------------
SynA            ------------------------------------------------------------
MleA            ------------------------------------------------------------
MleB            ------------------------------------------------------------
Ehi E Cu2+      ------------------------------------------------------------
EcoA            ------------------------------------------------------------
EcoB            ------------------------------------------------------------
SauB E Cd2+     ------------------------------------------------------------
SauA E Cd2+     ------------------------------------------------------------
Bfi E Cd2+      ------------------------------------------------------------
Lmo E Cd2+      ------------------------------------------------------------
Hpy             ------------------------------------------------------------
SynD            ------------------------------------------------------------
SynB            ------------------------------------------------------------
Syn I Ca2+      ------------------------------------------------------------
StyA I Mg2+     ------------------------------------------------------------
EcoE            ------------------------------------------------------------
StyB I Mg2+     ------------------------------------------------------------
EcoD            ------------------------------------------------------------
SynC            ------------------------------------------------------------
Hin             ------------------------------------------------------------
Hpy E Cu2+      ------------------------------------------------------------
Syn Ca2+        ------------------------------------------------------------
EcoC            ------------------------------------------------------------
Bja             ------------------------------------------------------------
Rme             ------------------------------------------------------------
Eco K+          ------------------------------------------------------------
```

```
HsaB Cu2+       EQGSATVKYVPSVVCLQQVCHQIGDMGFEASIAEGKAASWPSR-SLPAQEAVVKLRVEGM
HsaA Cu2+       QKRTVAVTIIPSIVNANQIKELVPELSLDTGTLEKKSGACEDHSMAQAGEVVLKMKVEGM
Ehi I Cu2+      ------------------------------------------------------------
Lmo I Cu2+      ------------------------------------------------------------
Syn E Cu2+      ------------------------------------------------------------
Pmi             ------------------------------------------------------------
Syn Cu2+        ------------------------------------------------------------
SynA            ------------------------------------------------------------
MleA            ------------------------------------------------------------
MleB            ------------------------------------------------------------
Ehi E Cu2+      ------------------------------------------------------------
EcoA            ------------------------------------------------------------
EcoB            ------------------------------------------------------------
SauB E Cd2+     ------------------------------------------------------------
SauA E Cd2+     ------------------------------------------------------------
Bfi E Cd2+      ------------------------------------------------------------
Lmo E Cd2+      ------------------------------------------------------------
Hpy             ------------------------------------------------------------
SynD            ------------------------------------------------------------
SynB            ------------------------------------------------------------
Syn I Ca2+      ------------------------------------------------------------
StyA I Mg2+     ------------------------------------------------------------
EcoE            ------------------------------------------------------------
StyB I Mg2+     ------------------------------------------------------------
EcoD            ------------------------------------------------------------
SynC            ------------------------------------------------------------
Hin             ------------------------------------------------------------
Hpy E Cu2+      ------------------------------------------------------------
Syn Ca2+        ------------------------------------------------------------
EcoC            ------------------------------------------------------------
Bja             ------------------------------------------------------------
Rme             ------------------------------------------------------------
Eco K+          ------------------------------------------------------------

HsaB Cu2+       TCQSCVSSIEGKVRKLQGVVRVKVSLSNQEAVITYQPYLIQPEDLRDHVNDMGFEAAIKS
HsaA Cu2+       TCHSCTSTIEGKIGKLQGVQRIKVSLDNQEATIVYQPHLISVEEMKKQIEAMGFPAFVKK
Ehi I Cu2+      ------------------------------------------------------------
Lmo I Cu2+      ------------------------------------------------------------
Syn E Cu2+      ------------------------------------------------------------
Pmi             ------------------------------------------------------------
Syn Cu2+        ------------------------------------------------------------
SynA            ------------------------------------------------------------
MleA            ------------------------------------------------------------
MleB            ------------------------------------------------------------
Ehi E Cu2+      ------------------------------------------------------------
EcoA            ------------------------------------------------------------
EcoB            ------------------------------------------------------------
SauB E Cd2+     ------------------------------------------------------------
SauA E Cd2+     ------------------------------------------------------------
Bfi E Cd2+      ------------------------------------------------------------
Lmo E Cd2+      ------------------------------------------------------------
Hpy             ------------------------------------------------------------
SynD            ------------------------------------------------------------
SynB            ------------------------------------------------------------
Syn I Ca2+      ------------------------------------------------------------
StyA I Mg2+     ------------------------------------------------------------
EcoE            ------------------------------------------------------------
StyB I Mg2+     ------------------------------------------------------------
EcoD            ------------------------------------------------------------
SynC            ------------------------------------------------------------
Hin             ------------------------------------------------------------
Hpy E Cu2+      ------------------------------------------------------------
Syn Ca2+        ------------------------------------------------------------
EcoC            ------------------------------------------------------------
Bja             ------------------------------------------------------------
Rme             ------------------------------------------------------------
Eco K+          ------------------------------------------------------------
```

```
HsaB Cu2+       KVAPLSLGPIDIERLQSTNPKRPLSSANQNFNNSETLGHQGSHVVTLQLRIDGMHCKSCV
HsaA Cu2+       QPKYLKLGAIDVERLKNTPVKS--SEGSQQRSPS------YTNDSTATFIIDGMHCKSCV
Ehi I Cu2+      ------------------------------------------------------------
Lmo I Cu2+      ------------------------------------------------------------
Syn E Cu2+      ------------------------------------------------------------
Pmi             ------------------------------------------------------------
Syn Cu2+        ------------------------------------------------------------
SynA            ------------------------------------------------------------
MleA            ------------------------------------------------------------
MleB            ------------------------------------------------------------
Ehi E Cu2+      ------------------------------------------------------------
EcoA            ------------------------------------------------------------
EcoB            ------------------------------------------------------------
SauB E Cd2+     ------------------------------------------------------------
SauA E Cd2+     ------------------------------------------------------------
Bfi E Cd2+      ------------------------------------------------------------
Lmo E Cd2+      ------------------------------------------------------------
Hpy             ------------------------------------------------------------
SynD            ------------------------------------------------------------
SynB            ------------------------------------------------------------
Syn I Ca2+      ------------------------------------------------------------
StyA I Mg2+     ------------------------------------------------------------
EcoE            ------------------------------------------------------------
StyB I Mg2+     ------------------------------------------------------------
EcoD            ------------------------------------------------------------
SynC            ------------------------------------------------------------
Hin             ------------------------------------------------------------
Hpy E Cu2+      ------------------------------------------------------------
Syn Ca2+        ------------------------------------------------------------
EcoC            ------------------------------------------------------------
Bja             ------------------------------------------------------------
Rme             ------------------------------------------------------------
Eco K+          ------------------------------------------------------------

HsaB Cu2+       LNIEENIGQLLGVQSIQVSLENKTAQVKYDPSCISPVALQRAIEALPPGNFKVSLPDGAE
HsaA Cu2+       SNIESTLSALQYVSSIVVSLENRSAIVKYNASSVTPESLRKAIEAVSPGLYRVSITSEVE
Ehi I Cu2+      ------------------------------------------------------------
Lmo I Cu2+      ------------------------------------------------------------
Syn E Cu2+      ------------------------------------------------------------
Pmi             ------------------------------------------------------------
Syn Cu2+        ------------------------------------------------------------
SynA            ------------------------------------------------------------
MleA            ------------------------------------------------------------
MleB            ------------------------------------------------------------
Ehi E Cu2+      ------------------------------------------------------------
EcoA            ------------------------------------------------------------
EcoB            ------------------------------------------------------------
SauB E Cd2+     ------------------------------------------------------------
SauA E Cd2+     ------------------------------------------------------------
Bfi E Cd2+      ------------------------------------------------------------
Lmo E Cd2+      ------------------------------------------------------------
Hpy             ------------------------------------------------------------
SynD            ------------------------------------------------------------
SynB            ------------------------------------------------------------
Syn I Ca2+      ------------------------------------------------------------
StyA I Mg2+     ------------------------------------------------------------
EcoE            ------------------------------------------------------------
StyB I Mg2+     ------------------------------------------------------------
EcoD            ------------------------------------------------------------
SynC            ------------------------------------------------------------
Hin             ------------------------------------------------------------
Hpy E Cu2+      ------------------------------------------------------------
Syn Ca2+        ------------------------------------------------------------
EcoC            ------------------------------------------------------------
Bja             ------------------------------------------------------------
Rme             ------------------------------------------------------------
Eco K+          ------------------------------------------------------------
```

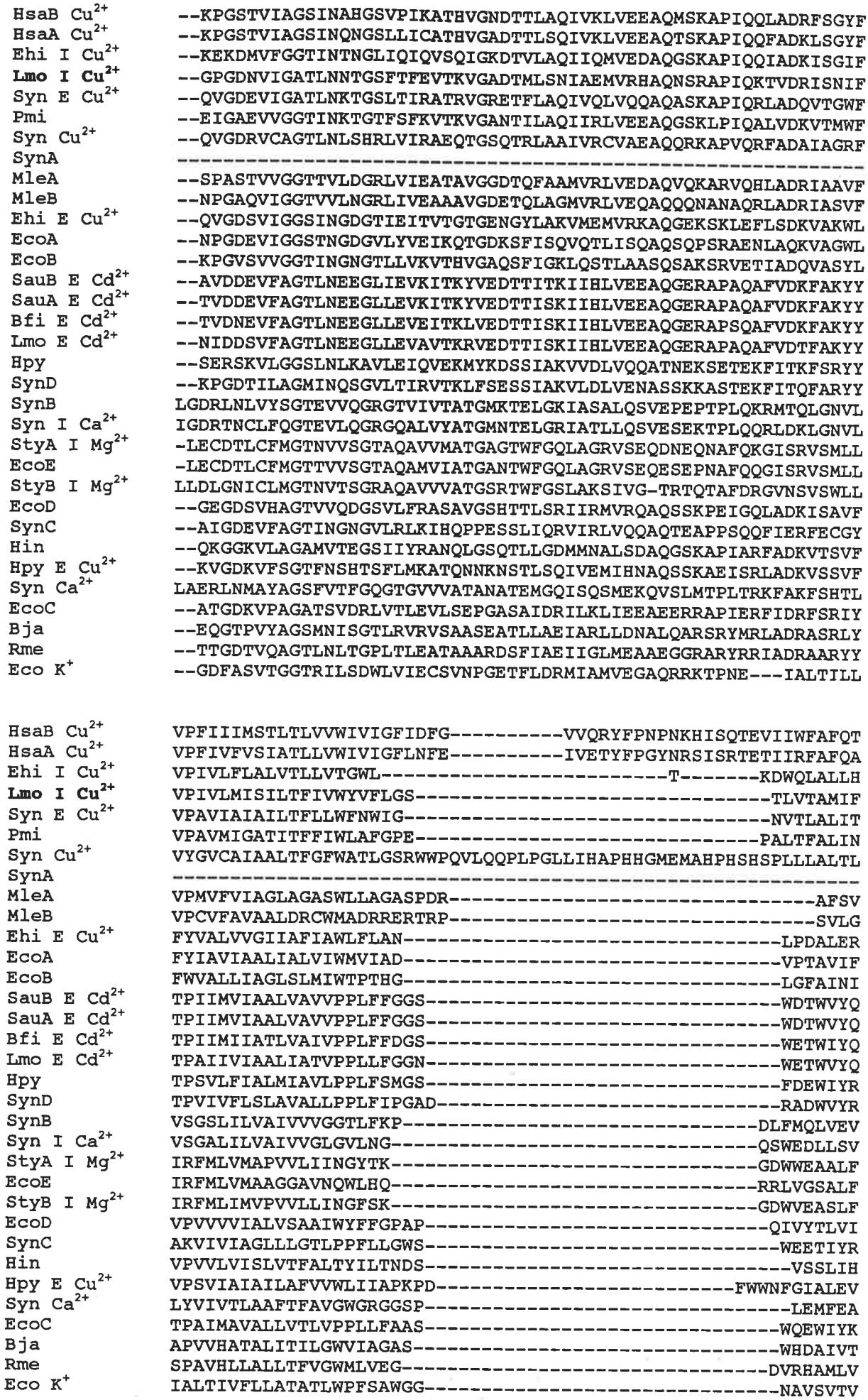
```
HsaB Cu2+       GSGTDHRSSSSHSPGLPLRENQVQGTCSTTLIAIAGMTCASCVHSIEGMISQLEGVQQIS
HsaA Cu2+       STSNSP--SSSSLQKIPLN-VVSQPLTQETVINIDGMTCNSCVQSIEGVISKKPGVKSIR
Ehi I Cu2+      ------------------------------------------------------------
Lmo I Cu2+      ------------------------------------------------------------
Syn E Cu2+      ------------------------------------------------------------
Pmi             ------------------------------------------------------------
Syn Cu2+        ------------------------------------------------------------
SynA            ------------------------------------------------------------
MleA            ------------------------------------------------------------
MleB            ------------------------------------------------------------
Ehi E Cu2+      ------------------------------------------------------------
EcoA            ------------------------------------------------------------
EcoB            ------------------------------------------------------------
SauB E Cd2+     ------------------------------------------------------------
SauA E Cd2+     ------------------------------------------------------------
Bfi E Cd2+      ------------------------------------------------------------
Lmo E Cd2+      ------------------------------------------------------------
Hpy             ------------------------------------------------------------
SynD            ------------------------------------------------------------
SynB            ------------------------------------------------------------
Syn I Ca2+      ------------------------------------------------------------
StyA I Mg2+     ------------------------------------------------------------
EcoE            ------------------------------------------------------------
StyB I Mg2+     ------------------------------------------------------------
EcoD            ------------------------------------------------------------
SynC            ------------------------------------------------------------
Hin             ------------------------------------------------------------
Hpy E Cu2+      ------------------------------------------------------------
Syn Ca2+        ------------------------------------------------------------
EcoC            ------------------------------------------------------------
Bja             ------------------------------------------------------------
Rme             ------------------------------------------------------------
Eco K+          ------------------------------------------------------------

HsaB Cu2+       VSLAEGTATVLYNPAVISPEELRAAIEDMGFEASVVSESCSTNPLGNHSAGNSMVQTTDG
HsaA Cu2+       VSLANSNGTVEYDPLLTSPETLRGAIEDMGFDATLSDTNEPLVVIAQPSSEMPLLTSTNE
Ehi I Cu2+      ------------------------------------------------------------
Lmo I Cu2+      ------------------------------------------------------------
Syn E Cu2+      ------------------------------------------------------------
Pmi             ------------------------------------------------------------
Syn Cu2+        ------------------------------------------------------------
SynA            ------------------------------------------------------------
MleA            ------------------------------------------------------------
MleB            ------------------------------------------------------------
Ehi E Cu2+      ------------------------------------------------------------
EcoA            ------------------------------------------------------------
EcoB            ------------------------------------------------------------
SauB E Cd2+     ------------------------------------------------------------
SauA E Cd2+     ------------------------------------------------------------
Bfi E Cd2+      ------------------------------------------------------------
Lmo E Cd2+      ------------------------------------------------------------
Hpy             ------------------------------------------------------------
SynD            ------------------------------------------------------------
SynB            ------------------------------------------------------------
Syn I Ca2+      ------------------------------------------------------------
StyA I Mg2+     ------------------------------------------------------------
EcoE            ------------------------------------------------------------
StyB I Mg2+     ------------------------------------------------------------
EcoD            ------------------------------------------------------------
SynC            ------------------------------------------------------------
Hin             ------------------------------------------------------------
Hpy E Cu2+      ------------------------------------------------------------
Syn Ca2+        ------------------------------------------------------------
EcoC            ------------------------------------------------------------
Bja             ------------------------------------------------------------
Rme             ------------------------------------------------------------
Eco K+          ------------------------------------------------------------
```

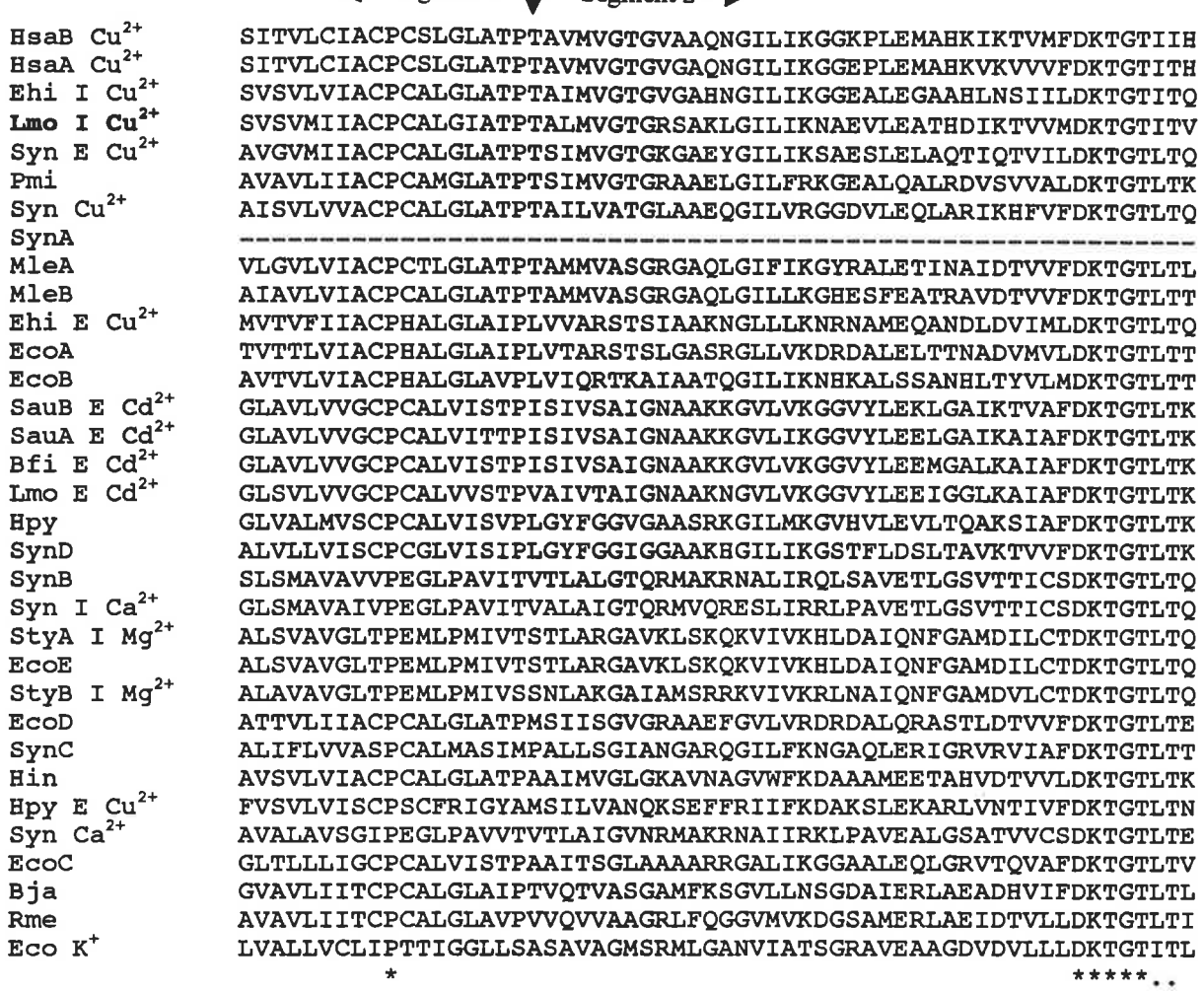

HsaB Cu2+ TPTSLQEVAPHTGRLPANHAPDILAKSPQSTRAVAPQKCFLQIKGMTCASCVSNIERNLQ
HsaA Cu2+ FYT-------KG-MTPVQDKEEGKN---SS-------KCYIQVTGMTCASCVANIERNLR
Ehi I Cu2+
Lmo I Cu2+
Syn E Cu2+
Pmi -----------------------------------MNTPTTLSSANRLSLPVEGMTCASC
Syn Cu2+
SynA
MleA
MleB
Ehi E Cu2+
EcoA
EcoB
SauB E Cd2+
SauA E Cd2+ ----------------------MDSSTKTLTEDKQVYRVEGFSCANCAGKFEKNVKEL
Bfi E Cd2+
Lmo E Cd2+
Hpy
SynD
SynB
Syn I Ca2+
StyA I Mg2+
EcoE
StyB I Mg2+
EcoD -------------MSQTIDLTLDGLSCGHCVKRVKESLEQRPDVEQADVSITEAHVTGT
SynC
Hin
Hpy E Cu2+
Syn Ca2+
EcoC
Bja
Rme
Eco K+
HsaB Cu2+ KEAGVLSVLVALMAGKAEIKYDPEVIQPLEIAQFIQDLGFEAAVMEDYAGSDGSIELTIT
HsaA Cu2+ REEGIYSILVALMAGKAEVRYNPAVIQPPMIAEFIRELGFGATVIENADEGDGVLELVVR
Ehi I Cu2+ ---MATNTKMETFVIT
Lmo I Cu2+
Syn E Cu2+ ---MVNQQTLTLR
Pmi VGRVERALKAVPEIKDAVVNLATERADITFSSTPNPVLAVSAIESSGYKVPEEITELAIE
Syn Cu2+ ------------------------------------MPAAIVHSADPSSTSILVEVE
SynA
MleA ----------------------------------MTASLVEDTNNNHESVRRIQLDVA
MleB ---MQRIQLNIT
Ehi E Cu2+ ----------------------------------MNNGIDPENETNKKGAIGKNPEEKI
EcoA ----------------------------------MRNNKQHSSHSHHNHGDMEHSKHDHN
EcoB --MIAYHIKKRRRHPMRDEH
SauB E Cd2+ ------------------------------------MSEQKVKLMEEEMNVYRVQ
SauA E Cd2+ SGVHDAKVNFGASKIDVFGSATVEDLEKAGAFENLKVAPEKARRRVEPVVTEDKNVYRVE
Bfi E Cd2+ ------------------------------------MSDQKAITSEQEMKAYRVQ
Lmo E Cd2+ ---MAEKTVYRVD
Hpy --MQEYHIH
SynD --MTQSSPLKTQQMQVG
SynB
Syn I Ca2+
StyA I Mg2+ ---MLKIIT
EcoE --MF
StyB I Mg2+
EcoD ASAEQLIETIKQAGYDASVSHPKAKPLAESSIPSEALTAVSEALPAATADDDDSQQLLLS
SynC
Hin --MLDLTPQSKKISIQIG
Hpy E Cu2+
Syn Ca2+
EcoC -----MSTPDNHGKKAPQFAAFKPLTTVQNANDCCCDGACSSTPTLSENVSGTRYSWKVS
Bja ----------------------------------MHVTRDFSHYVRTAGEGIKHIDLAVE
Rme ---------------MSCCASSAAIMVAEGGQASPASEELWLASRDLGGGLRQTELSVP
Eco K+


HsaB Cu2+ GMTCASCVHNIESKLTRTNGITYASVALATSKALVKFDPEI----IGPRDIIIIIEEIGF
HsaA Cu2+ GMTCASCVHKIESSLTKHRGILYCSVALATNKAHIKYDPEI----IGPRDIIHTIESLGF
Ehi I Cu2+ GMTCANCSARIEKELNEQPGVMSATVNLATEKASVKYTDTT----T--ERLIKSVENIGY
Lmo I Cu2+
Syn E Cu2+ GMGCAACAGRIEALIQALPGVQECSVNFGAEQAQVCYDPAL----TQVAAIQAAIEAAGY
Pmi EMTCASCVGRVEKALAQIPGVLEATVNLATERARVRHLSGV----VSITDLEVAVVHAGY
Syn Cu2+ GMKCAGCVAAVERRLQQTAGVEAVSVNLITRLAKVDYDAAL----IE-DPTVLTTEITGL
SynA
MleA GMLCAACASRVETKLN-KIPGVRASVNFATRVATIDAVDVA----VD--ELRQVIEQAGY
MleB GMSCSCCAPNGWNNLPNKLSDFSTLVNSATRVARLTSARSP----R---PLRYVKAVRRA
Ehi E Cu2+ TVEQTNTKNNLQEHGKMENMDQHHTHGHMER-HQQMDHGHM----SGMDHSHMDHEDMSG
EcoA EMEHSQMDHSAMGHCAMG-GHAHHHHGDMD--HSKHDHNEM----K---HSQMDHSKMD-
EcoB EHQHDHEHHQHQDHTAMSGHNMHHEHHEMAMTHDDHDASHT----MR--HDHAA---MA-
SauB E Cd2+ GFTCANCAGKFEKNVKKIPGVQDAKVNFGASKIDVYG-NAS----VEELEKAGAFENLKV
SauA E Cd2+ GFSCANCAGKFEKNVKQLAGVQDAKVNFGASKIDVYG-NAS----VEELEKAGAFENLKV
Bfi E Cd2+ GFTCANCAGKFEKNVKQLSGVEDAKVNFGASKIAVYG-NAT----IEELEKAGAFENLKV
Lmo E Cd2+ GLSCTNCAAKFERNVKEIEGVTEAIVNFGASKITVTG-EAS----IQQVEQAGAFEHLKI
Hpy NLDCPDCASKLERDLNKLDYVKKAQINFSTSRLFLDTSDFE----KVKAFIKQNEPHLSL
SynD GMDCTSCKLKIEGSLERLKGVAEASVTVATGRLTVTYDPKQ----VSEITIQERIAALGY
SynB -------MKACCYFFISMPPSASFQ-----GQ-PLITWHTL----S-------ADQVVSD
Syn I Ca2+ ------------MKGAIVSASLTDV-----RQ-PIAHWHSL----T-------VEECHQQ
StyA I Mg2+ RQLFARLNRHLPYRLVHRDPLPGAQTAVNATIPPSLSERCL----KV---AAMEQETLWR
EcoE KEIFTRLIRHLPSRLVHRDPLPGAQQTVNTVVPPSLSAHCL----KM---AVMPEEELWK
StyB I Mg2+ -----MTDMNIENRKLNRPASENDKQHKKVFPIEAEAFHS-------------PEETLAR
EcoD GMSCASCVTRVQNALQSVPGVTQARVNLAERTALVMGSASP----Q---DLVQAVEKAGY
SynC ---------------------------------MVVTPPSSA----FRFSNLFKDHPDAVA
Hin GMTCQSCANRIEKVLNKKPFVQQAGVNFAAEEAQVVFDATQ----ASKXEAQIIEIIHKT
Hpy E Cu2+
Syn Ca2+ ------------------------------MGAFPLPPNQ----YGFPHLKFLPPSPST
EcoC GMDCAACARKVENAVRQLAGVNQVQVLFATEKLVVDADNDI----RAQVESALQKAGYSL
Bja GVHCAGCMAKIERGLSAIPDVTLARVNLTDRRVALEWKAGT----LDPGRFIDRLEELGY
Rme NAYCGTCIATIEGALRAKPEVERARVNLSSRRVSIVWKEEVGGRRTNPCDFLHAIAERGY
Eco K+
HsaB Cu2+ HASLAQR------NPNAHHLDHKMEIKQWKKSFLCSLVFGIPVMALMIYMLIP-------
HsaA Cu2+ EASLVKK------DRSASHLDHKREIRQWRRSFLVSLFFCIPVMGLMTYMMVMDHHFATL
Ehi I Cu2+ GAILYD---------EA----HKQKIAEEKQTYLRKMKFDLIFSAILTLPLMLA------
Lmo I Cu2+ -------------------------------MSIKNRFIIGVIGSVPLLINMF------
Syn E Cu2+ HAFPLQD------PWDNEVEAQERHRRARSQRQLAQRVWVSGLIASLLVIGSLP------
Pmi KPRRLSD------NPANTRDLSEE-RR----EKEARSLRRALLIATIFTLPVFV------
Syn Cu2+ GFRAQLR------QDDNPLTLPIAEIPPLQQQRLQLAIAAFLLIVSSWGHLGHW------
SynA
MleA RATAHA-------ESAVEEIDPDADYARNLLRRLIVAALLFVPLADLSTMFAIV------
MleB ALCTDG-------GEALQRRQADADNARYLLIRLAVAAALFVPLAHLSVMFAVL------
Ehi E Cu2+ MNHSHMG------HENMSGMDHSMHMGNFKQKFWLSLILAIPIILFSPMMGMSFP-----
EcoA --YSEMD------HGAMGGHAH-HHHGSFKDIFLKSLPLGIAILLITPLMGIQLP-----
EcoB --HHHMH------MSDDPGMAH-MDMTDMGRRFWWSLALMVPIIIITPLMGMTFP-----
SauB E Cd2+ SPEKLA-------NQTIQRVKDDTKAHKEEKTPFYKKHST----LLFATLLIAFG-----
SauA E Cd2+ IPEKLA-------NPSIQAVKEDTKAPKEEKIPFYKKHST----LLFATLLIAFG-----
Bfi E Cd2+ TPEKSA-------RQASQEVKEDTKE---DKVPFYKKHST----LLYASLLITFG-----
Lmo E Cd2+ IPEKES-------FTDPE-HFTDHQS------FIRKNWR-----LLLSGLFIAVG-----
Hpy SFKEAA--------EKPLSFT-------------------PLIVTIAVFLGAIL-----
SynD TLAEPKSSVTLNGHKHPHSHREEGHSHSHGAGEFNLKQELLPVLTAIALFTIAIL-----
SynB LHGDRQQ-----------------------------------GLSQQQVAEN------
Syn I Ca2+ LDAHRN-------------------------------------GLTAEVAADR------
StyA I Mg2+ VFDTHPE-----------------------------------GLNAAEVTRA------
EcoE TFDTHPE-----------------------------------GLNQAEVESA------
StyB I Mg2+ -LNSHRQ-----------------------------------GLTIEEASER------
EcoD GAKRLKMT-----LNAASASKKPPSLAMKRFRWQAIVALAVGIPVMVWGMIGDN------
SynC AIACGG-----------------------------------LVFLGWQMLNLG-----
Hin GFSAHIK--------QANELPIEENTSIPWRLIVLWIINIPFLIGMLGMIGGSHN-----
Hpy E Cu2+
Syn Ca2+ RGRHSCR-----------------------------------FAHRSRFRSDSG-----
EcoC RDEQAAE----------------------EPQASRLKENLPLITLIVMMAISWG-----
Bja KAYPFET-------------ESAEVAEVAESRFLLRCLGVAAFATMNVMMLSIPVWSG-
Rme QTHLFSPG-----EEEGDDLLKQLILAVAVSGFAATNIMLLSVSVWSGADAATRD-----
Eco K+

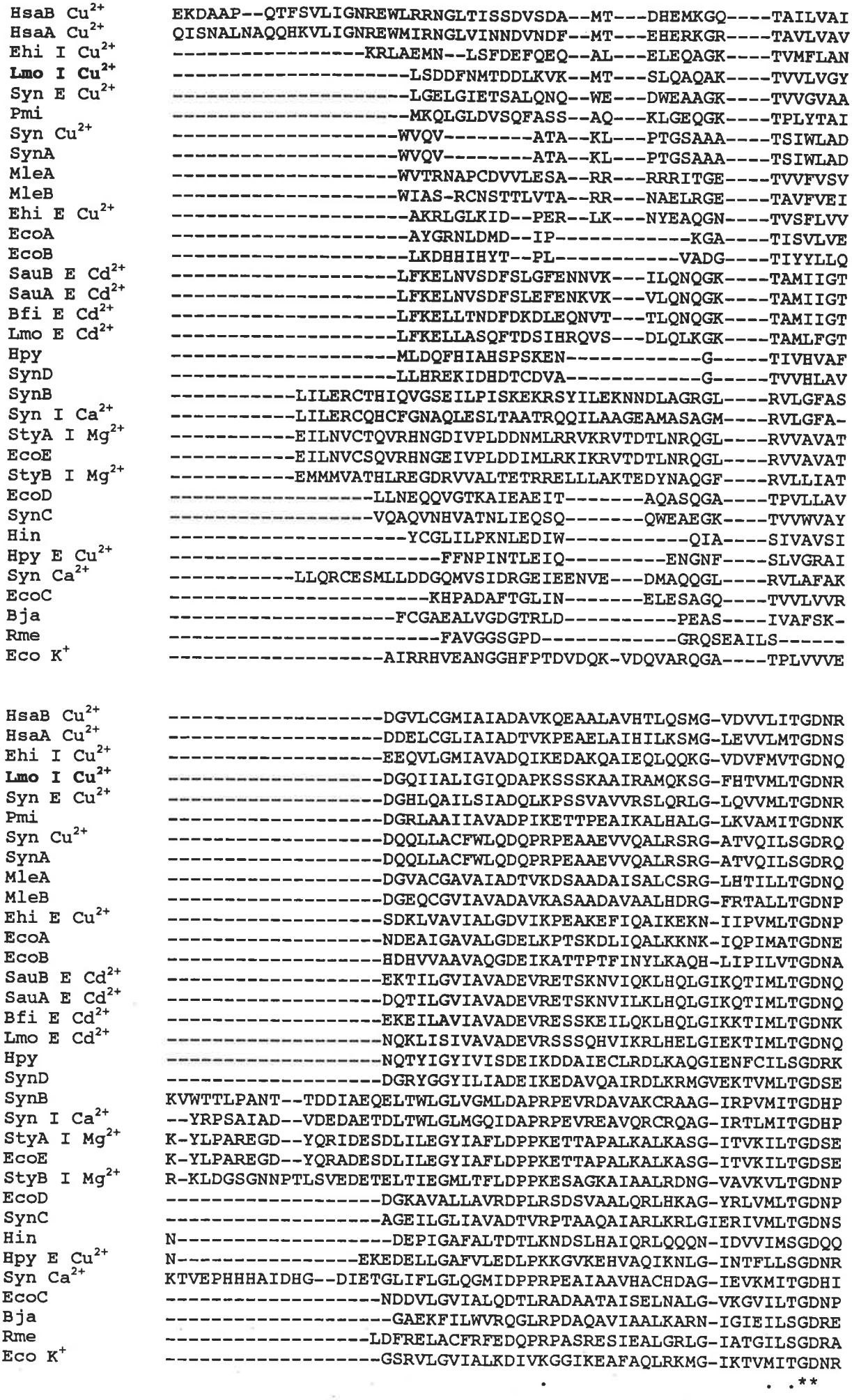

HsaB Cu2+
HsaA Cu2+
Ehi I Cu2+
Lmo I Cu2+
Syn E Cu2+
Pmi
Syn Cu2+
SynA
MleA
MleB
Ehi E Cu2+
EcoA
EcoB
SauB E Cd2+
SauA E Cd2+
Bfi E Cd2+
Lmo E Cd2+
Hpy
SynD
SynB
Syn I Ca2+
StyA I Mg2+
EcoE
StyB I Mg2+
EcoD
SynC
Hin
Hpy E Cu2+
Syn Ca2+
EcoC
Bja
Rme
Eco K+

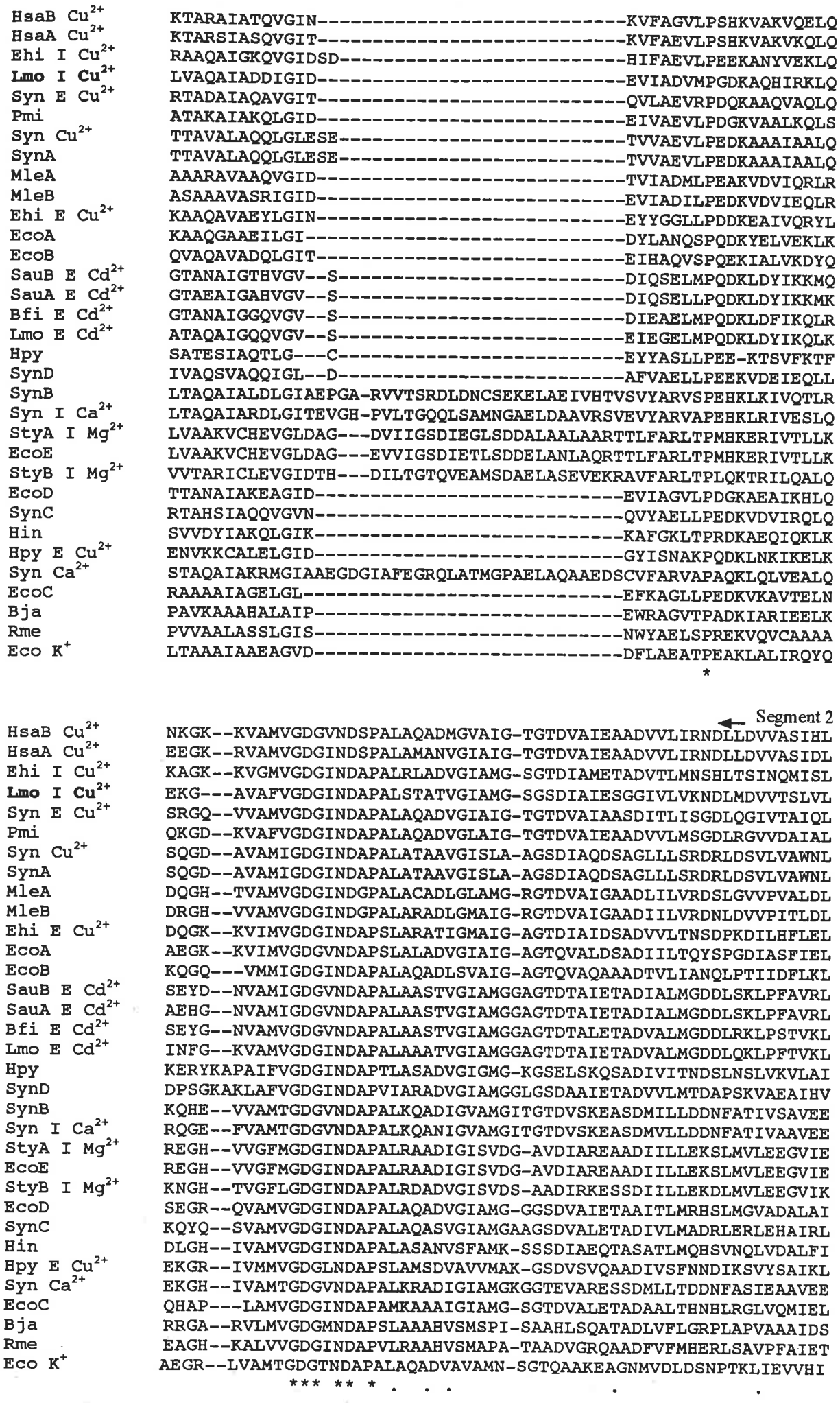
```
HsaB Cu2+       IALGRWLEHLAKSKTSEALAKLMSLQATEATVVTLGEDN-----LIIREEQVPMELVQRG
HsaA Cu2+       IALGRWLEHIAKGKTSEALAKLISLQATEATIVTLDSDN-----ILLSEEQVDVELVQRG
Ehi I Cu2+      ILLGKYLEHTAKSKTGDAIKQMMSLQTKTAQVLRDG----------K-EETIAIDEVMID
Lmo I Cu2+      ILLGSYFEERMKASASSAVDKLMGLQAKDAEVLRDGE----------FIKLPIEEIIVG
Syn E Cu2+      LLLGRSLEERAKGQTSAAIRQLIGLQAKTARVLRQGQ----------ELTLPITEVQVE
Pmi             ILLGRNLEAKAKGNTSQAIKRLVGLQAKTARVSRHGE----------ILEIPLDQVMMG
Syn Cu2+        ILLGRTLEEQARFRSQAALQNLLALQPETTQLLTAPSSIAPQDLLEAPAQIWPVAQLRAG
SynA            ------------------------------------------------------------
MleA            VLAGRFFEARAKSKAGSALRALAARGAKNVEVLLP----------NGAELTIPAGELKKQ
MleB            VLAGKYYTARAKSHASIALLALAALSAKDAAVLQP----------DGSEMVIPANELNEQ
Ehi E Cu2+      MLLGHWIEMNAVSNASDALQKLAELLPESVKRLKKDGT----------EETVSLKEVHEG
EcoA            MLLGHWIEMKALGEAGNAQKALAELVPKDAHVVLEDDS----------IETRPVADLQVG
EcoB            MLLGHRIETAATMEAGDATAKLRALLPNTAHVQHGDH----------FMDMPVSALKPD
SauB E Cd2+     FAISEALERFSMDRSRQSIRSLMDIAPKEALVRRNGQ----------EIIIHVDDIAVG
SauA E Cd2+     FAISEALERFSMDRARQSIRSLMDIAPKEALVRRNGQ----------EIMIHVDDIAVG
Bfi E Cd2+      FAISEALERFSMDRARQSIRSLMDIAPKEALVKRNGQ----------EIMIHVDDIAVG
Lmo E Cd2+      FAVSEALERYSMDKARQSIRSLMDIAPKEALVRRSGT----------DRMVHVDDIQIG
Hpy             YSAGEFLQKLAIARSKKSLKALVDVAPNLAYLKKGDA----------LVSVAPEDLRIN
SynD            FRVGELFQEYSVGRSRRSIKALLEARPDTANLKRNGT----------VQQVSPETVQVD
SynB            VLLNGILGYVQERGAEKALAALKDLSTSRVRVIREGKTT----------EVESTELVPG
Syn I Ca2+      VVLNAVLGYLQESRAEKALAALKGMAAPLVRVRRDNRDQ----------EIPVAGLVPG
StyA I Mg2+     VGISTLLNFVQEARSTKAADALKAMVSNTATVLRVINENG-----ENAWLELPIDQLVPG
EcoE            VAISTLLNFIQEARSTKAADALKAMVSNTATVLRVINDKG-----ENGWLEIPIDQLVPG
StyB I Mg2+     VSLSGLLRFWQEFRTNRAAQALKKMVRTTATVLRRGPGNI-----GAVQEEIPIEELVPG
EcoD            INLGHMLEARARQRSSKALEKLLDLTPPTARLVTDEG----------EKSVPLAEVQPG
SynC            FAISGALEGYAMQRTERSIQGLMSLTADVARVLRNGQ----------EQTIPISELKMG
Hin             VSLGKFLEDRTKKHSLNSLSMLLQLTPKKVTVLRNEK----------WIEIALDQVNIG
Hpy E Cu2+      VMVGKRIENVSKDKALDAMQALMKNAPKTALKIQNDQ----------QIEVLVDSIVVG
Syn Ca2+        TLVNAIIGYIQEAKAEGAIASLAKAVTTEATVLRDGQ----------NLRIPSQDLVIG
EcoC            FLIGERLEGWAASRARQGVSALMALKPETATRLRKGE----------REEVAINSLRPG
Bja             LLVGRFLDQNMRRRTRAVAGNLAALKAETAAKFVGPDE----------ISQVPVAAISPG
Rme             LLIGRTLDHMMRGRARTAISGLARLSPRGATVVHPDGS----------REYRAVDEINPG
Eco K+          VLFANFAEALAEGRSKAQANSLKGVKKTAFARKLRDAKYG------AAADKVPADQLRKG

HsaB Cu2+       DIVKVVPGGKFPVDGKVLEGNTM-ADESLITGEAMPVTK----------------------
HsaA Cu2+       DIIKVVPGGKFPVDGRVIEGHSM-VDESLITGEAMPVAK----------------------
Ehi I Cu2+      DILVIRPGEQVPTDGRIIAGTSA-LDESMLTGESVPVEK----------------------
Lmo I Cu2+      DLIRVKPGEKVAVDGQIVEGTST-LDESMVTGESMPVEK----------------------
Syn E Cu2+      DWVRVRPGEKVPVDGEVIDGRST-VDESMVTGESLPVQK----------------------
Pmi             DIVVVRPGEKIPVDGEVVEGHSY-VDESMITGEPVPVAK----------------------
Syn Cu2+        DYVQVLPGDRIPVDGCIVAGQST-LDTAMLTGEPLPQPC----------------------
SynA            ------------------------------------------------------------
MleA            QHFLVRPGETITADGVVIDGTAT-IDMSAITGEARPVHA----------------------
MleB            QRFVVRPGQTIAADGLVIDGSAT-VSMSPITGEAKPVRV----------------------
Ehi E Cu2+      DRLIVRAGDKMPTDGTIDKGHTI-VDESAVTGESKGVKK----------------------
EcoA            DLIRVQAGENVPADGTIQRGESR-VNEALVTGESKPIEK----------------------
EcoB            MVVQVLAGEAFPADGVILSGESQ-VDESLMTGESRLIDK----------------------
SauB E Cd2+     DIMIVKPGEKIAMDGIIVNGLSA-VNQAAITGESVPVSK----------------------
SauA E Cd2+     DIMIVKPGEKIAMDGIIINGVSA-VNQAAITGESVPVAK----------------------
Bfi E Cd2+      DIMIVKPGQKIAMDGVVVSGYSA-VNQTAITGESVPVEK----------------------
Lmo E Cd2+      DIMIIKPGQKIAMDGHVVKGYSA-VNQAAITGESIPVEK----------------------
Hpy             DIVVVKVGEKVPVDGVVIKGESL-LDERALSGESMPVNV----------------------
SynD            DLILVKPGEKVPLDGEILGGTSQ-VDTSALTGESVPGTV----------------------
SynB            DLILLEAGVKVPADGRILEGANLQIREAALTGEAEAVMKQGDVLLPADSA-----------
Syn I Ca2+      DLILLEAGDQVPADARLVESANLQVKESALTGEAEAVQKLADQQLPTDVV-----------
StyA I Mg2+     DIIKLAAGDMIPADLRIIQARDLFVAQASLTGESLPVEKVAATREPRQNN----P------
EcoE            DIIKLAAGDMIPADLRILQARDLFVAQASLTGESLPVEKAATTRQPEHSN----P------
StyB I Mg2+     DVVFLAAGDLVPADVRLLASRDLFISQSILSGESLPVEKYDVMADVAGKDSEQLPDKDKS
EcoD            MLLRLTTGDRVPVDGEITQGEAW-LDEAMLTGEPIPQQK----------------------
SynC            DQVLVKPGELVPTDGLVIEGFST-LNQASITGESMPVEK----------------------
Hin             EIIRANQGERIAADGVIESGNGW-CDESHLTGESRPEEK----------------------
Hpy E Cu2+      DILKVLPGTLIAVDGEIIEGEGE-LDESMLSGEALPVYK----------------------
Syn Ca2+        DIVSLASGDKVPADLRLLKVRNLQVDESALTGEAVPVEKAVELLPEETP-------------
EcoC            DVIEVAAGGRLPADGKLLSPFAS-FDESALTGESIPVER----------------------
Bja             DIVLLRPGERCAVDGTVIEGRSE-IDQSLITGETLYVTA----------------------
Rme             DRLIVAAGERVPVDGRVLSGTSD-LDRSVVNGESSPTVV----------------------
Eco K+          DIVLVEAGDIIPCDGEVIEGGAS-VDESAITGESAPVIR------ES--G-----------
                                        **
```

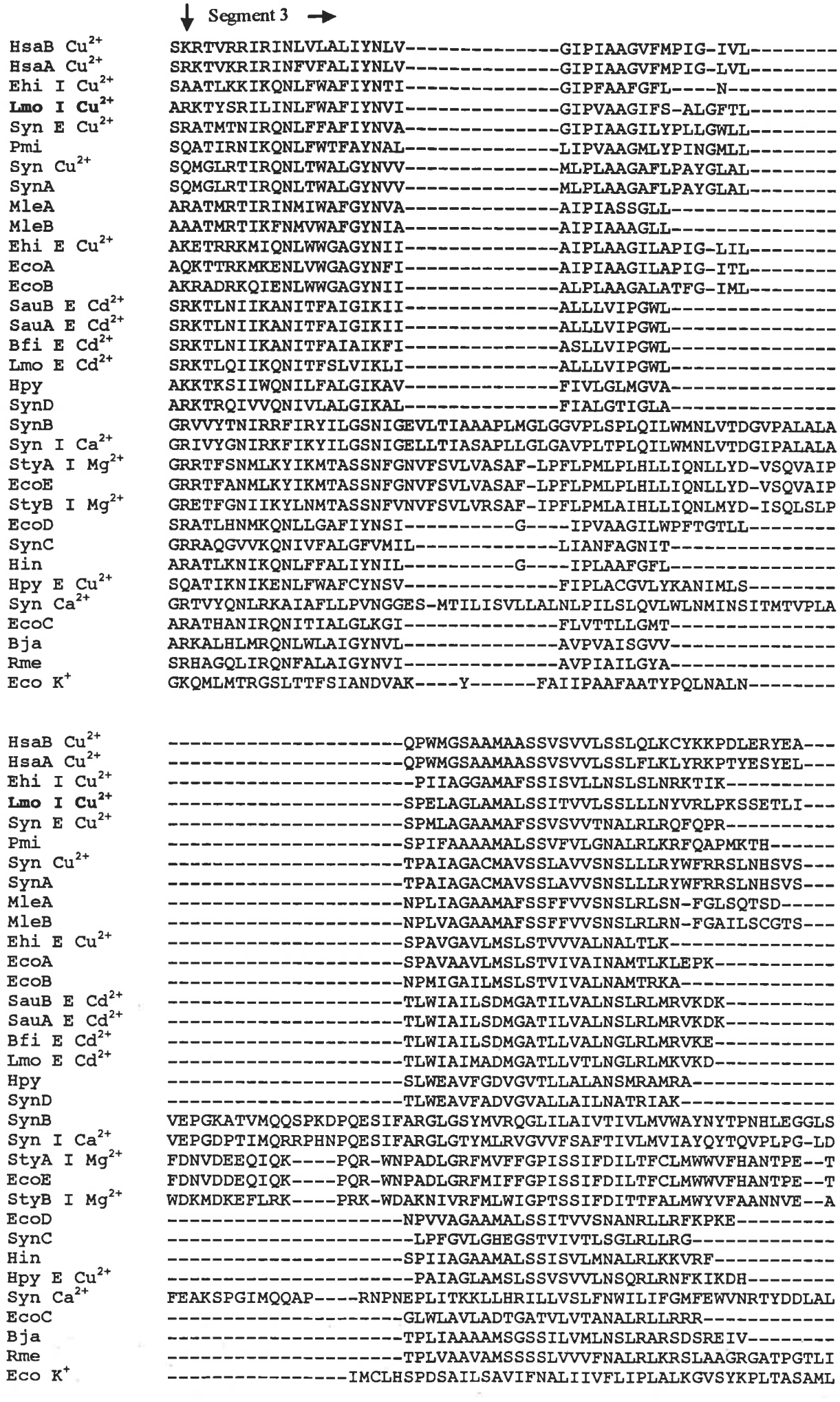

HsaB Cu2+ --KPGSTVIAGSINAHGSVPIKATHVGNDTTLAQIVKLVEEAQMSKAPIQQLADRFSGYF
HsaA Cu2+ --KPGSTVIAGSINQNGSLLICATHVGADTTLSQIVKLVEEAQTSKAPIQQFADKLSGYF
Ehi I Cu2+ --KEKDMVFGGTINTNGLIQIQVSQIGKDTVLAQIIQMVEDAQGSKAPIQQIADKISGIF
Lmo I Cu2+ --GPGDNVIGATLNNTGSFTFEVTKVGADTMLSNIAEMVRHAQNSRAPIQKTVDRISNIF
Syn E Cu2+ --QVGDEVIGATLNKTGSLTIRATRVGRETFLAQIVQLVQQAQASKAPIQRLADQVTGWF
Pmi --EIGAEVVGGTINKTGTFSFKVTKVGANTILAQIIRLVEEAQGSKLPIQALVDKVTMWF
Syn Cu2+ --QVGDRVCAGTLNLSHRLVIRAEQTGSQTRLAAIVRCVAEAQQRKAPVQRFADAIAGRF
SynA
MleA --SPASTVVGGTTVLDGRLVIEATAVGGDTQFAAMVRLVEDAQVQKARVQHLADRIAAVF
MleB --NPGAQVIGGTVVLNGRLIVEAAAVGDETQLAGMVRLVEQAQQQNANAQRLADRIASVF
Ehi E Cu2+ --QVGDSVIGGSINGDGTIEITVTGTGENGYLAKVMEMVRKAQGEKSKLEFLSDKVAKWL
EcoA --NPGDEVIGGSTNGDGVLYVEIKQTGDKSFISQVQTLISQAQSQPSRAENLAQKVAGWL
EcoB --KPGVSVVGGTINGNGTLLVKVTHVGAQSFIGKLQSTLAASQSAKSRVETIADQVASYL
SauB E Cd2+ --AVDDEVFAGTLNEEGLIEVKITKYVEDTTITKIIHLVEEAQGERAPAQAFVDKFAKYY
SauA E Cd2+ --TVDDEVFAGTLNEEGLLEVKITKYVEDTTISKIIHLVEEAQGERAPAQAFVDKFAKYY
Bfi E Cd2+ --TVDNEVFAGTLNEEGLLEVEITKLVEDTTISKIIHLVEEAQGERAPSQAFVDKFAKYY
Lmo E Cd2+ --NIDDSVFAGTLNEEGLLEVAVTKRVEDTTISKIIHLVEEAQGERAPAQAFVDTFAKYY
Hpy --SERSKVLGGSLNLKAVLEIQVEKMYKDSSIAKVVDLVQQATNEKSETEKFITKFSRYY
SynD --KPGDTILAGMINQSGVLTIRVTKLFSESSIAKVLDLVENASSKKASTEKFITQFARYY
SynB LGDRLNLVYSGTEVVQGRGTVIVTATGMKTELGKIASALQSVEPEPTPLQKRMTQLGNVL
Syn I Ca2+ IGDRTNCLFQGTEVLQGRGQALVYATGMNTELGRIATLLQSVESEKTPLQQRLDKLGNVL
StyA I Mg2+ -LECDTLCFMGTNVVSGTAQAVVMATGAGTWFGQLAGRVSEQDNEQNAFQKGISRVSMLL
EcoE -LECDTLCFMGTTVVSGTAQAMVIATGANTWFGQLAGRVSEQESEPNAFQQGISRVSMLL
StyB I Mg2+ LLDLGNICLMGTNVTSGRAQAVVVATGSRTWFGSLAKSIVG-TRTQTAFDRGVNSVSWLL
EcoD --GEGDSVHAGTVVQDGSVLFRASAVGSHTTLSRIIRMVRQAQSSKPEIGQLADKISAVF
SynC --AIGDEVFAGTINGNGVLRLKIHQPPESSLIQRVIRLVQQAQTEAPPSQQFIERFECGY
Hin --QKGGKVLAGAMVTEGSIIYRANQLGSQTLLGDMMNALSDAQGSKAPIARFADKVTSVF
Hpy E Cu2+ --KVGDKVFSGTFNSHTSFLMKATQNNKNSTLSQIVEMIHNAQSSKAEISRLADKVSSVF
Syn Ca2+ LAERLNMAYAGSFVTFGQGTGVVVATANATEMGQISQSMEKQVSLMTPLTRKFAKFSHTL
EcoC --ATGDKVPAGATSVDRLVTLEVLSEPGASAIDRILKLIEEAEERRAPIERFIDRFSRIY
Bja --EQGTPVYAGSMNISGTLRVRVSAASEATLLAEIARLLDNALQARSRYMRLADRASRLY
Rme --TTGDTVQAGTLNLTGPLTLEATAAARDSFIAEIIGLMEAAEGGRARYRRIADRAARYY
Eco K+ --GDFASVTGGTRILSDWLVIECSVNPGETFLDRMIAMVEGAQRRKTPNE---IALTILL
HsaB Cu2+ VPFIIIMSTLTLVVWIVIGFIDFG----------VVQRYFPNPNKHISQTEVIIWFAFQT
HsaA Cu2+ VPFIVFVSIATLLVWIVIGFLNFE----------IVETYFPGYNRSISRTETIIRFAFQA
Ehi I Cu2+ VPIVLFLALVTLLVTGWL----------T-------KDWQLALLH
Lmo I Cu2+ VPIVLMISILTFIVWYVFLGS----------TLVTAMIF
Syn E Cu2+ VPAVIAIAILTFLLWFNWIG----------NVTLALIT
Pmi VPAVMIGATITFFIWLAFGPE----------PALTFALIN
Syn Cu2+ VYGVCAIAALTFGFWATLGSRWWPQVLQQPLPGLLIHAPHHGMEMAHPHSHSPLLLALTL
SynA
MleA VPMVFVIAGLAGASWLLAGASPDR----------AFSV
MleB VPCVFAVAALDRCWMADRRERTRP----------SVLG
Ehi E Cu2+ FYVALVVGIIAFIAWLFLAN----------LPDALER
EcoA FYIAVIAALIALVIWMVIAD----------VPTAVIF
EcoB FWVALLIAGLSLMIWTPTHG----------LGFAINI
SauB E Cd2+ TPIIMVIAALVAVVPPLFFGGS----------WDTWVYQ
SauA E Cd2+ TPIIMVIAALVAVVPPLFFGGS----------WDTWVYQ
Bfi E Cd2+ TPIIMIIATLVAIVPPLFFDGS----------WETWIYQ
Lmo E Cd2+ TPAIIVIAALIATVPPLLFGGN----------WETWVYQ
Hpy TPSVLFIALMIAVLPPLFSMGS----------FDEWIYR
SynD TPVIVFLSLAVALLPPLFIPGAD----------RADWVYR
SynB VSGSLILVAIVVVGGTLFKP----------DLFMQLVEV
Syn I Ca2+ VSGALILVAIVVGLGVLNG----------QSWEDLLSV
StyA I Mg2+ IRFMLVMAPVVLIINGYTK----------GDWWEAALF
EcoE IRFMLVMAAGGAVNQWLHQ----------RRLVGSALF
StyB I Mg2+ IRFMLIMVPVVLLINGFSK----------GDWVEASLF
EcoD VPVVVVIALVSAAIWYFFGPAP----------QIVYTLVI
SynC AKVIVIAGLLLGTLPPFLLGWS----------WEETIYR
Hin VPVVLVISLVTFALTYILTNDS----------VSSLIH
Hpy E Cu2+ VPSVIAIAILAFVVWLIIAPKPD----------FWWNFGIALEV
Syn Ca2+ LYVIVTLAAFTFAVGWGRGGSP----------LEMFEA
EcoC TPAIMAVALLVTLVPPLLFAAS----------WQEWIYK
Bja APVVHATALITILGWVIAGAS----------WHDAIVT
Rme SPAVHLLALLTFVGWMLVEG----------DVRHAMLV
Eco K+ IALTIVFLLATATLWPFSAWGG----------NAVSVTV

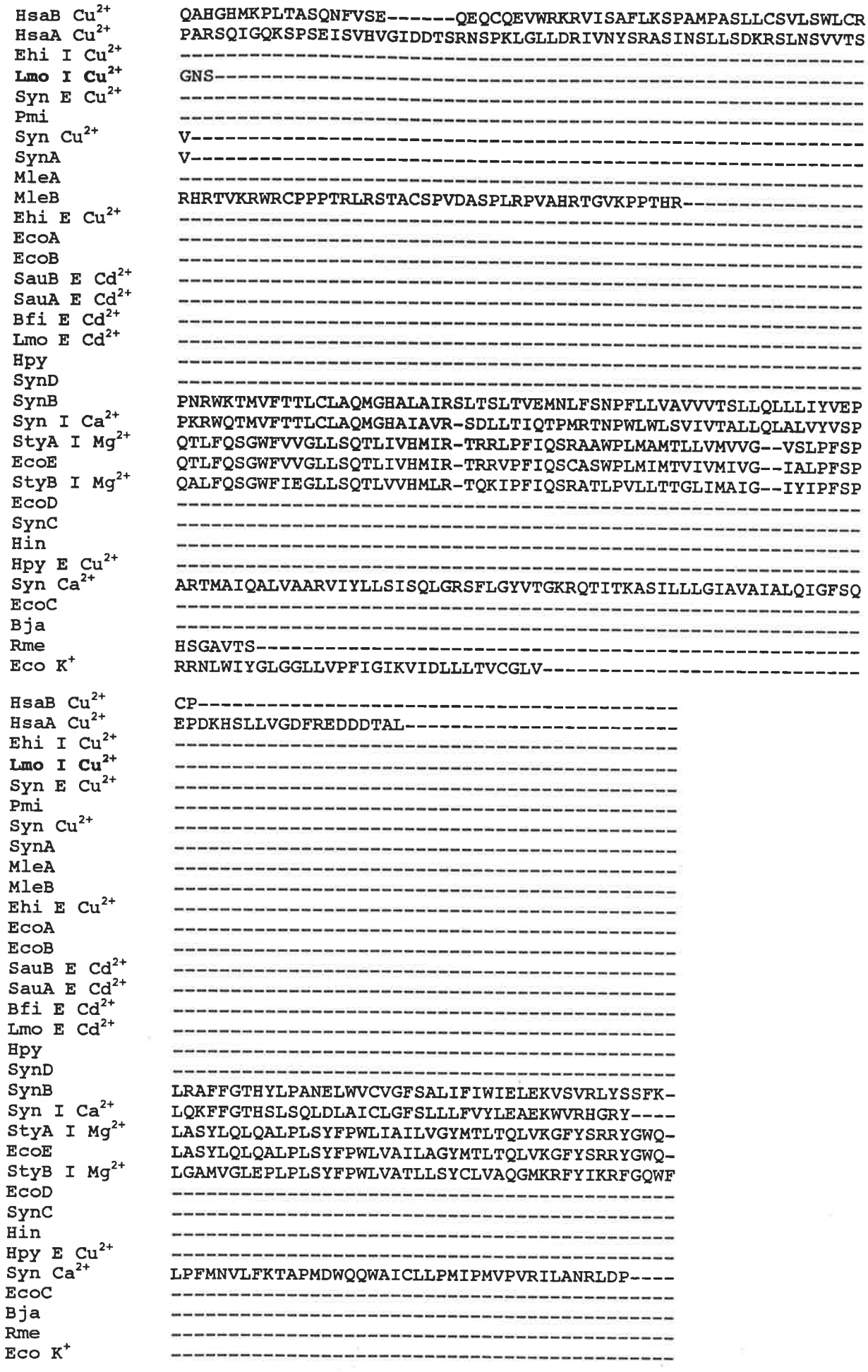

← Segment 1
Segment 2 →
HsaB Cu2+ SITVLCIACPCSLGLATPTAVMVGTGVAAQNGILIKGGKPLEMAHKIKTVMFDKTGTIIH
HsaA Cu2+ SITVLCIACPCSLGLATPTAVMVGTGVGAQNGILIKGGEPLEMAHKVKVVVFDKTGTITH
Ehi I Cu2+ SVSVLVIACPCALGLATPTAIMVGTGVGAHNGILIKGGEALEGAAHLNSIILDKTGTITQ
Lmo I Cu2+ SVSVMIIACPCALGIATPTALMVGTGRSAKLGILIKNAEVLEATHDIKTVVMDKTGTITV
Syn E Cu2+ AVGVMIIACPCALGLATPTSIMVGTGKGAEYGILIKSAESLELAQTIQTVILDKTGTLTQ
Pmi AVAVLIIACPCAMGLATPTSIMVGTGRAAELGILFRKGEALQALRDVSVVALDKTGTLTK
Syn Cu2+ AISVLVVACPCALGLATPTAILVATGLAAEQGILVRGGDVLEQLARIKHFVFDKTGTLTQ
SynA ----------
MleA VLGVLVIACPCTLGLATPTAMMVASGRGAQLGIFIKGYRALETINAIDTVVFDKTGTLTL
MleB AIAVLVIACPCALGLATPTAMMVASGRGAQLGILLKGHESFEATRAVDTVVFDKTGTLTT
Ehi E Cu2+ MVTVFIIACPHALGLAIPLVVARSTSIAAKNGLLLKNRNAMEQANDLDVIMLDKTGTLTQ
EcoA TVTTLVIACPHALGLAIPLVTARSTSLGASRGLLVKDRDALELTTNADVMVLDKTGTLTT
EcoB AVTVLVIACPHALGLAVPLVIQRTKAIAATQGILIKNHKALSSANHLTYVLMDKTGTLTT
SauB E Cd2+ GLAVLVVGCPCALVISTPISIVSAIGNAAKKGVLVKGGVYLEKLGAIKTVAFDKTGTLTK
SauA E Cd2+ GLAVLVVGCPCALVITTPISIVSAIGNAAKKGVLIKGGVYLEELGAIKAIAFDKTGTLTK
Bfi E Cd2+ GLAVLVVGCPCALVISTPISIVSAIGNAAKKGVLVKGGVYLEEMGALKAIAFDKTGTLTK
Lmo E Cd2+ GLSVLVVGCPCALVVSTPVAIVTAIGNAAKNGVLVKGGVYLEEIGGLKAIAFDKTGTLTK
Hpy GLVALMVSCPCALVISVPLGYFGGVGAASRKGILMKGVHVLEVLTQAKSIAFDKTGTLTK
SynD ALVLLVISCPCGLVISIPLGYFGGIGGAAKHGILIKGSTFLDSLTAVKTVVFDKTGTLTK
SynB SLSMAVAVVPEGLPAVITVTLALGTQRMAKRNALIRQLSAVETLGSVTTICSDKTGTLTQ
Syn I Ca2+ GLSMAVAIVPEGLPAVITVALAIGTQRMVQRESLIRRLPAVETLGSVTTICSDKTGTLTQ
StyA I Mg2+ ALSVAVGLTPEMLPMIVTSTLARGAVKLSKQKVIVKHLDAIQNFGAMDILCTDKTGTLTQ
EcoE ALSVAVGLTPEMLPMIVTSTLARGAVKLSKQKVIVKHLDAIQNFGAMDILCTDKTGTLTQ
StyB I Mg2+ ALAVAVGLTPEMLPMIVSSNLAKGAIAMSRRKVIVKRLNAIQNFGAMDVLCTDKTGTLTQ
EcoD ATTVLIIACPCALGLATPMSIISGVGRAAEFGVLVRDRDALQRASTLDTVVFDKTGTLTE
SynC ALIFLVVASPCALMASIMPALLSGIANGARQGILFKNGAQLERIGRVRVIAFDKTGTLTT
Hin AVSVLVIACPCALGLATPAAIMVGLGKAVNAGVWFKDAAAMEETAHVDTVVLDKTGTLTK
Hpy E Cu2+ FVSVLVISCPSCFRIGYAMSILVANQKSEFFRIIFKDAKSLEKARLVNTIVFDKTGTLTN
Syn Ca2+ AVALAVSGIPEGLPAVVTVTLAIGVNRMAKRNAIIRKLPAVEALGSATVVCSDKTGTLTE
EcoC GLTLLLIGCPCALVISTPAAITSGLAAAARRGALIKGGAALEQLGRVTQVAFDKTGTLTV
Bja GVAVLIITCPCALGLAIPTVQTVASGAMFKSGVLLNSGDAIERLAEADHVIFDKTGTLTL
Rme AVAVLIITCPCALGLAVPVVQVVAAGRLFQGGVMVKDGSAMERLAEIDTVLLDKTGTLTI
Eco K+ LVALLVCLIPTTIGGLLSASAVAGMSRMLGANVIATSGRAVEAAGDVDVLLLDKTGTITL
* *****..
HsaB Cu2+ GVP----RVMR-------VLLLGDVA----------TLPLRKVLAVV
HsaA Cu2+ GTP----VVNQ-------VKVLTESN----------RISHHKILAIV
Ehi I Cu2+ GRP----EVTD-------VIGPKE----------IISLF
Lmo I Cu2+ GKP----QVTD-------IISIG----------RISENEILRIA
Syn E Cu2+ GQP----SVTD-------FLAIGD----------RDQQQTLLGWA
Pmi GRP----ELTD-------LIPAE----------KFEYNEILSLV
Syn Cu2+ GQF----ELIE-------IQPLAD----------VDPDRLLQWA
SynA ----------
MleA GQL----SVST-------VTSTGG----------WCSGEVLALA
MleB GQL----KVSA-------VTAAPG----------WQANEVLQMA
Ehi E Cu2+ GKF----TVTG-------IEILDEAY----------QE--EEILKYI
EcoA GEF----KVLD-------VELFNDKY----------TK--DEIVALL
EcoB GQF----KVM---------QVVTDNF----------DQ--KEALGIM
SauB E Cd2+ GVP----VVTD-------FEVLNDQV----------EEKE--LFSII
SauA E Cd2+ GVP----VVTD-------FKVLNDQV----------EEKE--LFSII
Bfi E Cd2+ GVP----AVTD-------YNVLNKQI----------NEKE--LLSII
Lmo E Cd2+ GVP----VVTD-------YIELTEAT----------NIQHNKNYIIM
Hpy GVF----KVTD-------IVPQNGHS----------KE-E--VLHYA
SynD GTF----KVTQ-------VVTKNGFS----------ES-E--LLTLA
SynB NKM----VVQSIISDRHRLVVTGEGYNPVG--EFQAGEGEDLKIENIPEIEKLLMACILC
Syn I Ca2+ NKM----VVQQIHTLDHDFTVTGEGYVPAG--HFLIG-GEIIVPNDYRDLMLLLAAGAVC
StyA I Mg2+ DKI----VLE------NHTDISGK----------PSEHVLHCAWL
EcoE DKI----VLE------NHTDISGK----------TSERVLHSAWL
StyB I Mg2+ DNI----FLE------HHLDVSGV----------KSSRVLMLAWL
EcoD GKP----QVVA-------VKTFAD----------VDEAQALRLA
SynC GKP----EVVN---------ILATQP----------STDKLLQIA
Hin GEL----EISALWQ--PQSAVYSE----------DDLYRFA
Hpy E Cu2+ GKP----VVKS---------VHSK----------IELLELLSLA
Syn Ca2+ NQM----TVQAVYAGGKHYEVSGGGYSPKGEFWQVMGEEVDNVLLDGLPPVLEECLLTGM
EcoC GKPRVTA----------IHPATG----------ISESELLTLA
Bja PDL----EVMN------AADIPAD----------IFELA
Rme GKP----RLVN------AHEISPG----------RLATA
Eco K+ GNR----QASE-------FIPAQG----------VDEKTLADA

```
HsaB Cu2+       GTAEAS----------SEHPLGVAVTKYCKEELGTETLG-YCTDFQAVPG----CGIGC
HsaA Cu2+       GTAESN----------SEHPLGTAITKYCKQELDTETLG-TCIDFQVVPG----CGISC
Ehi I Cu2+      YSLEHA----------SEHPLGKAIVAYGAKVGAKTQP---ITDFVAHPG----AGISG
Lmo I Cu2+      AGLEDS----------SEHPLALAVINEAKDKKITPAV---AKNFTAISG----KGVQA
Syn E Cu2+      ASLENY----------SEHPLAEAIVRYGEAQGITLST---VTDFEAIPG----SGVQG
Pmi             ASIETY----------SEHPIAQSIVNAANEAKLTLAS---VDNFEAIPG----FGVSA
Syn Cu2+        AALEAD----------SRHPLATALQTAAQAANLAPIAAS--D-RQQVPG----LGVSG
SynA            AALEAD----------SRHPLATALQTAAQAANLAPIAAS--D-RQQVPG----LGVSG
MleA            SAVEAA----------SEHSVATAI--VAAYADPRPVAD-----FVAFAG----CGVSG
MleB            ATVESA----------SEHAVALAI--AASTTHREPVAN-----FRAVPG----HGVSG
Ehi E Cu2+      GALEAH----------ANHPLAIGIMNYLKEKKITPYQAQ--E-QKNLAG----VGLEA
EcoA            SGIEGG----------SSHPIAQSIISYAEQQGIRPVSFD--S-IDVMSG----AGVEG
EcoB            AALDAQ----------STHPLAQGIVSYAKQQQAPVLSAT--D-VENMAG----YGIAG
SauB E Cd2+     TALEYR----------SQHPLASAIMKKAEQDNIPYS-NVQVEEFTSITG----RGIKG
SauA E Cd2+     TALEYR----------SQHPLASAIMKKAEQDNITYS-DVRVKDFTSITG----RGIQG
Bfi E Cd2+      TALEYR----------SQHPLASAIMKKAEEENITYS-DVQVEDFSSITG----KGIKG
Lmo E Cd2+      AALEQL----------SQHPLASAIIKYGETREMDLT-SINVNDFTSITG----KGIRG
Hpy             SCSQLL----------STHPIALSIQEACEEMLKDDKHQHDIKNYEELSG----MGVKA
SynD            AKAESH----------STHPIALSIREAYAQSIADS----EVADYEEIAG----HGIRA
SynB            NDAILQKENG--QWAILGDPTEGALLALAGKANIFKHEQEQYFPRITEFPFSSERKRMSV
Syn I Ca2+      NDAALVASGE--HWSIVGDPTEGSLLTVAAKAGIDPEGLQRVLPRQDEIPFTSERKRMSV
StyA I Mg2+     NSHYQT----------GLKNLLDTAVLEGVDETAARQLSGRWQKIDEIPFDFERRRMSV
EcoE            NSHYQT----------GLKNLLDTAVLEGTDEESARSLASRWQKIDEIPFDFERRRMSV
StyB I Mg2+     NSSSQS----------GARNVMDRAILRFGEGRIAPSTKARFIKRDELPFDFVRRRVSV
EcoD            AALEQG----------SSHPLARAILDKAGDMRLPQVNG-----FRTLRG----LGVSG
SynC            AALESL----------SEHPIGEAIADFTRQQNQAWATAR---NVQAQAG----QGIIG
Hin             AAVERQ----------ANHPIAKAIVQAAEXKMLEIPT----ALFSKMEVG---QGIQA
Hpy E Cu2+      NSIEKS----------SEHVIAKGIVEYAKEHNAPLKEMS---EVKVKTG----FGISA
Syn Ca2+        LCNDSQLEHRGDDWAVVGDPTEGALLASAAKAGFSQAGLASQKPRLDSIPFESDYQYMAT
EcoC            AAVEQG----------ATHPLAQAIVREAQVAELAIPTAE---SQRALVG----SGIEA
Bja             GRLALS----------SHHPVAAAVAQAAGARSPIVG-------AVEEAG----QGVRA
Rme             AAIAVH----------SRHPIAVAIQNSAGAASPIAG------DIREIPG----AGIEV
Eco K+          AQLASLA---------DETPEGRSIVILAKQRFNLRERDVQSLHATFVPFTAQSRMSGI

HsaB Cu2+       KVSNA-----------EDILAHS--------------ERPLSAPASHLNEAGSLPA
HsaA Cu2+       KVTNI-----------EGLLHKNNWNIEDNNIKNASLVQIDASNEQSSTSSSMIIDA
Ehi I Cu2+      TINGV-----------HYFAGTR-----------------------------------
Lmo I Cu2+      LIDGK-----------QAFIGNDR-----------------------------------
Syn E Cu2+      QVEGI-----------WLQIGTQRW----------------------------------
Pmi             TVDGR-----------SVSVGADRF----------------------------------
Syn Cu2+        TCDGR-----------SLRLGNPT-----------------------------------
SynA            TCDGR-----------SLRLGNPT-----------------------------------
MleA            VVAEH-----------HVKIGKPS-----------------------------------
MleB            TVAER-----------AVRVGKPS-----------------------------------
Ehi E Cu2+      TVEDK-----------DVKIINEKE----------------------------------
EcoA            QANGH-----------RYQLISQK-----------------------------------
EcoB            MVNDK-----------HYLLVSERY----------------------------------
SauB E Cd2+     IVNGT-----------TYYIGSPK-----------------------------------
SauA E Cd2+     NIDGT-----------TYYIGSPR-----------------------------------
Bfi E Cd2+      IVNGT-----------TYYIGSPK-----------------------------------
Lmo E Cd2+      TVDGN-----------TYYVGSPV-----------------------------------
Hpy             QCHTD-----------LIIAGNEK----------------------------------
SynD            VVQNQ-----------VVIAGNDR-----------------------------------
SynB            IVQDG---QGKINTPDSYVMFVKGSPE--------------------------------
Syn I Ca2+      VVADLGETTLTIREGQPYVLFVKGSAE--------------------------------
StyA I Mg2+     VVAED--------SN-VHQLVCKGALQ---------------------------------
EcoE            VVAEN--------TE-HHQLVCKGALQ---------------------------------
StyB I Mg2+     LVEDA--------QHGDRCLICKGAVE---------------------------------
EcoD            EAEGH-----------ALLLGNQA-----------------------------------
SynC            DIEGQ-----------QAIVGKAVF----------------------------------
Hin             ELEQVG----------TIKVGKPD-----------------------------------
Hpy E Cu2+      KTDYQG----------TKEIIKVGNSE--------------------------------
Syn Ca2+        LHDGDG----------RTIYVKGSVES--------------------------------
EcoC            QVNGE-----------RVLICAAG-----------------------------------
Bja             DVDGA-----------EIRLGRPS-----------------------------------
Rme             KTEDG-----------VYRLGSRD-----------------------------------
Eco K+          NIDNR-----------MIRKGSVD-----------------------------------
```


HsaB Cu2+ EKDAAP--QTFSVLIGNREWLRRNGLTISSDVSDA--MT---DHEMKGQ----TAILVAI
HsaA Cu2+ QISNALNAQQHKVLIGNREWMIRNGLVINNDVNDF--MT---EHERKGR----TAVLVAV
Ehi I Cu2+ ----------KRLAEMN--LSFDEFQEQ--AL---ELEQAGK----TVMFLAN
Lmo I Cu2+ ----------LSDDFNMTDDLKVK--MT---SLQAQAK----TVVLVGY
Syn E Cu2+ ----------LGELGIETSALQNQ--WE---DWEAAGK----TVVGVAA
Pmi ----------MKQLGLDVSQFASS--AQ---KLGEQGK----TPLYTAI
Syn Cu2+ ----------WVQV--------ATA--KL---PTGSAAA----TSIWLAD
SynA ----------WVQV--------ATA--KL---PTGSAAA----TSIWLAD
MleA ----------WVTRNAPCDVVLESA--RR---RRRITGE----TVVFVSV
MleB ----------WIAS-RCNSTTLVTA--RR---NAELRGE----TAVFVEI
Ehi E Cu2+ ----------AKRLGLKID--PER--LK---NYEAQGN----TVSFLVV
EcoA ----------AYGRNLDMD--IP----------KGA----TISVLVE
EcoB ----------LKDHHIHYT--PL----------VADG----TIYYLLQ
SauB E Cd2+ ----------LFKELNVSDFSLGFENNVK---ILQNQGK----TAMIIGT
SauA E Cd2+ ----------LFKELNVSDFSLEFENKVK---VLQNQGK----TAMIIGT
Bfi E Cd2+ ----------LFKELLTNDFDKDLEQNVT---TLQNQGK----TAMIIGT
Lmo E Cd2+ ----------LFKELLASQFTDSIHRQVS---DLQLKGK----TAMLFGT
Hpy ----------MLDQFHIAHSPSKEN----------G-----TIVHVAF
SynD ----------LLHREKIDHDTCDVA----------G-----TVVHLAV
SynB ----------LILERCTHIQVGSEILPISKEKRSYILEKNNDLAGRGL----RVLGFAS
Syn I Ca2+ ----------LILERCQHCFGNAQLESLTAATRQQILAAGEAMASAGM----RVLGFA-
StyA I Mg2+ ----------EILNVCTQVRHNGDIVPLDDNMLRRVKRVTDTLNRQGL----RVVAVAT
EcoE ----------EILNVCSQVRHNGEIVPLDDIMLRKIKRVTDTLNRQGL----RVVAVAT
StyB I Mg2+ ----------EMMMVATHLREGDRVVALTETRRELLLAKTEDYNAQGF----RVLLIAT
EcoD ----------LLNEQQVGTKAIEAEIT-------AQASQGA----TPVLLAV
SynC ----------VQAQVNHVATNLIEQSQ-------QWEAEGK----TVVWVAY
Hin ----------YCGLILPKNLEDIW----------QIA----SIVAVSI
Hpy E Cu2+ ----------FFNPINTLEIQ---------ENGNF----SLVGRAI
Syn Ca2+ ----------LLQRCESMLLDDGQMVSIDRGEIEENVE---DMAQQGL----RVLAFAK
EcoC ----------KHPADAFTGLIN-------ELESAGQ----TVVLVVR
Bja ----------FCGAEALVGDGTRLD----------PEAS----IVAFSK-
Rme ----------FAVGGSGPD----------GRQSEAILS------
Eco K+ ----------AIRRHVEANGGHFPTDVDQK-VDQVARQGA----TPLVVVE
HsaB Cu2+ ----------DGVLCGMIAIADAVKQEAALAVHTLQSMG-VDVVLITGDNR
HsaA Cu2+ ----------DDELCGLIAIADTVKPEAELAIHILKSMG-LEVVLMTGDNS
Ehi I Cu2+ ----------EEQVLGMIAVADQIKEDAKQAIEQLQQKG-VDVFMVTGDNQ
Lmo I Cu2+ ----------DGQIIALIGIQDAPKSSSKAAIRAMQKSG-FHTVMLTGDNR
Syn E Cu2+ ----------DGHLQAILSIADQLKPSSVAVVRSLQRLG-LQVVMLTGDNR
Pmi ----------DGRLAAIIAVADPIKETTPEAIKALHALG-LKVAMITGDNK
Syn Cu2+ ----------DQQLLACFWLQDQPRPEAAEVVQALRSRG-ATVQILSGDRQ
SynA ----------DQQLLACFWLQDQPRPEAAEVVQALRSRG-ATVQILSGDRQ
MleA ----------DGVACGAVAIADTVKDSAADAISALCSRG-LHTILLTGDNQ
MleB ----------DGEQCGVIAVADAVKASAADAVAALHDRG-FRTALLTGDNP
Ehi E Cu2+ ----------SDKLVAVIALGDVIKPEAKEFIQAIKEKN-IIPVMLTGDNP
EcoA ----------NDEAIGAVALGDELKPTSKDLIQALKKNK-IQPIMATGDNE
EcoB ----------HDHVVAAVAQGDEIKATTPTFINYLKAQH-LIPILVTGDNA
SauB E Cd2+ ----------EKTILGVIAVADEVRETSKNVIQKLHQLGIKQTIMLTGDNQ
SauA E Cd2+ ----------DQTILGVIAVADEVRETSKNVILKLHQLGIKQTIMLTGDNQ
Bfi E Cd2+ ----------EKEILAVIAVADEVRESSKEILQKLHQLGIKKTIMLTGDNK
Lmo E Cd2+ ----------NQKLISIVAVADEVRSSSQHVIKRLHELGIEKTIMLTGDNQ
Hpy ----------NQTYIGYIVISDEIKDDAIECLRDLKAQGIENFCILSGDRK
SynD ----------DGRYGGYILIADEIKEDAVQAIRDLKRMGVEKTVMLTGDSE
SynB KVWTTLPANT--TDDIAEQELTWLGLVGMLDAPRPEVRDAVAKCRAAG-IRPVMITGDHP
Syn I Ca2+ --YRPSAIAD--VDEDAETDLTWLGLMGQIDAPRPEVREAVQRCRQAG-IRTLMITGDHP
StyA I Mg2+ K-YLPAREGD--YQRIDESDLILEGYIAFLDPPKETTAPALKALKASG-ITVKILTGDSE
EcoE K-YLPAREGD--YQRADESDLILEGYIAFLDPPKETTAPALKALKASG-ITVKILTGDSE
StyB I Mg2+ R-KLDGSGNNPTLSVEDETELTIEGMLTFLDPPKESAGKAIAALRDNG-VAVKVLTGDNP
EcoD ----------DGKAVALLAVRDPLRSDSVAALQRLHKAG-YRLVMLTGDNP
SynC ----------AGEILGLIAVADTVRPTAAQAIARLKRLGIERIVMLTGDNS
Hin N----------DEPIGAFALTDTLKNDSLHAIQRLQQQN-IDVVIMSGDQQ
Hpy E Cu2+ N----------EKEDELLGAFVLEDLPKKGVKEHVAQIKNLG-INTFLLSGDNR
Syn Ca2+ KTVEPHHHAIDHG--DIETGLIFLGLQGMIDPPRPEAIAAVHACHDAG-IEVKMITGDHI
EcoC ----------NDDVLGVIALQDTLRADAATAISELNALG-VKGVILTGDNP
Bja ----------GAEKFILWVRQGLRPDAQAVIAALKARN-IGIEILSGDRE
Rme ----------LDFRELACFRFEDQPRPASRESIEALGRLG-IATGILSGDRA
Eco K+ ----------GSRVLGVIALKDIVKGGIKEAFAQLRKMG-IKTVMITGDNR

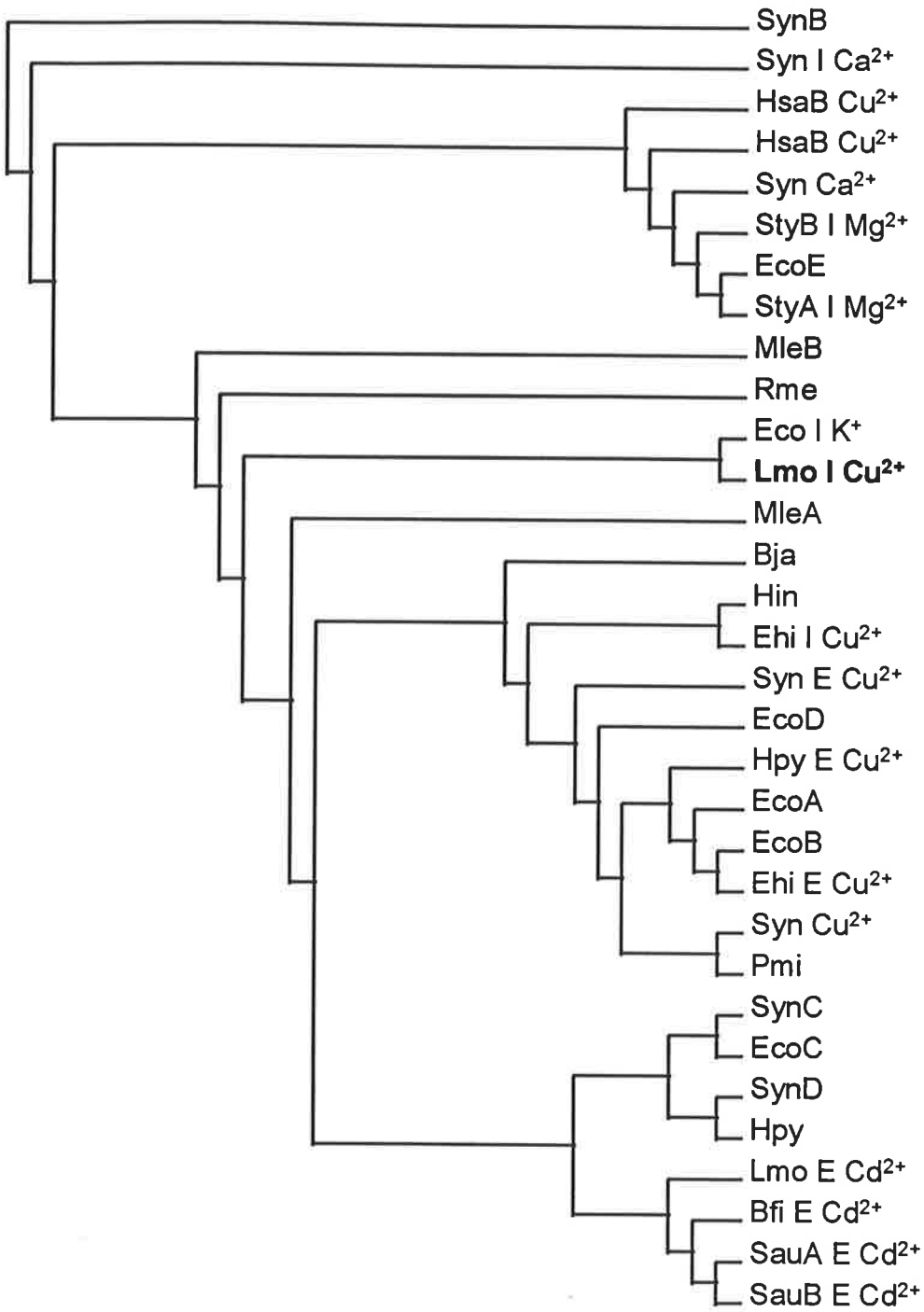

HsaB Cu2+ KTARAIATQVGIN----------KVFAGVLPSHKVAKVQELQ
HsaA Cu2+ KTARSIASQVGIT----------KVFAEVLPSHKVAKVKQLQ
Ehi I Cu2+ RAAQAIGKQVGIDSD----------HIFAEVLPEEKANYVEKLQ
Lmo I Cu2+ LVAQAIADDIGID----------EVIADVMPGDKAQHIRKLQ
Syn E Cu2+ RTADAIAQAVGIT----------QVLAEVRPDQKAAQVAQLQ
Pmi ATAKAIAKQLGID----------EIVAEVLPDGKVAALKQLS
Syn Cu2+ TTAVALAQQLGLESE----------TVVAEVLPEDKAAAIAALQ
SynA TTAVALAQQLGLESE----------TVVAEVLPEDKAAAIAALQ
MleA AAARAVAAQVGID----------TVIADMLPEAKVDVIQRLR
MleB ASAAAVASRIGID----------EVIADILPEDKVDVIEQLR
Ehi E Cu2+ KAAQAVAEYLGIN----------EYYGGLLPDDKEAIVQRYL
EcoA KAAQGAAEILGI----------DYLANQSPQDKYELVEKLK
EcoB QVAQAVADQLGIT----------EIHAQVSPQEKIALVKDYQ
SauB E Cd2+ GTANAIGTHVGV--S----------DIQSELMPQDKLDYIKKMQ
SauA E Cd2+ GTAEAIGAHVGV--S----------DIQSELLPQDKLDYIKKMK
Bfi E Cd2+ GTANAIGGQVGV--S----------DIEAELMPQDKLDFIKQLR
Lmo E Cd2+ ATAQAIGQQVGV--S----------EIEGELMPQDKLDYIKQLK
Hpy SATESIAQTLG---C----------EYYASLLPEE-KTSVFKTF
SynD IVAQSVAQQIGL--D----------AFVAELLPEEKVDEIEQLL
SynB LTAQAIALDLGIAEPGA-RVVTSRDLDNCSEKELAEIVHTVSVYARVSPEHKLKIVQTLR
Syn I Ca2+ LTAQAIARDLGITEVGH-PVLTGQQLSAMNGAELDAAVRSVEVYARVAPEHKLRIVESLQ
StyA I Mg2+ LVAAKVCHEVGLDAG---DVIIGSDIEGLSDDALAALAARTTLFARLTPMHKERIVTLLK
EcoE LVAAKVCHEVGLDAG---EVVIGSDIETLSDDELANLAQRTTLFARLTPMHKERIVTLLK
StyB I Mg2+ VVTARICLEVGIDTH---DILTGTQVEAMSDAELASEVEKRAVFARLTPLQKTRILQALQ
EcoD TTANAIAKEAGID----------EVIAGVLPDGKAEAIKHLQ
SynC RTAHSIAQQVGVN----------QVYAELLPEDKVDVIRQLQ
Hin SVVDYIAKQLGIK----------KAFGKLTPRDKAEQIQKLK
Hpy E Cu2+ ENVKKCALELGID----------GYISNAKPQDKLNKIKELK
Syn Ca2+ STAQAIAKRMGIAAEGDGIAFEGRQLATMGPAELAQAAEDSCVFARVAPAQKLQLVEALQ
EcoC RAAAAIAGELGL----------EFKAGLLPEDKVKAVTELN
Bja PAVKAAAHALAIP----------EWRAGVTPADKIARIEELK
Rme PVVAALASSLGIS----------NWYAELSPREKVQVCAAAA
Eco K+ LTAAAIAAEAGVD----------DFLAEATPEAKLALIRQYQ
*
Segment 2
HsaB Cu2+ NKGK--KVAMVGDGVNDSPALAQADMGVAIG-TGTDVAIEAADVVLIRNDLLDVVASIHL
HsaA Cu2+ EEGK--RVAMVGDGINDSPALAMANVGIAIG-TGTDVAIEAADVVLIRNDLLDVVASIDL
Ehi I Cu2+ KAGK--KVGMVGDGINDAPALRLADVGIAMG-SGTDIAMETADVTLMNSHLTSINQMISL
Lmo I Cu2+ EKG---AVAFVGDGINDAPALSTATVGIAMG-SGSDIAIESGGIVLVKNDLMDVVTSLVL
Syn E Cu2+ SRGQ--VVAMVGDGINDAPALAQADVGIAIG-TGTDVAIAASDITLISGDLQGIVTAIQL
Pmi QKGD--KVAFVGDGINDAPALAQADVGLAIG-TGTDVAIEAADVVLMSGDLRGVVDAIAL
Syn Cu2+ SQGD--AVAMIGDGINDAPALATAAVGISLA-AGSDIAQDSAGLLLSRDRLDSVLVAWNL
SynA SQGD--AVAMIGDGINDAPALATAAVGISLA-AGSDIAQDSAGLLLSRDRLDSVLVAWNL
MleA DQGH--TVAMVGDGINDGPALACADLGLAMG-RGTDVAIGAADLILVRDSLGVVPVALDL
MleB DRGH--VVAMVGDGINDGPALARADLGMAIG-RGTDVAIGAADIILVRDNLDVVPITLDL
Ehi E Cu2+ DQGK--KVIMVGDGINDAPSLARATIGMAIG-AGTDIAIDSADVVLTNSDPKDILHFLEL
EcoA AEGK--KVIMVGDGVNDAPSLALADVGIAIG-AGTQVALDSADIILTQYSPGDIASFIEL
EcoB KQGQ---VMMIGDGINDAPALAQADLSVAIG-AGTQVAQAAADTVLIANQLPTIIDFLKL
SauB E Cd2+ SEYD--NVAMIGDGVNDAPALAASTVGIAMGGAGTDTAIETADIALMGDDLSKLPFAVRL
SauA E Cd2+ AEHG--NVAMIGDGVNDAPALAASTVGIAMGGAGTDTAIETADIALMGDDLSKLPFAVRL
Bfi E Cd2+ SEYG--NVAMVGDGVNDAPALAASTVGIAMGGAGTDTALETADVALMGDDLRKLPSTVKL
Lmo E Cd2+ INFG--KVAMVGDGINDAPALAAATVGIAMGGAGTDTAIETADVALMGDDLQKLPFTVKL
Hpy KERYKAPAIFVGDGINDAPTLASADVGIGMG-KGSELSKQSADIVITNDSLNSLVKVLAI
SynD DPSGKAKLAFVGDGINDAPVIARADVGIAMGGLGSDAAIETADVVLMTDAPSKVAEAIHV
SynB KQHE--VVAMTGDGVNDAPALKQADIGVAMGITGTDVSKEASDMILLDDNFATIVSAVEE
Syn I Ca2+ RQGE--FVAMTGDGVNDAPALKQANIGVAMGITGTDVSKEASDMVLLDDNFATIVAAVEE
StyA I Mg2+ REGH--VVGFMGDGINDAPALRAADIGISVDG-AVDIAREAADIILLEKSLMVLEEGVIE
EcoE REGH--VVGFMGDGINDAPALRAADIGISVDG-AVDIAREAADIILLEKSLMVLEEGVIE
StyB I Mg2+ KNGH--TVGFLGDGINDAPALRDADVGISVDS-AADIRKESSDIILLEKDLMVLEEGVIK
EcoD SEGR--QVAMVGDGINDAPALAQADVGIAMG-GGSDVAIETAAITLMRHSLMGVADALAI
SynC KQYQ--SVAMVGDGINDAPALAQASVGIAMGAAGSDVALETADIVLMADRLERLEHAIRL
Hin DLGH--IVAMVGDGINDAPALASANVSFAMK-SSSDIAEQTASATLMQHSVNQLVDALFI
Hpy E Cu2+ EKGR--IVMMVGDGLNDAPSLAMSDVAVVMAK-GSDVSVQAADIVSFNNDIKSVYSAIKL
Syn Ca2+ EKGH--IVAMTGDGVNDAPALKRADIGIAMGKGGTEVARESSDMLLTDDNFASIEAAVEE
EcoC QHAP---LAMVGDGINDAPAMKAAAIGIAMG-SGTDVALETADAALTHNHLRGLVQMIEL
Bja RRGA--RVLMVGDGMNDAPSLAAAHVSMSPI-SAAHLSQATADLVFLGRPLAPVAAAIDS
Rme EAGH--KALVVGDGINDAPVLRAAHVSMAPA-TAADVGRQAADFVFMHERLSAVPFAIET
Eco K+ AEGR--LVAMTGDGTNDAPALAQADVAVAMN-SGTQAAKEAGNMVDLDSNPTKLIEVVHI
*** ** * . . . . .


Segment 3
HsaB Cu2+ SKRTVRRIRINLVLALIYNLV----------GIPIAAGVFMPIG-IVL--------
HsaA Cu2+ SRKTVKRIRINFVFALIYNLV----------GIPIAAGVFMPIG-LVL--------
Ehi I Cu2+ SAATLKKIKQNLFWAFIYNTI----------GIPFAAFGFL----N----------
Lmo I Cu2+ ARKTYSRILINLFWAFIYNVI----------GIPVAAGIFS-ALGFTL--------
Syn E Cu2+ SRATMTNIRQNLFFAFIYNVA----------GIPIAAGILYPLLGWLL--------
Pmi SQATIRNIKQNLFWTFAYNAL----------LIPVAAGMLYPINGMLL--------
Syn Cu2+ SQMGLRTIRQNLTWALGYNVV----------MLPLAAGAFLPAYGLAL--------
SynA SQMGLRTIRQNLTWALGYNVV----------MLPLAAGAFLPAYGLAL--------
MleA ARATMRTIRINMIWAFGYNVA----------AIPIASSGLL----------
MleB AAATMRTIKFNMVWAFGYNIA----------AIPIAAAGLL----------
Ehi E Cu2+ AKETRRKMIQNLWWGAGYNII----------AIPLAAGILAPIG-LIL--------
EcoA AQKTTRKMKENLVWGAGYNFI----------AIPIAAGILAPIG-ITL--------
EcoB AKRADRKQIENLWWGAGYNII----------ALPLAAGALATFG-IML--------
SauB E Cd2+ SRKTLNIIKANITFAIGIKII----------ALLLVIPGWL----------
SauA E Cd2+ SRKTLNIIKANITFAIGIKII----------ALLLVIPGWL----------
Bfi E Cd2+ SRKTLNIIKANITFAIAIKFI----------ASLLVIPGWL----------
Lmo E Cd2+ SRKTLQIIKQNITFSLVIKLI----------ALLLVIPGWL----------
Hpy AKKTKSIIWQNILFALGIKAV----------FIVLGLMGVA----------
SynD ARKTRQIVVQNIVLALGIKAL----------FIALGTIGLA----------
SynB GRVVYTNIRRFIRYILGSNIGEVLTIAAAPLMGLGGVPLSPLQILWMNLVTDGVPALALA
Syn I Ca2+ GRIVYGNIRKFIKYILGSNIGELLTIASAPLLGLGAVPLTPLQILWMNLVTDGIPALALA
StyA I Mg2+ GRRTFSNMLKYIKMTASSNFGNVFSVLVASAF-LPFLPMLPLHLLIQNLLYD-VSQVAIP
EcoE GRRTFANMLKYIKMTASSNFGNVFSVLVASAF-LPFLPMLPLHLLIQNLLYD-VSQVAIP
StyB I Mg2+ GRETFGNIIKYLNMTASSNFVNVFSVLVRSAF-IPFLPMLAIHLLIQNLMYD-ISQLSLP
EcoD SRATLHNMKQNLLGAFIYNSI----------G----IPVAAGILWPFTGTLL--------
SynC GRRAQGVVKQNIVFALGFVMIL----------LIANFAGNIT----------
Hin ARATLKNIKQNLFFALIYNIL----------G----IPLAAFGFL----------
Hpy E Cu2+ SQATIKNIKENLFWAFCYNSV----------FIPLACGVLYKANIMLS--------
Syn Ca2+ GRTVYQNLRKAIAFLLPVNGGES-MTILISVLLALNLPILSLQVLWLNMINSITMTVPLA
EcoC ARATHANIRQNITIALGLKGI----------FLVTTLLGMT----------
Bja ARKALHLMRQNLWLAIGYNVL----------AVPVAISGVV----------
Rme SRHAGQLIRQNFALAIGYNVI----------AVPIAILGYA----------
Eco K+ GKQMLMTRGSLTTFSIANDVAK----Y------FAIIPAAFAATYPQLNALN--------
HsaB Cu2+ ----------QPWMGSAAMAASSVSVVLSSLQLKCYKKPDLERYEA---
HsaA Cu2+ ----------QPWMGSAAMAASSVSVVLSSLFLKLYRKPTYESYEL---
Ehi I Cu2+ ----------PIIAGGAMAFSSISVLLNSLSLNRKTIK----------
Lmo I Cu2+ ----------SPELAGLAMALSSITVVLSSLLLNYVRLPKSSETLI---
Syn E Cu2+ ----------SPMLAGAAMAFSSVSVVTNALRLRQFQPR----------
Pmi ----------SPIFAAAAMALSSVFVLGNALRLKRFQAPMKTH------
Syn Cu2+ ----------TPAIAGACMAVSSLAVVSNSLLLRYWFRRSLNHSVS---
SynA ----------TPAIAGACMAVSSLAVVSNSLLLRYWFRRSLNHSVS---
MleA ----------NPLIAGAAMAFSSFFVVSNSLRLSN-FGLSQTSD-----
MleB ----------NPLVAGAAMAFSSFFVVSNSLRLRN-FGAILSCGTS---
Ehi E Cu2+ ----------SPAVGAVLMSLSTVVVALNALTLK----------
EcoA ----------SPAVAAVLMSLSTVIVAINAMTLKLEPK----------
EcoB ----------NPMIGAILMSLSTVIVALNAMTRKA----------
SauB E Cd2+ ----------TLWIAILSDMGATILVALNSLRLMRVKDK----------
SauA E Cd2+ ----------TLWIAILSDMGATILVALNSLRLMRVKDK----------
Bfi E Cd2+ ----------TLWIAILSDMGATLLVALNGLRLMRVKE----------
Lmo E Cd2+ ----------TLWIAIMADMGATLLVTLNGLRLMKVKD----------
Hpy ----------SLWEAVFGDVGVTLLALANSMRAMRA----------
SynD ----------TLWEAVFADVGVALLAILNATRIAK----------
SynB VEPGKATVMQQSPKDPQESIFARGLGSYMVRQGLILAIVTIVLMVWAYNYTPNHLEGGLS
Syn I Ca2+ VEPGDPTIMQRRPHNPQESIFARGLGTYMLRVGVVFSAFTIVLMVIAYQYTQVPLPG-LD
StyA I Mg2+ FDNVDEEQIQK----PQR-WNPADLGRFMVFFGPISSIFDILTFCLMWWVFHANTPE--T
EcoE FDNVDDEQIQK----PQR-WNPADLGRFMIFFGPISSIFDILTFCLMWWVFHANTPE--T
StyB I Mg2+ WDKMDKEFLRK----PRK-WDAKNIVRFMLWIGPTSSIFDITTFALMWYVFAANNVE--A
EcoD ----------NPVVAGAAMALSSITVVSNANRLLRFKPKE----------
SynC ----------LPFGVLGHEGSTVIVTLSGLRLLRG----------
Hin ----------SPIIAGAAMALSSISVLMNALRLKKVRF----------
Hpy E Cu2+ ----------PAIAGLAMSLSSVSVVLNSQRLRNFKIKDH--------
Syn Ca2+ FEAKSPGIMQQAP----RNPNEPLITKKLLHRILLVSLFNWILIFGMFEWVNRTYDDLAL
EcoC ----------GLWLAVLADTGATVLVTANALRLLRRR----------
Bja ----------TPLIAAAAMSGSSILVMLNSLRARSDSREIV--------
Rme ----------TPLVAAVAMSSSSLVVVFNALRLKRSLAAGRGATPGTLI
Eco K+ ----------IMCLHSPDSAILSAVIFNALIIVFLIPLALKGVSYKPLTASAML



The approximate location of each amino acid segment division used in examination of evolutionary relatedness is indicated by an arrow. Identical amino acids are indicated by an asterisk and similar amino acids by a dot.

Appendix B



Unrooted parsimonious phylogenetic tree derived from Segment 3 amino acid sequences from bacterial P-type ATPases. The putative Cu^{2+} P-type ATPases associated with Human Menkes and Wilson disease were included for comparison given their high degree of identity to CtpA over a 300 amino acid overlap (in the order of 40% identity, data not shown). Abbreviations are as indicated in Table 5.7. The distances are considered to reflect actual evolutionary distances in arbitrary units. The CtpA ATPase identified in this study is highlighted in bold typeface.

Appendix C

Prediction of transmembrane segments for CtpA amino acid sequence using secondary structure prediction algorithms

α-Helix domain	SSP	SOPMA	nnPredict	SSPRED	TMpred	PHDsec
1	NP	NP	NP	Met_1-Phe_{16} (16)	NP	NP
2	NP	NP	NP	NP	Phe_7-Gly_{32} (26)	NP
3	NP	NP	NP	NP	Tyr_{35}-Leu_{53} (19)	Ile_{39}-Phe_{55} (17)
4	NP	Leu_{56}-Asp_{71} (16)	NP	NP	NP	NP
5	NP	NP	NP	NP	His_{67}-Ala_{91} (25)	Gly_{75}-Tyr_{87} (13)
6	NP	NP	Tyr_{84}-Ile_{105} (22)	NP	NP	NP
7	NP	NP	NP	NP	Thr_{96}-Glu_{115} (20)	Phe_{98}-Gly_{111} (14)
8	Phe_{114}-Asp_{142} (23)	Tyr_{113}-Arg_{141} (29)	Phe_{114}-Leu_{140} (27)	Met_{118}-Glu_{138} (21)	NP	NP
9	NP	NP	NP	Gly_{171}-Val_{185} (15)	NP	NP
10	Ala_{220}-Asn_{250} (31)	Ala_{220}-Ile_{248} (29)	NP	NP	NP	NP
11	NP	NP	NP	NP	Ile_{248}-Leu_{271} (24)	Ile_{251}-Gly_{272} (22)
12	NP	NP	NP	NP	Ile_{261}-Ala_{289} (29)	NP
13	NP	NP	NP	NP	NP	Ile_{280}-Gly_{307} (28)
14	NP	NP	Leu_{312}-Lys_{329} (18)	Gly_{305}-Lys_{329} (25)	NP	NP
15	Glu_{357}-Lys_{384} (28)	NP	Glu_{357}-Glu_{382} (26)	Ile_{360}-Thr_{389} (30)	NP	NP
16	NP	Phe_{374}-Asn_{395} (22)	NP	NP	NP	NP
17	Ala_{392}-Ile_{408} (18)	NP	NP	NP	NP	NP
18	NP	Asp_{423}-Lys_{442} (20)	NP	NP	NP	NP
19	Gly_{486}-Met_{509} (24)	NP	NP	NP	NP	NP
20	NP	NP	NP	Ser_{540}-Val_{564} (25)	Ala_{536}-Gly_{559} (24)	NP
21	NP	Met_{569}-Val_{597} (29)	Leu_{568}-Phe_{593} (25)	NP	NP	NP
22	NP	NP	NP	NP	Ile_{585}-Phe_{607} (23)	Leu_{589}-Phe_{607} (19)
23	Val_{602}-Ala_{624} (23)	Ala_{603}-Leu_{625} (23)	NP	NP	NP	NP
24	NP	NP	Glu_{617}-Val_{640} (24)	NP	Leu_{621}-Val_{640} (23)	Ser_{615}-Ser_{633} (19)

NP indicates segments not predicted by algorithms. Numbers in parentheses indicates the length of each segment. PHDsec (Rost and Sander, 1993; Rost and Sander, 1994), TMpred (Hofmann K. and Stoffel W., Swiss Institute for Experimental Cancer Research, Lausanne, Switzerland), SSP (Solovyev and Salamov, 1994), SOPMA (Geourjon and Deleage, 1994), nnPredict (Kneller *et al.*, 1990), SSPRED (Mehta *et al.*, 1995).

Chapter 8 Literature cited

Adams T.J., Vartivarian S., and R.E. Cowart. (1990). Iron acquisition systems of *Listeria monocytogenes. Infect. Immun.* **58:**2715-2718.

Adhikari P., Kirby S.D., Nowalk A.J., Veraldi K.L., Schryvers A.B., and T.A. Mietzner. (1995). Biochemical characterization of a *Haemophilus influenzae* periplasmic iron transport operon. *J. Biol. Chem.* **270:**25142-25149.

Aggarwal S., Kumar A., Jaffe R., Hone D., Gross M., and J. Sadoff. (1990). Oral *Salmonella*: malaria circumsporocoite recombinants induce specific CD8 cytotoxic T cells. *J. Exp. Med.* **172:**1083-1090.

Aiba H., Adhya S., and B. de-Crombrugghe. (1981). Evidence for two functional *gal* promoters in intact *Escherichia coli* cells. *J. Biol. Chem.* **256:**11905-11910

Aldovini A., and R.A. Young. (1991). Humoral and cell-mediated immune resposes to live recombinant BCG-HIV vaccines. *Nature* **351:**479-482.

Alexander J.E., Andrew P.W., Jones D., and I.S. Roberts. (1990). Development of an optimized system for electroporation of *Listeria* species. *Lett. Appl. Microbiol.* **10:**179-181.

Alexander J.E., Andrew P.W., Jones D., and I.S. Roberts. (1993). Characterisation of an aromatic amino acid-dependent *Listeria monocytogenes* mutant: attenuation, persistence, and ability to induce protective immunity in mice. *Infect. Immun.* **61:**2245-2248.

Allaoui, A., Mounier J., Prevost C., Sansonetti P.J., and C. Parsot. (1992). *icsB*: a *Shigella flexneri* virulence gene necessary for the lysis of protrusions during intracellular spread. *Mol. Microbiol.* **6:**1605-1616.

Alouf J.E. (1980). Streptococcal toxins (streptolysin O, streptolysin S, erythrogenic toxins). *Pharmacol. Ther.* **11:**661-717.

Alouf J.E., and C. Geoffroy. (1984). Structure activity relationships in sulphydryl-activated toxins. In *Bacterial protein toxins.* (J.E. Alouf, F.J. Fehrenbach, J.H. Freer, and J. Jeljaszewicz, eds.). Academic Press, Inc., London, pp. 165-171.

An L.-L., Pamer E., and J.L. Whitton. (1996). A recombinant minigene vaccine containing a nonameric cytotoxic-T-lymphocyte epitope confers limited protection against *L. monocytogenes* infection. *Infect. Immun.* **64:**1685-1693.

Anderson J.R., Cossart P., Sheehan B., and B.W. Wren. (1995a). Characterization of a two-component regulatory system which may control potassium transport in *L. monocytogenes.* In *Proceedings of the 12th International Symposium on Problems of Listeriosis.* 2-6 October, Perth, Australia, pp. 204.

Anderson J.R., Stabler R., and B.W. Wren. (1995b). A strategy for constructing defined *Listeria monocytogenes* mutants. In *Proceedings of the 12th International Symposium on Problems of Listeriosis.* 2-6 October, Perth, Australia, pp. 457-462.

Ang D., Liberek K., Skowyra D., Zylicz M., and C. Georgeopolous. (1991). Biological role and regulation of the universially conserved heat shock proteins. *J. Biol. Chem.* **266:**24233-24236.

Angerer A., Klupp B., and V. Braun. (1992). Iron transport systems of *Serratia marcescens. J. Bacteriol.* **174:**1378-1387.

Aranha H., Evans S.L., Arcebeaux J.E.L., and B.R. Byers. (1986). Calcium modulation of growth of *Streptococcus mutans*. *J. Gen. Microbiol.* **132:**2661-2663.

Armstrong B.A., and C.P. Sword. (1966). Electron microscopy of *Listeria monocytogenes*-infected mouse spleen. *J. Bacteriol.* **91:**1346-1355.

Arroyo J., Hurley M.C., Wolf M., McClain M.S., Eisenstein B.I., and N.C. Engleberg. (1994). Shuttle mutagenesis of *Legionella pnuemophila*: identification of a gene associated with host cell cytopathicity. *Infect. Immun.* **62:**4075-4080.

Atkinson M.R., and S.H. Fisher. (1991). Identification of genes and gene products whose expression is activated during nitrogen-limited growth in *Bacillus subtilis*. *J. Bacteriol.* **173:**23-27.

Atkinson M.R., Wray L.V. Jr, and S.H. Fisher. (1990). Regulation of histidine and proline degradation enzymes by amino acid availability in *Bacillus subtilis*. *J. Bacteriol.* **172:**4758-4765.

Audurier A., and C. Martin. (1989). Phage typing of *Listeria monocytogenes*. *Int. J. Food Microbiol.* **8:**251-257.

Audurier A., Pardon P., Marly J., and F. Lautier. (1980). Experimental infection of mice with *Listeria monocytogenes* and *Listeria innocua*. *Ann. Inst. Pasteur Microbiol.* **131B:**47-57.

Audurier A., Pardon P., Marly J., Lautier F., and J. Loulergue. (1981). Mesure de la virulence chez la souris de différentes bactéries appartenant au genre *Listeria*. *Ann. Inst. Pasteur Immunol.* **132:**191-200.

Babior B.M., Kipnes R.S., and J.T. Curnutte. (1973). The production by leukocytes of superoxide, a potential bacteriocidal agent. *J. Clin. Invest.* **52:**741-744.

Bach M.C., and K.M. Davis. (1987). *Listeria* rhombencephalitis mimicking tuberculous meningitis. *Rev. Infect. Dis.* **9:**130-133.

Balke V.L., and J.D. Gralla. (1987). Changes in the linking number of supercoiled DNA accompany growth transitions in *Escherichia coli*. *J. Bacteriol.* **169:**4499-4506.

Barclay R., Threlfall D.R., and I. Leighton. (1989). Haemolysins and extracellular enzymes of *Listeria monocytogenes* and *L. ivanovii*. *J. Med. Microbiol.* **30:**111-118.

Barry R.A., Bouwer H.G.A., Portnoy D.A., and D.J. Hinrichs. (1992). Pathogenicity and immunogenicity of *Listeria monocytogenes* small-plaque mutants defective for intracellular growth and cell-to-cell spread. *Infect. Immun.* **60:**1625-1632.

Barve S.S., and S.C. Straley. (1990). *lcrR*, a low-Ca^{2+} response locus with dual Ca^{2+}-dependent functions in *Yersinia pestis*. *J. Bacteriol.* **172:**4661-4671.

Beaman L., and B.L. Beaman. (1984). The role of oxygen and its derivatives in microbial pathogenesis and host defense. *Annu. Rev. Microbiol.* **38:**27-48.

Beisel W.R. (1977). Magnitude of the host nutritional responses to infection. *Am. J. Clin. Nutr.* **30:**1236-1247.

Bellinger-Kawahara K.C., and M.A. Horwitz. (1990). Complement component C3 fixes selectively to the major outer membrane protein (MOMP) of *Legionella pneumophila* and mediates phagocytosis of liposome-MOMP complexes by human monocytes. *J. Exp. Med.* **172:**1201-1210.

Berche P. (1995). Bacteremia is required for invasion of the murine central nervous system by *Listeria monocytogenes. Microb. Pathog.* **18:**323-336.

Berche P., Gaillard J.-L., Geoffroy C., and J.E. Alouf. (1987a). T cell recognition of listeriolysin O is induced during infection with *Listeria monocytogenes. J. Immunol.* **139:**3813-3821.

Berche P., Gaillard J.-L., and P.J. Sansonetti. (1987b). Intracellular growth of *Listeria monocytogenes* as a prerequisite for an *in vivo* induction of T cell-mediated immunity. *J. Immunol.* **138:**2266-2271.

Bernardini M., Mounier J., d'Hauteville H., Coquis-Rondon M., and P.J. Sansonetti. (1989). Identification of *icsA,* a plasmid locus of *Shigella flexneri* that governs bacterial intra and intercellular spread through interaction with F-actin. *Proc. Natl. Acad. Sci.* USA **86:**3867-3871.

Bernheimer A.W. (1974). Interaction between membranes and cytolytic bacterial toxins. *Biochim. Biophys. Acta* **344:**27-50.

Bernheimer A.W., and B. Rudy. (1986). Interactions between membranes and cytolytic peptides. *Biochim. Biophys. Acta* **864:**123-141.

Bessesen M.T., Luo Q., Rotbart H.A., Blaser M.J., and R.T. Ellison III. (1990). Detection of *Listeria monocytogenes* by using the polymerase chain reaction. *Appl. Environ. Microbiol.* **56:**2930-2932.

Bever R.A., and B.H. Iglewski. (1988). Molecular characterization and nucleotide sequence of the *Pseudomonas aeruginosa* elastase structural gene. *J. Bacteriol.* **170:**4309-4314.

Bewsey K.E., Johnson M.E., and P. Huff. (1991). Rapid isolation and purification of DNA from agarose gels: the phenol-freeze-fracture method. *BioTechniques* **10:**724-725.

Bhakdi S., and J. Tranum-Jenson. (1986). Membrane damage by pore-forming bacterial cytolysins. *Microb. Pathog.* **1:**5-14.

Bhakdi S., Tranum-Jensen J., and A. Sziegoleit. (1985). Mechanism of membrane damage by streptolysin O. *Infect. Immun.* **47:**52-60.

Bhunia A.K., Steele P.J., Westbrook D.G., Bly L.A., Maloney T.P., and M.G. Johnson. (1994). A six-hour *in vitro* virulence assay for *Listeria monocytogenes* using myeloma and hybridoma cells from murine and human sources. *Microb. Pathog.* **16:**99-110.

Bibb W.F., Schwartz B., Gellin B.G., Plikaytis B.D., and R.E. Weaver. (1989). Analysis of *Listeria monocytogenes* by multilocus enzyme electrophoresis and application of the method to epidemiologic investigations. *Int. J. Food Microbiol.* **8:**233-239.

Bibb W.F., Gellin B.G., Weaver R.E., Schwartz B., Plikaytis B.D., Reeves M.W., Pinner R.W., and C.V. Broome. (1990). Analysis of foodborne and clinical isolates of *Listeria monocytogenes* in United States by multilocus enzyme electrophoreis and application of the method to epidemiologic investigations. *Appl. Environ. Microbiol.* **56:**2133-2141.

Bielecki J., Youngman P., Connelly P., and D.A. Portnoy. (1990). *Bacillus subtilis* expressing a haemolysin gene from *Listeria monocytogenes* can grow in mammalian cells. *Nature* **345:**175-176

Bille J., and M.P. Doyle. (1991). *Listeria* and *Erysipelothrix.* In *Manual of Clinical Microbiology.* (E.H. Lennette, A. Balows, W.J. Hausler Jr, and H.J., Shadomy, eds.), American Society for Microbiology, Washington, DC., pp. 287-295.

Bischoff D.S., and G.W. Ordal. (1991). Sequence and characterization of *Bacillus subtilis* CheB, a homolog of *Escherichia coli* CheY, and its role in a different mechanism of chemotaxis. *J. Biol. Chem.* **266:**12301-12305.

Black W.J., Quinn F.D., and L.S. Tompkins. (1990). *Legionella pneumophila* zinc metalloprotease is structurally and functionally homologous to *Pseudomonas aeruginosa* elastase. *J. Bacteriol.* **172:**2608-2613.

Bohne J., Sokolovic Z., and W. Goebel. (1994). Transcriptional regulation of *prfA* and PrfA-regulated virulence genes in *Listeria monocytogenes. Mol. Microbiol.* **11:**1141-1150.

Bortolussi R., Schlech III W.F., and W.L. Albritton. (1985). *Listeria.* In *Manual of Clinical Microbiology* 4th ed. (E.H. Lennette, A. Balows, W.J. Hausler Jr, and H.J. Shadomy, eds.), American Society of Microbiology, Washington, DC., pp. 205-208.

Boulnois G.J., and Mitchell T.J., and P.W. Andrew. (1991). Structure and function of pneumolysin, the multifunctional, thiol-activated toxin of *Streptococcus pneumoniae. Mol. Microbiol.* **5:**2611-2616.

Bouwer H.G.A., Nelson C.S., Gibbins B.L., Portnoy D.A., and D.J. Hinrichs. (1992). Listeriolysin O is a target of the immune response to *Listeria monocytogenes. J. Exp. Med.* **175:**1467-1471.

Bradbeer C., Reynolds P.R., Bauler G.M., and M.T. Fernandez. (1986). A requirement for calcium in the transport of cobalamin across the outer membrane of *Escherichia coli. J. Biol. Chem.* **261:**2520-2523.

Branden C., and J. Tooze. (1991). Membrane proteins. In *Introduction to protein structure.* Garland Publishing, Inc., New York, pp. 201-216.

Brandl C.J., and C.M. Deber. (1986). Hypothesis about the function of membrane-buried proline residues in transport proteins. *Proc. Natl. Acad. Sci.* USA **83:**917-921.

Brandl C.J., Green N.M., Korczak B., and D.H. MacLennan. (1986). Two Ca^{2+} ATPase genes: homologies and mechanistic implications of deduced amino acid sequences. *Cell* **44:**597-607.

Brehm K., Haas A., Goebel W., and J. Kreft. (1992). A gene encoding a superoxide dismutase of the facultative intracellular bacterium *Listeria monocytogenes. Gene* **118:**121-125.

Brosch R., Buchrieser C., and J. Rocourt. (1991). Subtyping of *Listeria monocytogenes* serotype 4b by use of low-frequency-cleavage restriction endonucleases and pulse-field gel electrophoresis. *Res. Microbiol.* **142:**667-675.

Brosch R., Chen J., and J.B. Luchansky. (1994). Pulsed field fingerprinting of *listeriae*: identification of genomic divisions for *Listeria monocytogenes* and their correlation with serovar. *Appl. Environ. Microbiol.* **60:**2584-2592.

Brown N.L., Rouch D.A., and B.T.O. Lee. (1992). Copper resistance determinants in bacteria. *Plasmid* **27:**41-51.

Brown N.L., Lee B.T.O., and S. Silver. (1994). In *Metal ions in biological systems,* (H. Sigel, and A. Sigel, eds.), Dekker, New York, pp. 405-434.

Brubaker R.R. (1983). The Vwa^{+} virulence factor of *Yersiniae*: the molecular basis of the attendant nutritional requirement for Ca^{2+}. *Rev. Infect. Dis.* **5:**S748-S758.

Bruce J.L., Hubner R.J., Cole E.M., McDowell C.I., and J.A. Webster. (1995). Sets of *Eco*R1 fragments containing ribosomal RNA sequences are conserved among different strains of *Listeria monocytogenes. Proc. Natl. Acad. Sci.* USA **92:**5229-5233.

Bruchez N., and J.-L. Cordier. (1995). Detection of *Listeria monocytogenes* using immunomagnetic beads (Vicam-Listertest). In *Proceedings of the 12th International Symposium on Problems of Listeriosis,* 2-6 October, Perth, Australia, pp. 251-252.

Brundage R.A., Smith G.A., Camilli A., Theriot J.A., and D.A. Portnoy. (1993). Expression and phosphorylation of the *Listeria monocytogenes* ActA protein in mammalian cells. *Proc. Natl. Acad. Sci.* USA **90:**11890-11894.

Brunt L.M., Portnoy D.A., and E.R. Unanue. (1990). Presentation of *Listeria monocytogenes* to $CD8^+$ T cells requires secretion of hemolysin and intracellular bacterial growth. *J. Immunol.* **145:**3540-3546.

Bubert A., Köhler S., and W. Goebel. (1992a). The homologous and heterologous regions within the *iap* gene allow genus- and species-specific identification of *Listeria* spp. by polymerase chain reaction. *Appl. Environ. Microbiol.* **58:**2625-2632.

Bubert A., Kuhn M., Goebel W., and S. Köhler. (1992b). Structural and functional properties of the p60 proteins from different *Listeria* species. *J. Bacteriol.* **174:**8166-8171.

Bubert A., Schubert P., Köhler S., and W. Goebel. (1995). Specific immunological detection of all *Listeria monocytogenes* serotypes using antibodies raised against p60-derived synthetic peptides. In *Proceedings of the 12th International Symposium on Problems of Listeriosis.* 2-6 October, Perth, Australia, pp. 65-70.

Bull P.C., and D.W. Cox. (1994). Wilson disease and Menkes disease: new handles on heavy-metal transport. *Trends Genet.* **10:**246-252.

Bull P.C., Thomas G.R., Rummens J.M., Forbes J.R., and D.W. Cox. (1993). The Wilson disease gene is a putative copper transporting P-type ATPase similar to the Menkes gene. *Nature Genet.* **5:**327-337.

Butman B.T., Plank M.C., Durham R.J., and J.A. Mattingly. (1988). Monoclonal antibodies which identify a genus-specific *Listeria* antigen. *Appl. Environ. Microbiol.* **54:**1564-1569.

Buysse J.M., Stover C.K., Oaks E.V., Venkatesan M., and D.J. Kopecko. (1987). Molecular cloning of invasion plasmid antigen (*ipa*) genes from *Shigella flexneri*: analysis of *ipa* gene products and genetic mapping. *J. Bacteriol.* **169:**2561-2569.

Calderwood S.B., and J.J. Mekalanos. (1987). Iron regulation of Shiga-like toxin expression in *Escherichia coli* is mediated by the *fur* locus. *J. Bacteriol.* **169:**4759-4764.

Calmette A., Guérin C., Negre L., and A. Boquet. (1927). Sur la vaccination prevente des enfants nouveau-nés contre la tuberculose par le BCG. *Ann. Inst. Pasteur* **3:**201.

Camilli A., Paynton C.R., and D.A. Portnoy. (1989). Intracellular methicillin selection of *Listeria monocytogenes* mutants unable to replicate in a macrophage cell line. *Proc. Natl. Acad. Sci.* USA **86:**5522-5526.

Camilli A., Portnoy D.A., and P. Youngman. (1990). Insertional mutagenesis of *Listeria monocytogenes* with a novel Tn*917* derivative that allows direct cloning of DNA flanking transposon insertions. *J. Bacteriol.* **172:**3738-3744.

Camilli A., Goldfine H., and D.A. Portnoy. (1991). *Listeria monocytogenes* mutants lacking phosphatidylinositol-specific phospholipase C are avirulent. *J. Exp. Med.* **173:**751-754.

Camilli A., Tilney L.G., and D.A. Portnoy. (1993). Dual roles of *plcA* in *Listeria monocytogenes* pathogenesis. *Mol. Microbiol.* **8:**143-157.

Campbell A., Berg D.E., Botstein D., Lederberg E.M., Novick R.P., Starlinger P., and W. Szybalski. (1979). Nomenclature of transposable elements in prokaryotes. *Gene* **5:**197-206.

Cardoso de Almeida M.L., and M.J. Turner. (1983). The membrane form variant surface glycoprotein of *T. brucei. Nature* **302:**349-352.

Carvajal A., and W. Frederiksen. (1988). Fatal endocarditis due to *Listeria monocytogenes. Rev. Infect. Dis.* **10:**616-623.

Chakraborty T., and W. Goebel. (1988). Recent developments in the study of virulence in *Listeria monocytogenes. Curr. Topics Microbiol. Immunol.* **138:**41-58.

Chakraborty T., Leimeister-Wächter M., Domann E., Hartl M., Goebel W., Nichterlein T., and S. Notermans. (1992). Coordinate regulation of virulence genes in *Listeria monocytogenes* requires the product of the *prfA* gene. *J. Bacteriol.* **174:**568-574.

Chakraborty T., Domann E., Ebel F., Hain T., Pistor S., Gerstel B., Niebuhr K., Lingnau A., and J. Wehland. (1995). Invasion and intracellular motility: two fundamental properties of pathogenic *Listeria.* In *Proceedings of the 12th International Symposium on Problems of Listeriosis.* 2-6 October, Perth, Australia, pp. 495-499.

Chelly J., Tümer Z., Tonnesen T., Petterson A., Ishikava-Brush Y., Tommerrup N., Horn N., and A.P. Monaco. (1993). Isolation of a candidate gene for Menkes disease that encodes a potential heavy metal binding protein. *Nature Genet.* **3:**14-19.

Chen J., Brosch R., and J.B. Luchansky. (1993). Isolation and characterization of *Listeria monocytogenes*-specific nucleotide sequences. *Appl. Environ. Microbiol.* **59:**4367-4370.

Chenevert J., Mengaud J., Gormley E., and P. Cossart. (1989). A DNA probe specific for *L. monocytogenes* in the genus *Listeria. Int. J. Food. Microbiol.* **8:**317-319.

Cheo D.L., Bayles K.W., and R.E. Yasbin. (1991). Cloning and characterization of DNA damage-inducible promoter regions from *Bacillus subtilis. J. Bacteriol.* **173:**1696-1703.

Christensen D.P., and R.W. Hutkins. (1992). Collapse of the proton motive force in *Listeria monocytogenes* caused by a bacteriocin produced by *Pediococcus acidilactici. Appl. Environ. Microbiol.* **58:**3312-3315.

Clewell D.B. (1981). Plasmids, drug resistance, and gene transfer in the genus *Streptococcus. Microbiol. Rev.* **45:**409-436.

Conlan J.W., and R.J. North. (1992). Roles of *Listeria monocytogenes* virulence factors in survival: virulence factors distinct from listeriolysin are needed for the organism to survive an early neutrophil-mediated host defense mechanism. *Infect. Immun.* **60:**951-957.

Corbisier P., Ji G., Nuyts G., Mergeay M., and S. Silver. (1993). *luxAB* gene fusions with the arsenic and cadmium resistance operons of *Staphylococcus aureus* plasmid pI258. FEMS *Microbiol. Letts.* **110:**231-238.

Cossart P., and J. Mengaud. (1989). *Listeria monocytogenes*: a model system for the molecular study of intracellular parasitism. *Mol. Biol. Med.* **6**:463-474.

Cossart P., Vicente M.F., Mengaud J., Baquero F., Perez-Diaz J.C., and P. Berche. (1989). Listeriolysin O is essential for virulence of *Listeria monocytogenes*: direct evidence obtained by gene complementation. *Infect. Immun.* **57**:3629-3636.

Costa G.L., and M.P. Weiner. (1994). Polishing with T4 or *Pfu* polymerase increases the efficiency of cloning of PCR fragments. *Nucl. Acid Res.* **22**:2423.

Cowart R.E. (1987). Iron regulation of growth and haemolysin production by *Listeria monocytogenes*. *Ann. Inst. Pasteur Microbiol.* **138**:246-249.

Cowart R.E., and B.G. Foster. (1981). The role of iron in the production of hemolysin by *Listeria monocytogenes*. *Curr. Microbiol.* **6**:287-290.

Cowart R.E., and B.G. Foster. (1985). Differential effects of iron on the growth of *Listeria monocytogenes*: minimum requirements and mechanism of acquistion. *J. Infect. Dis.* **151**:721-730.

Cozzarelli N.R. (1980). DNA topoisomerases. *Cell* **22**:327-328.

Crichton R.R. and M. Charloteaux-Wauters. (1987). Iron transport and storage. *Eur. J. Biochem.* **164**:485-506.

Crocker A., Lee C., Aboko-Cole G., and C. Durham. (1992). Interaction of nutrition and infection: effect of copper deficiency on resistance to *Trypanosoma lewisi*. *J. Natl. Med. Assoc.* **84**:697-706.

Crosa J.H. (1989). Genetics and molecular biology of siderophore-mediated iron transport in bacteria. *Microbiol. Rev.* **53**:517-530.

Czuprynski C.J., Brown J.F., and J.T. Roll. (1989). Growth at reduced temperatures increase the virulence of *Listeria monocytogenes* for intravenously but not intragastrically inoculated mice. *Microb. Pathog.* **7**:213-223.

Dabiri G.A., Sanger J.M., Portnoy D.A., and F.S. Southwick. (1990). *Listeria monocytogenes* moves rapidly through the host-cell cytoplasm by inducing directional actin assembly. *Proc. Natl. Acad. Sci.* USA **87**:6068-6072.

Dai J.-H., Lee Y.-S., and H.-C. Wong. (1992). Effects of iron limitation on production of a siderophore, outer membrane proteins, and hemolysin and on hydrophobicity, cell adherance, and lethality for mice of *Vibrio parahaemolyticus*. *Infect. Immun.* **60**:2952-2956.

Dallmier A.W., and S.E. Martin. (1988). Catalase and superoxide dismutase activities after heat injury of *Listeria monocytogenes*. *Appl. Environ. Microbiol.* **54**:581-582.

Dallmier A.W., and S.E. Martin. (1990). Catalase, superoxide dismutase, and hemolysin activities and heat susceptibility of *Listeria monocytogenes* after growth in media containing sodium chloride. *Appl. Environ. Microbiol.* **56**:2807-2810.

Danks D.M. (1989). In *The metabolic basis of inherited disease* (C.R. Scriver, A.L. Beaudet, W.S. Sly, and D. Valle, eds.), McGraw-Hill, pp. 1411-1431.

Darwish H.M., Hoke J.E., and M.J. Ettinger. (1983). Kinetics of Cu^{2+} transport and accumulation by hepatocytes from copper-deficient mice and the brindled mouse model of Menkes disease. *J. Biol. Chem.* **258**:13621-13626.

Datta A.R., and M.A. Benjamin. (1995). Advances in nucleic acid-based techniques for detection of *Listeria monocytogenes.* In *Proceedings of the 12th International Symposium on Problems of Listeriosis.* 2-6 October, Perth, Australia, pp. 71-83.

Datta A.R., Wentz B.A., and W.E. Hill. (1987). Detection of hemolytic *Listeria monocytogenes* by using DNA colony hybridization. *Appl. Environ. Microbiol.* **53:**2256-2259.

Datta A.R., Wentz B.A., Shook D., and M.W. Trucksess. (1988). Synthetic oligodeooxyribonucleotide probes for detection of *Listeria monocytogenes. Appl. Environ. Microbiol.* **54:**2933-2937.

Datta A.R., Wentz B.A., and J. Russell. (1990). Cloning of the listeriolysin O gene and development of specific gene probes for *Listeria monocytogenes. Appl. Environ. Microbiol.* **56:**3874-3877.

Dawson R.M.C., Elliot D.C., Elliot W.H., and K.M. Jones. (1986). Data for biochemical research, 3rd edn. Oxford University Press, New York.

Dayhoff M.O., Barker W.C., and L.Y. Hunt. (1983). Establishing homologies in protein sequences. *Methods Enzymol.* **91:**524-545.

De Chastellier C., and P. Berche. (1994). Fate of *Listeria monocytogenes* in murine macrophages: evidence for simultaneous killing and survival of intracellular bacteria. *Infect. Immun.* **62:**543-553.

Deber C.M., Glibowicka M., and G.A. Woolley. (1990). Confirmations of proline residues in membrane environments. *Biopolymers* **29:**149-157.

Dee R.R., and B. Lorber. (1986). Brain abscess due to *Listeria monocytogenes*: case report and literature review. *Rev. Infect. Dis.* **8:**968-977.

DeLange R.J., Chang J.Y., Shaper J.H., and A.N. Glazer. (1976). Amino acid sequence of flagellin of *Bacillus subtilis* 168. *J. Biol. Chem.* **251:**705-711.

Delves-Broughton J. (1990). Nisin and its uses as a food preservative. *Food Technol.* **44:**110-112.

Deneer H.G., and I. Boychuk. (1993). Reduction of ferric iron by *Listeria monocytogenes* and other species of *Listeria. Can. J. Microbiol.* **39:**480-485.

Deneer H.G., Healey V., and I. Boychuk. (1995). Reduction of exogenous ferric iron by a surface-associated ferric reductase of *Listeria* spp. *Microbiol.* **141:**1985-1992.

Derbise A., Dyke K.G.H., and N. El Solh. (1994). Isolation and characterisation of IS*1181*, an insertion sequence from *Staphylococcus aureus. Plasmid* **31:**252-264.

Devereux J., Haeberli P., and O. Smithies. (1984). A comprehensive set of sequence analysis programs for the VAX. *Nucl. Acids Res.* **12:**387-395.

Dold F.G., Sanger J.M., and J.W. Sanger. (1994). Intact alpha-actinin molecules are needed for both the assembly of actin into the tails and the locomotion of *Listeria monocytogenes* inside infected cells. *Cell Motil. Cytoskeleton* **28:**97-107.

Domann E., Leimeister-Wächter M., Goebel W., and T. Chakraborty. (1991). Molecular cloning, sequencing, and identification of a metalloprotease gene from *Listeria monocytogenes* that is species specific and physically linked to the listeriolysin gene. *Infect. Immun.* **59:**65-72.

Domann E., Wehland J., Rohde M., Pistor S., Hartl M., Goebel W., Leimeister-Wächter M., Wuenscher M., and T. Chakraborty. (1992). A novel bacterial virulence gene in *Listeria monocytogenes* required for host cell microfilament interaction with homology to the proline-rich region of vinculin. *EMBO J.* **11:**1981-1990.

Domann E., Wehland J., Neibuhr K., Haffner C., Leimeister-Wächter M., and T. Chakraborty. (1993). Detection of a *prfA*-independent promoter responsible for listeriolysin gene expression in mutant *Listeria monocytogenes* strains lacking the PrfA regulator. *Infect. Immun.* **61:**3073-3075.

Domann E., Ebel F., Nichterlein T., and T. Chakraborty. (1995). Identification of a 32-kDa lipoprotein of *Listeria monocytogenes* with strong sequence homologies to iron-binding and iron-transport components of pathogenic organisms. In *Proceedings of the 12th International Symposium on Problems of Listeriosis.* 2-6 October, Perth, Australia, pp. 301-305.

Dominguez Rodriguez L., Vazquez Boland J.A., Fernandez Garayzabal J.F., Echalecu Tranchant P., Gomez-Lucia E., Rodriguez Ferri E.F., and G. Suarez Fernandez. (1986). Microplate technique to determine hemolytic activity for routine typing of *Listeria* species. *J. Clin. Microbiol.* **24:**99-103.

Dons L., Rasmussen O.F., and J.E. Olsen. (1992). Cloning and characterization of a gene encoding flagellin of *Listeria monocytogenes. Mol. Microbiol.* **6:**2919-2929.

Dons L., Olsen J.E., and O.F. Rasmussen. (1994). Characterisation of two putative *Listeria monocytogenes* genes encoding polypeptides homologous to the sensor protein CheA and the response regulator CheY of chemotaxis. *DNA Sequence* **4:**301-311.

Dons L., Rasmussen O.F., and J.E. Olsen. (1995). Sequence diversity of the flagellin gene of *Listeria monocytogenes.* In *Proceedings of the 12th International Symposium on Problems of Listeriosis.* 2-6 October, Perth, Australia, pp. 205-207.

Dorman C.J. (1991). DNA supercoiling and environmental regulation of gene expression in pathogenic bacteria. *Infect. Immun.* **59:**745-749.

Dorman C.J. (1995). DNA topology and the global control of bacterial gene expression: implications for the regulation of virulence gene expression. *Microbiol.* **141:**1271-1280.

Dorman C.J., Barr G.C., Ní Bhriain N., and C.F. Higgins. (1988). DNA supercoiling and the anaerobic and growth phase regulation of *tonB* gene expression. *J. Bacteriol.* **170:**2816-2826.

Dorman C.J., Ní Bhriain N., and C.F. Higgins. (1990). DNA supercoiling and environmental regulation of virulence gene expression in *Shigella flexneri. Nature* **344:**789-792.

Dower W.J. (1990). In *Genetic engineering: principles and methods,* (J.K. Setlow, ed.). Plenum Press, New York, pp. 275-296.

Dramsi S., Dehoux P., and P. Cossart. (1993a). Common features of Gram-positive bacterial proteins involved in cell recognition. *Mol. Microbiol.* **9:**1119-1122.

Dramsi S., Kocks C., Forestier C., and P. Cossart. (1993b). Internalin-mediated invasion of epithelial cells by *Listeria monocytogenes* is regulated by bacterial growth state, temperature and the pleiotropic activator *prfA. Mol. Microbiol.* **9:**931-941.

Dramsi S., Biswas I., Maguin E., Braun L., Mastroeni P., and P. Cossart. (1995). Entry of *Listeria monocytogenes* into hepatocytes requires expression of InlB, a surface protein of the internalin multigene family. *Mol. Microbiol.* **16:**251-261.

Drevets D.A., Canono B.P., Leenen P.J.M., and P.A. Campbell. (1994). Gentamycin kills intracellular *Listeria monocytogenes. Infect. Immun.* **62:**2222-2228.

Drlica K. (1984). Biology of bacterial deoxyribonucleic acid topoisomerases. *Microbiol. Rev.* **48:**273-289.

Drlica K. (1987). The nucleoid. In Escherichia coli *and* Salmonella typhimurium *cellular and molecular biology* (F.C. Neidhardt, J.L. Ingraham, K.B. Low, B. Magasanik, M. Schaechter, and H.E. Umbarger, eds.), American Society for Microbiology, Washington, DC., pp. 91-103.

Dunny G.M., Lee L.N., and D.J. Leblanc. (1991). Improved electroporation and cloning vector system for Gram-positive bacteria. *Appl. Environ. Microbiol.* **57:**1194-1201.

Ebel F., Gerstel B., Pistor S., Niebuhr K., Domann E., Wehland J., and T. Chakraborty. (1995). A novel eukaryotic protein associated with intracytoplasmic motility of *Listeria monocytogenes.* In *Proceedings of the 12th International Symposium on Problems of Listeriosis.* 2-6 October, Perth, Australia, pp. 309-313.

Endo G., and S. Silver. (1995). CadC, the transcriptional regulatory protein of the cadmium resistance system of *Staphylococcus aureus* plasmid pI258. *J. Bacteriol.* **177:**4437-4441.

Ericsson H., Danielsson-Tham M., and W. Tham. (1995). *Listeria monocytogenes* serovar 4b strains divided into two groups using polymerase chain reaction (PCR) and restriction enzyme analysis (REA). In *Proceedings of the 12th International Symposium on Problems of Listeriosis.* 2-6 October, Perth, Australia, p. 421.

Espaze E.P., Gautreaum D., Catimel B., Miegeville A.F., Rocourt J., and A.L. Courtieu. (1989). An epidemiological survey of human listeriosis in France during 1987, using serotyping and phagetyping. *Acta Microbiol. Hung.* **36:**231-234.

Facinelli B., Varaldo P.E., Toni M., Casolari C., and U. Fabio. (1989). Ignorance about *Listeria. Br. Med J.* **299:**738.

Fagan M.J., and M.H. Saier. (1994). P-type ATPases of eukaryotes and bacteria: sequence analyses and construction of phylogenetic trees. *J. Mol. Evol.* **38:**57-99.

Falkow S. (1991). Bacterial entry into eukaryotic cells. *Cell* **65:**1099-1102.

Farber J.M., and J.I. Speirs. (1987). Monoclonal antibodies directed against the flagellar antigens of *Listeria* spp. and their potential in EIA-based methods. *J. Food Protect.* **50:**479-484.

Farber J.M., and P.I. Peterkin. (1991). *Listeria monocytogenes,* a food-borne pathogen. *Microbiol. Rev.* **55:**476-511.

Farber J.M., Sanders G.W., and J.I. Speirs. (1988). Methodology for the isolation of *Listeria* in foods: a Canadian perspective. *J. Assoc. Off. Anal. Chem.* **71:**675-678.

Farber J.M., Sanders G.W., Dunfield S., and R. Prescott. (1989). The effect of various acidulants on the growth of *Listeria monocytogenes. Lett. Appl. Microbiol.* **9:**181-183.

Farber J.M., Daley E., Coates F., Beausoleil N., and J. Fournier. (1991). Feeding trials of *Listeria monocytogenes* with a non-human primate model. *J. Clin. Microbiol.* **29:**2606-2608.

Farley R.A., and L.D. Faller. (1985). The amino acid sequence of an active site peptide from the H,K-ATPase of gastric mucosa. *J. Biol. Chem.* **260:**3899-3901.

Feinberg A.P., and B. Vogelstein. (1983). A technique for radiolabelling DNA restriction enzyme fragments to high specifc activity. *Anal. Biochem.* **132:**6-13.

Fenlon D.R. (1985). Wild birds and silage as reservoirs of *Listeria* in the agricultural environment. *J. Appl. Bacteriol.* **59:**537-543.

Feresu S.B., and D. Jones. (1988). Taxonomic studies on *Brochothrix, Erysipelothrix, Listeria* and atypical Lactobacilli. *J. Gen. Microbiol.* **134:**1165-1183.

Fiedler F., Seger J., Schrettenbrunner A., and H.P.R. Seeliger. (1984). The biochemistry of murein and cell wall teichoic acids in the genus *Listeria. Syst. Appl. Microbiol.* **5:**360-376.

Figurski D.H., and D.R. Helinski. (1979). Replication of an origin-containing derivative of plasmid RK2 dependent on a plasmid function provided in *trans. Proc. Natl. Acad. Sci.* USA. **76:**1648-1652.

Finkelstein R.A., Sciortino C.V., and M.A. McIntosh. (1983). Role of iron in microbe-host interactions. *Rev. Infect. Dis.* **5:**S759-S777.

Finlay B.B., and S. Falkow. (1989a). Common themes in microbial pathogenicity. *Microbiol. Rev.* **53:**210-230.

Finlay B.B., and S. Falkow. (1989b). *Salmonella* as an intracellular parasite. *Mol. Microbiol.* **3:**1833-1841.

Fitter S., Heuzenroeder M., and C.J. Thomas. (1992). A combined PCR and selective enrichment method for rapid detection of *Listeria monocytogenes. J. Appl. Bacteriol.* **73:**53-59.

Flamm R.K., Hinrichs D.J., and M.F. Thomashow. (1984). Introduction of pAMβ1 into *Listeria monocytogenes* by conjugation and homology between native *L. monocytogenes* plasmids. *Infect. Immun.* **44:**157-161.

Fleming D.W., Cochi S.L., MacDonald K.L., Brondum J., Hayes P.S., Plikaytis B.D., Holmes M.B., Audurier A., Broome C.V., and A.L. Reingold. (1985). Pasteurized milk as a vehicle of infection in an outbreak of listeriosis. *N. Engl. J. Med.* **312:**404-407.

Flynn J.L., Weiss W.R., Norris K.A., Seifert H.S., Kumar S., and M. So. (1990). Generation of a cytotoxic T-lymphocyte response using a *Salmonella* antigen-delivery system. *Mol. Microbiol.* **4:**2111-2118.

Fowler A.V., and I. Zabin. (1978). Amino acid sequence of the beta-galactosidase. XI. Peptide ordering procedures and the complete sequence. *J. Biol. Chem.* **253:**5521-5525.

Francis K.P., Rees C.E.D., and G.S.A.B. Stewart. (1995). Identification of a major cold shock protein homologue in *Listeria monocytogenes.* In *Proceedings of the 12th International Symposium on Problems of Listeriosis,* 2-6 October, Perth, Australia, pp. 403-409.

Freitag N.E., and D.A. Portnoy. (1994). Dual promoters of the *Listeria monocytogenes prfA* transcriptional activator appear essential *in vitro* but are redundant *in vivo. Mol. Microbiol.* **12:**845-853.

Freitag N.E., Youngman P., and D.A. Portnoy. (1992). Transcriptional activation of the *Listeria monocytogenes* hemolysin gene in *Bacillus subtilis. J. Bacteriol.* **174:**1293-1298.

Freitag N.E., Rong L., and D.A. Portnoy. (1993). Regulation of the *prfA* transcriptional activator of *Listeria monocytogenes*: multiple promoter elements contribute to intracellular growth and cell-to-cell spread. *Infect. Immun.* **61:**2537-2544.

Freter R., Allweiss B., O'Brien P.C.M., Halstead S.A., and M.S. Macsai. (1981a). Role of chemotaxis in the association of motile bacteria with intestinal mucosa: *in vitro* studies. *Infect. Immun.* **34:**241-249.

Freter R., O'Brien P.C.M., and M.S. Macsai. (1981b). Role of chemotaxis in the association of motile bacteria with intestinal mucosa: *in vivo* studies. *Infect. Immun.* **34:**234-240.

Friederich E., Gouin E., Hellio R., Kocks C., Cossart P., and D. Louvard. (1995). Targeting of *Listeria monocytogenes* ActA protein to the plasma membrane as a tool to dissect both actin-based cell morphogenesis and ActA function. *EMBO J.* **14:**2731-2744.

Friedman D.I. (1988). Integration host factor: a protein for all reasons. *Cell* **55:**545-554.

Fsihi H., and S.T. Cole. (1995). The *Mycobacterium leprae* genome: systematic sequence analysis identifies key catabolic enzymes, ATP-dependent transport systems and a novel *polA* locus associated with genomic variability. *Mol. Microbiol.* **16:**909-919.

Fuhrer D.K., and G.W. Ordal. (1991). *Bacillus subtilis* CheN, a homolog of CheA, the central regulator chemotaxis in *Escherichia coli. J. Bacteriol.* **173:**7443-7448.

Fuzi M., and I. Pillis. (1962). Production of opacity in egg-yolk medium by *Listeria monocytogenes. Nature* **13:**195.

Gahan C.G.M., and J.K. Collins. (1995). Vaccination of mice with attenuated mutants of *Listeria monocytogenes*: requirement for induction of macrophage Ia expression. *Microb. Pathog.* **18:**417-422.

Gaillard J.-L., Berche P., and P. Sansonetti. (1986). Transposon mutagenesis as a tool to study the role of hemolysin in the virulence of *Listeria monocytogenes. Infect. Immun.* **52:**50-55.

Gaillard J.-L., Berche P., Mounier J., Richard S., and P. Sansonetti. (1987). *In vitro* model of penetration and intracellular growth of *Listeria monocytogenes* in the human enterocyte-like cell line Caco-2. *Infect. Immun.* **55:**2822-2829.

Gaillard J.-L., Berche P., Frehel C., Gouin E., and P. Cossart. (1991). Entry of *L. monocytogenes* into cells is mediated by internalin, a repeat protein reminiscent of surface antigens from Gram-positive cocci. *Cell.* **65:**1127-1141.

Galán J.E., and R. Curtiss III. (1990). Expression of *Salmonella typhimurium* genes required for invasion is regulated by changes in DNA supercoiling. *Infect. Immun.* **58:**1879-1885.

Galas D.J., and M. Chandler. (1989). Bacterial insertion sequences. In *Mobile DNA.* (D.E. Berg, and M.M. Howe, eds.), American Society for Microbiology, Washington, DC., pp. 109-162.

Galbraith N.S. (1988). Listeriosis. *Br. Med. J.* **297:**754-755.

Galsworthy S.B., Girdler S., and S.F. Koval. (1990). Chemotaxis in *Listeria monocytogenes. Acta Microbiol. Hung.* **37:**81-85.

Gandhi A.J., Perussia B., and H. Goldfine. (1993). *Listeria monocytogenes* phosphatidylinositol (PI)-specific phospholipase C has low activity on glycosy-PI-anchored proteins. *J. Bacteriol.* **175:**8014-8017.

Garcia-del Portillo F., Foster J.W., Maguire M.E., and B.B. Finlay. (1992). Characterization of the micro-environment of *Salmonella typhimurium*-containing vaculoles within MDCK epithelial cells. *Mol. Microbiol.* **6:**3289-3297.

Garduno R.A., Phipps B.M., Baumeister W., and W.W. Kay. (1992). Novel structural patterns in divalent cation-depleted surface layers of *Aeromonas salmonicida. J. Struct. Biol.* **109:**184-195.

Garger S.J., Griffith O.M., and L.K. Grill. (1983). Rapid purification of plasmid DNA by a single centrifugation in a two step caesium chloride-ethidium bromide gradient. *Biochem. Biophys. Res. Commun.* **117:**835-842.

Ge Z., Hiratsuka K., and D.E. Taylor. (1995). Nucleotide sequence and mutational analysis indicate that two *Helicobacter pylori* genes encode a P-type ATPase and a cation-binding protein associated with copper transport. *Mol. Microbiol.* **15:**97-106.

Gellin B.G., and C.V. Broome. (1989) Listeriosis. *J. Am. Med. Assoc.* **261:**1313-1320.

Gentschev I., Sokolovic Z., Köhler S., Krohne G.F., Hof H., Wagner J., and W. Goebel. (1992). Identification of p60 antibodies in human sera and presentation of this listerial antigen on the surface of attenuated *Salmonellae* by the HlyB-HlyD secretion system. *Infect. Immun.* **60:**5091-5098.

Gentschev I., Mollenkopf H.-J., Sokolovic Z., Ludwig A., Tengal C., Gross R., Hess J., Demuth A., and W. Goebel. (1994). Synthesis and secretion of bacterial antigens by attenuated *Salmonella* via the *Escherichia coli* hemolysin secretion system. *Behring. Inst. Mitt.* Dec(95):57-66

Gentschev I., Sokolovic Z., Mollenkopf H.-J., Hess J., Kaufmann S.H.E., Kuhn M., Krohne G., and W. Goebel. (1995). *Salmonella* strain secreting active listeriolysin changes its intracellular location. *Infect. Immun.* **63:**4202-4205.

Geoffroy C., Gaillard J., Alouf J.E., and P. Berche. (1987). Purification, charcterization, and toxicity of the sulfhydryl-activated hemolysin listeriolysin O from *Listeria monocytogenes. Infect. Immun.* **55:**1641-1646.

Geoffroy C., Gaillard J., Alouf J.E., and P. Berche. (1989). Production of thiol-dependent haemolysins by *Listeria monocytogenes* and related species. *J. Gen. Microbiol.* **135:**481-487.

Geoffroy C., Mengaud J., Alouf J.E., and P. Cossart. (1990). Alveolysin, the thiol-activated toxin of *Bacillus alvei*, is homologous to listeriolysin O, perfringolysin O, pneumolysin, and streptolysin O and contains a single cysteine. *J. Bacteriol.* **172:**7301-7305.

Geoffroy C., Raveneau J., Beretti J.-L., Lecroisey A., Vazquez-Boland J.-A., Alouf J.E., and P. Berche. (1991). Purification and characterization of an extracellular 29-kilodalton phospholipase C from *Listeria monocytogenes. Infect. Immun.* **59:**2382-2388.

Geourjon C., and G. Deleage. (1994). SOPM: a self-optimized method for protein secondary structure prediction. *Protein Eng.* **7:**157-164.

Germanier R. (1975). Isolation and characterization of *galE* mutant Ty21a of *Salmonella typhi*: a candidate for a live oral typhoid vaccine. *J. Infect. Dis.* **141:**553-558.

Germond J.E., Lapierre L., Delley M., and B. Mollet. (1995). A new mobile genetic element in *Lactobacillus delbrueckii* subsp. bulgaricus. *Mol. Gen. Genet.* **248:**407-416.

Gilmore M.S., Cruz-Rodz A.L., Leimeister-Wächter M., Kreft J., and W. Goebel. (1989). A *Bacillus cereus* cytolytic determinant, cereolysin AB, which comprises the phospholipase C and sphingomyelinase genes: nucleotide sequence and genetic linkage. *J. Bacteriol.* **171:**744-753.

Girard K.F., Sbarra A.J., and W.A. Bardawil. (1963). Serology of *Listeria monocytogenes*. I. Characteristics of soluble hemolysin. *J. Bacteriol.* **85:**349-355.

Göhmann S., Leimeister-Wächter M., Schiltz E., Goebel W., and T. Chakraborty. (1990). Characterization of *Listeria monocytogenes*-specific protein capable of inducing delayed hypersensitivity in *Listeria*-immune mice. *Mol. Microbiol.* **4:**1091-1099.

Goldberg M.B., Boyko S.A., and S.B. Calderwood. (1990). Transcriptional regulation by iron of a *Vibrio cholerae* virulence gene and homology of the gene to the *Escherichia coli* Fur system. *J. Bacteriol.* **172:**6863-6870.

Goldfine H., and C. Knob. (1992). Purification and characterization of *Listeria monocytogenes* phosphatidylinositol-specific phospholipase C. *Infect. Immun.* **60:**4059-4067.

Goldfine H., Johnston N.C., and C. Knob. (1993). Nonspecific phospholipase C of *Listeria monocytogenes*: activity on phospholipids in triton X-100-mixed micelles and in biological membranes. *J. Bacteriol.* **175:**4298-4306.

Goldstein E., and K. Drlica. (1984). Regulation of bacterial DNA supercoiling: plasmid linking numbers vary with growth temperature. *Proc. Natl. Acad. Sci.* USA **81:**4046-4050.

Gopalakrishna Y., Langley D., and N. Sarkar. (1981). Detection of high levels of polyadenylate-containing RNA in bacteria by the use of a single-step RNA isolation procedure. *Nucl. Acids Res.* **9:**3545-3554.

Gorgansson M., Sonden B., Nilsson P., Dagberg B., Forman K., Emanuelsson K., and B.E. Uhlin. (1990). Transcriptional silencing and thermoregulation of gene expression in *Escherichia coli. Nature* **344:**682-685.

Gormley E., Mengaud J., and P. Cossart. (1989). Sequences homologous to the listeriolysin O gene region of *Listeria monocytogenes* are present in virulent and avirulent haemolytic species of the genus *Listeria*. *Res. Microbiol.* **140:**631-643.

Gouin E., Mengaud J., and P. Cossart. (1994). The virulence gene cluster of *Listeria monocytogenes* is also present in *Listeria ivanovii,* an animal pathogen, and *Listeria seeligeri,* a nonpathogenic species. *Infect. Immun.* **62:**3550-3553.

Gouin E., Dehoux P., Mengaud J., Kocks C., and P. Cossart. (1995). *iactA* of *Listeria ivanovii,* although distantly related to *Listeria monocytogenes actA,* restores actin tail formation in an *L. monocytogenes actA* mutant. *Infect. Immun.* **63:**2729-2737.

Graves L.M., Swaminathan B., Reeves M.W., and J. Wegner. (1991). Ribosomal DNA fingerprinting of *Listeria monocytogenes* using a digoxigenin-labeled DNA probe. *Eur. J. Epidemiol.* **7:**77-82.

Green N.M. (1992). Evolutionary relationships within the family of P-type cation pumps. *Ann. N. Y. Acad. Sci.* **671:**104-112.

Gross R. (1993). Signal transduction and virulence regulation in human and animal pathogens. FEMS *Microbiol. Rev.* **10:**1-26.

Guzman C.A., Domann E., Rohde M., Bruder D., Darji A., Weiss S., Wehland J., Chakraborty T., and K.N. Timmis. (1996). Apoptosis of mouse dendritic cells is triggered by listeriolysin, the major virulence determinant of *Listeria monocytogenes*. *Mol. Microbiol.* **20:**119-126.

Gygi D., Nicolet J., Hughes C., and J. Frey. (1992). Functional analysis of the Ca^{2+}-regulated hemolysin I operon of *Actinobacillus pleuropneumoniae* serotype 1. *Infect. Immun.* **60:**3059-3064.

Haas A., and W. Goebel. (1992). Cloning of a superoxide dismutase gene from *Listeria ivanovii* by functional complementation in *Escherichia coli* and characterization of the gene product. *Mol. Gen. Genet.* **231:**313-322.

Haas A., Brehm K., Kreft J., and W. Goebel. (1991). Cloning, characterization, and expression in *Escherichia coli* of a gene encoding *Listeria seeligeri* catalase, a bacterial enzyme highly homologous to mammalian catalases. *J. Bacteriol.* **173:**5159-5167.

Haas A., Dumbsky M., and J. Kreft. (1992). Listeriolysin genes: complete sequence of *ilo* from *Listeria ivanovii* and *lso* from *Listeria seeligeri*. *Biochem. Biophys. Acta* **1130:**81-84.

Hahn H., and S.H.E. Kaufmann. (1981). The role of cell-mediated immunity in bacterial infections. *Rev. Infect. Dis.* **3:**1221-1250.

Halbert S.P. (1970). Streptolysin O. In *Microbial toxins.* vol. 3. (T.C. Montie, S. Kadis, and S.J. Ajil, eds.), Academic Press, Inc., New York, pp. 69-98.

Hale T.L., Oaks E.V., and S.B. Formal. (1985). Identification and antigenic characterization of virulence associated, plasmid-coded proteins of *Shigella* spp. and enteroinvasive *Escherichia coli*. *Infect. Immun.* **50:**620-629.

Hanahan D. (1983). Studies on transformation of *Escherichia coli* with plasmids. *J. Mol. Biol.* **166:**557-580.

Hanawa T., Yamamoto T., and S. Kamiya. (1995). *Listeria monocytogenes* can grow in macrophages without the aid of proteins induced by environmental stresses. *Infect. Immun.* **63:**4595-4599.

Handwerger S., Skoble J., Discotto L.F., and M.J. Pucci. (1995). Heterogeneity of the *vanA* gene cluster in clinical isolates of enterococci from the northeastern United States. *Antimicrob. Agents Chemother.* **39:**362-368.

d'Hauteville H., and P.J. Sansonetti. (1992). Phosphorylation of IcsA by cAMP-dependent protein kinase and its effect on intercellular spread of *Shigella flexneri*. *Mol. Microbiol.* **6:**833-841.

Harshey R.M., Estepa G., and H. Yanagi. (1989). Cloning and nucleotide sequence of a flagellin-coding gene (*hag*) from *Serratia marcescens* 274. *Gene* **79:**1-8.

Hartford T., O'Brien S., Andrew P.W., Jones D., and I.S. Roberts. (1993). Utilization of transferrin-bound iron by *Listeria monocytogenes*. FEMS *Microbiol. Letts.* **108:**311-318.

Hartman A.B., Venkatesan M., Oaks E.V., and J.M. Buysse. (1990). Sequence and molecular characterization of a multicopy invasion plasmid antigen gene, *ipaH,* of *Shigella flexneri*. *J. Bacteriol.* **172:**1905-1915.

Hastings J.W., Sailer M., Johnson K., Roy K.L., Vederas J.C., and M.E. Stiles. (1991). Characterization of leucocin A-UAL 187 and cloning of the bacteriocin gene from *Leuconostoc gelidum*. *J. Bacteriol.* **173:**7491-7500.

Havell E.A. (1986). Synthesis and secretion of interferon by murine fibroblasts in responese to intracellular *Listeria monocytogenes*. *Infect. Immun.* **54:**787-792.

Héchard Y., Dérijard B., Letellier F., and Y. Cenatiempo. (1992). Characterization and purification of mesentericin Y105, an anti-*Listeria* bacteriocin from *Leuconostoc mesenteroides*. *J. Gen. Microbiol.* **138:**2725-2731.

Henner D.J., Yang M., Chen E., Hellmiss R., Rodriguez H., and M.G. Low. (1988). Sequence of the *Bacillus thuringiensis* phosphatidylinositol-specific phospholipase C. *Nucl. Acids Res.* **16:**10383.

Hess J., and S.H.E. Kaufmann. (1993). Vaccination strategies against intracellular microbes. FEMS *Immunol. Med. Microbiol.* **7:**95-104.

Hess J., Gentschev I., Goebel W., and T. Jarchau. (1990). Analysis of the hemolysin secretion system by PhoA-HlyA fusion proteins. *Mol. Gen. Genet.* **224:**201-208.

Hess J., Gentschev I., Szalay G., Ladel C., Bubert A., Goebel W., and S.H.E. Kaufmann. (1995). *Listeria monocytogenes* p60 supports host cell invasion by and *in vivo* survival of attenuated *Salmonella typhimurium*. *Infect. Immun.* **63:**2047-2053.

Hesse J.E., Wieczorek L., Altendorf K., Reicin A.S., Dorus E., and W. Epstein. (1984). Sequence homology between two membrane ATPases, the Kdp-ATPase of *Escherichia coli* and the Ca^{2+}-ATPase of sarcoplasmic reticulum. *Proc. Natl. Acad. Sci.* USA **81:**4746-4750.

Higgins C.F., Dorman C.J., Stirling D.A., Waddell L., Booth I.R., May G., and E. Bremer. (1988). A physiological role for DNA supercoiling in the osmotic regulation of gene expression in *S. typhimurium* and *E. coli*. *Cell* **52:**569-584.

Higgins C.F., Hinton J.C.D., Hulton C.S.J., Owen-Hughes T., Pavitt G.D., and A. Seirafi. (1990). Protein H1: a role for chromatin structure in the regulation of bacterial gene expression and virulence? *Mol. Microbiol.* **4:**2007-2012.

High N., Mounier J., Prevost M.C., and P.J. Sansonetti. (1992). IpaB of *Shigella flexneri* causes entry into epithielial cells and escape from the phagocytic vacuole. *EMBO J.* **11:**1991-1999.

Hiltbold E.M., and H.K. Ziegler. (1994). Mechanisms of processing and presentation of the antigens of *Listeria monocytogenes*. *Infect. Agents Dis.* **2:**314-323.

Hird D.W., and C. Genigeorgis. (1990). Listeriosis in food animals: clinical signs and livestock as a potential source of direct (nonfoodborne) infection for humans. In *Foodborne Listeriosis*, (A.J. Miller, J.L. Smith, and G.A. Somkuti, eds.), Elsevier Science Publications Co., Amsterdam, pp. 31-39.

Hiromatsu K., Yoshikai Y., Matsuzaki G., Ohga S., Muramori K., Matsumoto K., Bluestone J., and K. Nomoto. (1992). A protective role of γ/δ T cells in primary infection with *Listeria monocytogenes* in mice. *J. Exp. Med.* **175:**49-56.

Hof H. (1984). Virulence of different strains of *Listeria monocytogenes* serovar 1/2a. *Med. Microbiol. Immunol.* **173:**207-218.

Hof H. (1991a). Microbial strategies for intracellular survival. *Infection* **19:**S202-S205.

Hof H. (1991b). Therapeutic activities of antibiotics in listeriosis. *Infection* **19:**S229-S233.

Hof H., and P. Hefner. (1988). Pathogenicity of *Listeria monocytogenes* in comparison to other *Listeria* species. *Infection* **16:**S141-S144.

Hof H., and J. Rocourt. (1992). Is any strain of *Listeria monocytogenes* detected in food a health risk? *Int. J. Food Microbiol.* **16:**173-182.

Hoiseth S.K., and B.A. Stocker. (1981). Aromatic-dependent *Salmonella typhimurium* are non-virulent and effective as live vaccine. *Nature* **291:**238-239.

Hollingshead S.K., Fischetti V.A., and J.R. Scott. (1986). Complete nucleotide sequence of type 6 M protein of the group A *Streptococcus*: repetitive structure and membrane anchor. *J. Biol. Chem.* **261:**1677-1686.

Hollingshead S.K., Fischetti V.A., and J.R. Scott. (1987). Size variation in group A streptococcal M protein is generated by homologous recombination between intragenic repeats. *Mol. Gen. Genet.* **207:**196-203.

Hoopes B.C., and W.R. McClure. (1987). Strategies in regulation of transcription initiation. In Escherichia coli *and* Salmonella typhimurium *cellular and molecular biology* (F.C. Neidhardt, J.L. Ingraham, K.B. Low, B. Magasanik, M. Schaechter, and H.E. Umbarger, eds.), American Society for Microbiology, Washington, DC., pp. 1231-1240.

Huang D.C., Huang X.F., Novel G., and M. Novel. (1993). Two genes present on a transposon-like structure in *Lactococcus lactis* are involved in a Clp-family proteolytic activity. *Mol. Microbiol.* **7:**957-965.

Hubner R.J., Cole E.M., Bruce J.L., McDowell C.I., and J.A. Webster. (1995). Types of *Listeria monocytogenes* predicted by the positions of *Eco*R1 cleavage sites relative to ribosomal RNA sequences. *Proc. Natl. Acad. Sci.* USA **92:**5234-5238.

Hurst A. (1981). Nisin. *Adv. Appl. Microbiol.* **27:**85-123.

Hynes R.O. (1992). Integrins: versatility, modulation, and signaling in cell adhesion. *Cell* **69:**11-25.

Iordanescu S. (1976). Three distinct plasmids originating in the same *Staphylococcus aureus* strain. *Arch. Roum. Pathol. Exp. Microbiol.* **35:**111-118.

Ireton K., Payrastre B., Chap H., Ogawa W., Sakaue H., Kasuga M., and P. Cossart. (1996). A role for phosphoinositide 3-kinase in bacterial invasion. *Science* **274:**780-782.

Isberg R.R. (1991). Discrimination between intracellular uptake and surface adhesion of bacterial pathogens. *Science* **252:**934-938.

Isberg R.R., and G. Tran Van Nhieu. (1994). Binding and internalization of microorganisms by integrin receptors. *Trends Microbiol.* **2:**10-14.

Israelsen H., and E.B. Hansen. (1993). Insertion of transposon Tn*917* derivatives into the *Lactococcus lactis* subsp. *lactis* chromosome. *Appl. Environ. Microbiol.* **59:**21-26.

Ivey D.M., Guffanti A.A., Shen Z., Kudyan N., and T.A. Krulwich. (1992). The *cadC* gene product of alkaliphilic *Bacillus firmus* OF4 partially restores Na^+ resistance to an *Escherichia coli* strain lacking an Na^+/H^+ antiporter (NhaA). *J. Bacteriol.* **174:**4878-4884.

Jacquet C.H., Bille J., and J. Rocourt. (1992). Typing of *Listeria monocytogenes* by restriction polymorphism of the ribosomal ribonucleic acid gene region. *Int. J. Med. Microbiol. Virol. Parasitol. Infect. Dis.* **276:**356-365.

Jacquet C.H., Catimel B., Goulet V., Lepoutre A., Veit P., Dehaumont P., and J. Rocourt. (1995). Typing of *Listeria monocytogenes* during epidemiological investigations of the french listeriosis outbreaks in 1992, 1993, and 1995. In *Proceedings of the 12th International Symposium on Problems of Listeriosis.* 2-6 October, Perth, Australia, pp. 161-176.

Jenkins E.M., Njoku-Obi A.N., and E.W. Adams. (1964). Purification of the soluble hemolysins of *Listeria monocytogenes. J. Bacteriol.* **88:**418-424.

Johansen E., and A. Kibenich. (1992). Isolation and characterisation of IS*1165*, an insertion sequence of *Leuconostic mesenteroides* subsp. cremoris and other lactic acid bacteria. *Plasmid* **27:**200-206.

Johansen T., Holm T., Guddal P.H., Sletten K., Haugli F.B., and C. Little. (1988). Cloning and sequencing of the gene encoding the phosphatidylcholine-preferring phospholipase C from *Bacillus cereus. Gene* **65:**293-304.

Johnson M.K., Geoffroy C., and J.E. Alouf. (1980). Binding of cholesterol by sulfhydryl-activated cytolysins. *Infect Immun.* **27:**97-101.

Jones B.D., Lee C.A., and S. Falkow. (1992). Invasion by *Salmonella typhimurium* is affected by the direction of flagellar rotation. *Infect. Immun.* **60:**2475-2480.

Jones C.E., Shama G., Jones D., Roberts I.S., and P.W. Andrew. (1995). Biochemical studies on psychrotolerance in *Listeria monocytogenes.* In *Proceedings of the 12th International Symposium on Problems of Listeriosis.* 2-6 October, Perth, Australia, pp. 393-396.

Jones S., and D.A. Portnoy. (1994). Intracellular growth of bacteria. *Meth. Enzymol.* **236:**463-467.

Kahn D., David M., Domergue O., Daveran M.-L., Ghai J., Hirsch P.R., and J. Batut. (1989). *Rhizobium meliloti fixGHI* sequence predicts involvement of a specific cation pump in symbiotic nitrogen fixation. *J. Bacteriol.* **171:**929-939.

Kalnins A., Otto K., Ruther U., and B. Muller-Hill. (1983). Sequence of the *lacZ* gene of *Escherichia coli. EMBO J.* **2:**593-597.

Kamisango K., Fujii H., Okumura J., Saiki I., Araki Y., Yamamura Y., and Z. Azuma. (1983). Structural and immunochemical studies of teichoic acids for *Listeria monocytogenes. J. Biochem.* **93:**1401-1409.

Kampelmacher E.H., and L.M. van Noorle-Jansen. (1972). Further studies on the isolation of *Listeria monocytogenes* in clinically healthy individuals. *Zentralbl. Bacteriol. Parasitenkd Infektionskr. Hgy.* **221:**70-77.

Kanamaru K., Kashiwagi S., and T. Mizuno. (1993). The cyanobacterium, *Synechococcus* sp. PCC7942, possesses two distinct genes encoding cation-transporting P-type ATPases. FEBS *Letts.* **330:**99-104.

Kanamaru K., Kashiwagi S., and T. Mizuno. (1994). A copper-transporting P-type ATPase found in the thylakoid membrane of the cyanobacterium *Synechococcus* species PCC7942. *Mol. Microbiol.* **13:**369-377.

Karunasagar I., Krohne G., and W. Goebel. (1993). *Listeria ivanovii* is capable of cell-to-cell spread involving actin polymerization. *Infect. Immun.* **61:**162-169.

Karunasagar I., Senghaas B., Krohne G., and W. Goebel. (1994). Ultrastructural study of *Listeria monocytogenes* entry into cultured human colonic epithelial cells. *Infect. Immun.* **62:**3554-3558

Kathariou S., Metz P., Hof H., and W. Goebel. (1987a). Tn*916*-induced mutations in the hemolysin determinant affecting virulence of *Listeria monocytogenes*. *J. Bacteriol.* **169:**1291-1297.

Kathariou S., Hacker J., Hof H., Then I., Wagner W., Kuhn M., and W. Goebel. (1987b). Bacterial cytolysins: extracellular proteins and virulence factors. In *Molecular basis of viral and microbial pathogenesis.* (R. Rott, and W. Goebel, eds.), Springer-Verlag, Berlin, pp. 141-150.

Kathariou S., Rocourt J., Hof H., and W. Goebel. (1988). Levels of *Listeria monocytogenes* hemolysin are not directly proportional to virulence in experimental infections of mice. *Infect. Immun.* **56:**534-536

Kathariou S., Pine L., George V., Carlone G.M., and B.P. Holloway. (1990). Nonhemolytic *Listeria monocytogenes* mutants that are also noninvasive for mammalian cells in culture: evidence for coordinate regulation of virulence. *Infect. Immun.* **58:**3988-3995.

Kathariou S., Kanenaka R., Allen R.D., Fok A.K., and C. Mizumoto. (1995). Repression of motility and flagellin production at 37°C is stronger in *Listeria monocytogenes* than in the nonpathogenic species *Listeria innocua. Can. J. Microbiol.* **41:**572-577.

Kaufmann S.H.E. (1988). $CD8^+$ T lymphocytes in intracellular microbial infections. *Immunol. Today* **9:**168-174.

Kaufmann S.H.E. (1990). Heat shock proteins and the immune response. *Immunol. Today* **11:**129-136.

Kaufmann S.H.E., Hug E., and G. De Libero. (1986). *Listeria monocytogenes* reactive T lymphocyte clones with cytolytic activity against infected target T cells. *J. Exp. Med.* **164:**363-368.

Kawamura F., Wang L.-F., and R.H. Doi. (1985). Catabolite-resistant sporulation (*crsA*) mutations in *Bacillus subtilis* RNA polymerase σ^{43} gene (*rpoD*) can suppress and be suppressed by mutations in *spo0* genes. *Proc. Natl. Acad. Sci.* USA **82:**8124-8128.

Kehoe M.A., Miller L., Walker J.A., and G.J. Boulnois. (1987). Nucleotide sequence of the streptolysin O (SLO) gene: structural homologies between SLO and other membrane damaging, thiol-activated toxins. *Infect Immun.* **55:**534-536.

Kerr K.G., and R.W. Lacey. (1991). Isolation and identification of *Listeria monocytogenes. J. Clin. Pathol.* **44:**624-627.

Khan S.A., Khalid S.M., and R. Siddiqui. (1993). The effect of pH and temperature on haemolysin production by *Listeria* species. *Letts. Appl. Microbiol.* **17:**14-16.

King W., Raposa S., Warshaw J., Johnson A., Halbert D., and J.D. Klinger. (1989). A new colorimetric nucleic acid hybridization assay for *Listeria* in foods. *Int. J. Food. Microbiol.* **8:**225-232.

Kingdon G.C., and C.P. Sword. (1970a). Biochemical and immunological effects of *Listeria monocytogenes* hemolysin. *Infect. Immun.* **1:**363-372.

Kingdon G.C., and C.P. Sword. (1970b). Carditoxic and lethal effects of *Listeria monocytogenes* hemolysin. *Infect. Immun.* **1:**373-379.

Kingdon G.C., and C.P. Sword. (1970c). Effects of *Listeria monocytogenes* hemolysin on phagocytic cells and lysosomes. *Infect. Immun.* **1:**356-362.

Klaenhammer T.R. (1988). Bacteriocins of lactic acid bacteria. *Biochimie* **70:**337-349.

Klarsfeld A.D., Goossens P.L., and P. Cossart. (1994). Five *Listeria monocytogenes* genes preferentially expressed in infected mammalian cells: *plcA, purH, purD, pyrE* and an arginine ABC transporter gene, *arpJ. Mol. Microbiol.* **13:**585-597.

Klinger J.D., and A.R. Johnson. (1988). A rapid nucleic acid hybridization assay for *Listeria* in foods. *Food. Technol.* **42:**66 & 68-70.

Klinger J.D., Johnson A., Croan D., Flynn P., Whippie K., Kimball M., Lawrie J., and M. Curiale. (1988). Comparative studies of nucleic acid hybridization assay for *Listeria* in foods. *J. Assoc. Off. Anal. Chem.* **71:**669-673.

Kneller D.G., Cohen F.E., and R. Langridge. (1990). Improvements in protein secondary structure prediction by an enhanced neural network. *J. Mol. Biol.* **214:**171-182.

Kocks C., and P. Cossart. (1993). Directional actin assembly by *Listeria monocytogenes* at the site of polar surface expression of the *actA* gene product involving the actin-bundling protein plastin (fimbrin). *Infect. Agents Dis.* **2:**207-209.

Kocks C., Gouin E., Tabouret M., Berche P., Ohayon H., and P. Cossart. (1992). *L. monocytogenes*-induced actin assembly requires the actA gene product, a surface protein. *Cell* **68:**521-531.

Kocks C., Hellio R., Gounon P., Ohayon H., and P. Cossart. (1993). Polarised distribution of *Listeria monocytogenes* surface protein ActA at the site of directional actin assembly. *J. Cell Sci.* **105:**699-710.

Kocks C., Marchand J.B., Gouin E., d'Hauteville H., Sansonetti P., Carlier M.F., and P. Cossart. (1995). The unrelated surface proteins ActA of *Listeria monocytogenes* and IscA of *Shigella flexneri* are sufficient to confer actin-based motility on *Listeria innocua* and *Escherichia coli* respectively. *Mol. Microbiol.* **18:**413-423.

Köhler S. Leimeister-Wächter M., Chakraborty T., Lottspeich F., and W. Goebel. (1990). The gene coding for protein p60 of *Listeria monocytogenes* and its use as a specific probe for *Listeria monocytogenes. Infect. Immun.* **58:**1943-1950.

Köhler S., Bubert A., Vogel M., and W. Goebel. (1991). Expression of the *iap* gene coding for protein p60 of *Listeria monocytogenes* is controlled on the posttranscriptional level. *J. Bacteriol.* **173:**4668-4674.

Kolstad J., Caugant D.A., and L.M. Rørvik. (1992). Differentiation of *Listeria monocytogenes* isolates by using plasmid profiling and multilocus enzyme electrophoresis. *Int. J. Food Microbiol.* **16:**247-260.

Kreft J., Funke D., Haas A., Lottspeich F., and W. Goebel. (1989). Production, purification and characterization of hemolysin from *L. ivanovii* and *L. monocytogenes* Sv4b. FEMS *Microbiol. Letts.* **57:**197-202.

Kreft J., Dumbsky M., and S. Theiss. (1995). The actin-polymerisation protein from *Listeria ivanovii* is a large repeat protein which shows only limited amino acid sequence homology to ActA from *Listeria monocytogenes.* FEMS *Microbiol. Letts.* **126:**113-122.

Kuhn M., and W. Goebel. (1989). Identification of an extracellular protein of *Listeria monocytogenes* possibly involved in intracellular uptake by mammalian cells. *Infect. Immun.* **57:**55-61.

Kuhn M., Kathariou S., and W. Goebel. (1988). Hemolysin supports survival but not entry of the intracellular bacterium *Listeria monocytogenes. Infect. Immun.* **56:**79-82.

Kuppe A., Evans L.M., McMillen D.A., and O.H. Griffith. (1989). Phoshatidylinositol-specific phospholipase C of *Bacillus cerus*: cloning, sequencing, and relationship to other phospholipases. *J. Bacteriol.* **171:**6077-6083.

Kyte J. (1995). Membranes. In *Structure in protein chemistry.* Garland Publishing, Inc., New York, pp. 501-567.

Kyte J., and F.R. Doolittle. (1982). A simple method for displaying the hydropathy character of a protein. *J. Mol. Biol.* **157:**105-132.

Lampidis R., Gross R., Sokolovic Z., Goebel W., and J. Kreft. (1994). The virulence regulator protein of *Listeria ivanovii* is highly homologous to PrfA from *Listeria monocytogenes* and both belong to the Crp-Fnr family of transcription regulators. *Mol. Microbiol.* **13:**141-151.

Laoudji D., Andersen C.L., Bras A., Goldberg M., Jacq A., and I.B. Holland. (1994). EGTA induces the synthesis in *Escherichia coli* of three proteins that cross-react with calmodulin antibodies. *Mol. Microbiol.* **13:**445-457.

Larsen H.E., and H.P.R. Seeliger. (1986). A mannitol fermenting *Listeria. Listeria grayi* sp. In *Proceedings of the 3rd International Symposium on Listeriosis,* 13 July, Bilthoven, The Netherlands, p. 35.

Lasa I., and P. Cossart. (1996). Actin-based bacterial motility: towards a definition of the minimal requirements. *Trends Cell Biol.* **6:**109-114.

Lasa I., David V., Gouin E., Marchand J.B., and P. Cossart. (1995). The amino-terminal part of ActA is critical for the actin-based motility of *Listeria monocytogenes*; the central proline-rich region acts as a stimulator. *Mol. Microbiol.* **18:**425-436.

Lebond-Francillard M., Gaillard J.-L., and P. Berche. (1989). Loss of catalase activity in Tn*1545*-induced mutants does not reduce growth of *Listeria monocytogenes in vivo. Infect. Immun.* **57:**2569-2573.

Lebrun M., Loulergue J., Chaslus-Dancla E., and A. Audurier. (1992). Plasmids in *Listeria monocytogenes* in relation to cadmium resistance. *Appl. Environ. Microbiol.* **58:**3183-3186.

Lebrun M., Audurier A., and P. Cossart. (1994). Plasmid-borne cadmium resistance genes in *Listeria monocytogenes* are similar to *cadA* and *cadC* of *Staphylococcus aureus* and are induced by cadmium. *J. Bacteriol.* **176:**3040-3048.

Lechner M., Kupke T., Stefanovic S., and F. Götz. (1989). Molecular characterization and sequence of phosphatidylinositol-specific phospholipase C of *Bacillus thuringiensis. Mol. Microbiol.* **3:**621-626.

Leighton I., Threlfall D.R., and C.L. Dakley. (1975). Phospholipase C activity in culture filtrates from *Listeria monocytogenes.* In *Problems of listeriosis,* (M. Woodbine, ed.), Leicester University Press, Leicester, England, pp. 239-241.

Leimeister-Wächter M., and T. Chakraborty. (1989a). Detection of listeriolysin, the thiol-dependent hemolysin in *Listeria monocytogenes, Listeria ivanovii,* and *Listeria seeligeri. Infect. Immun.* **57:**2350-2357.

Leimeister-Wächter M., and T. Chakraborty. (1989b). Gene probes for the detection of *Listeria* spp. *Acta Micriobiol. Hung.* **36:**211-213.

Leimeister-Wächter M., Goebel W., and T. Chakraborty. (1989). Mutations affecting hemolysin production in *Listeria monocytogenes* located outside the listeriolysin gene. FEMS *Microbiol. Letts.* **65:**23-30.

Leimeister-Wächter M., Haffner C., Domann E., Goebel W., and T. Chakraborty. (1990). Identification of a gene that positively regulates expression of listeriolysin, the major virulence factor of *Listeria monocytogenes. Proc. Natl. Acad. Sci.* USA **87:**8336-8340.

Leimeister-Wächter M., Domann E., and T. Chakraborty. (1991). Detection of a gene encoding a phosphatidylinositol-specific phospholipase C that is co-ordinately expressed with listeriolysin in *Listeria monocytogenes. Mol. Microbiol.* **5:**361-366.

Leimeister-Wächter M., Domann E., and T. Chakraborty. (1992). The expression of virulence genes in *Listeria monocytogenes* is thermoregulated. *J. Bacteriol.* **174:**947-952.

Leong J.M., Fournier R., and R.R. Isberg. (1990). Identification of the integrin binding domain of the *Yersinia pseudotuberculosis* invasin protein. *EMBO J.* **9:**1979-1989.

Leslie D., Fairweather N., Pickard D., Dougan G., and M. Kehoe. (1989). Phospholipase C and haemolytic activities of *Clostridium perfringens* alpha-toxin cloned in *Escherichia coli*: sequence and homology with a *Bacillus cereus* phospholipase C. *Mol. Microbiol.* **3:**383-392.

Lett M.C., Sasakawa C., Okada N., Sakai T., Makino S., Yamada M., Komatsu K., and M. Yoshikawa. (1989). *virG,* a plasmid-coded virulence gene of *Shigella flexneri*: identification of the *virG* protein and determination of the complete coding sequence. *J. Bacteriol.* **171:**353-359.

Lew A.E., and P.M. Desmarchelier. (1992). Restriction fragment length polymorphic analysis of *Listeria monocytogenes* and its application to epidemiological investigations. *Int. J. Food Microbiol.* **15:**347-356.

Linde K., Abraham A.A., and J. Beer. (1991). Stable *Listeria monocytogenes* live vaccine candidate strains with graded attenuation on the mouse model. *Vaccine.* **9:**101-105.

Lingnau A., Domann E., Hudel M., Bock M., Nichterlein T., Wehland J., and T. Chakraborty. (1995). Expression of the *Listeria monocytogenes* EGD *inlA* and *inlB* genes, whose products mediate bacterial entry into tissue culture cell lines, by PrfA-dependent and -independent mechanisms. *Infect. Immun.* **63:**3896-3903.

Linnan M.J., Mascola L., Lou X.D., Goulet V., May S., Salminen C., Hird D.W., Yonekura M.L., Hayes P., Weaver R., Audurier A., Plikaytis B.D., Fannin S.L., Kleks A., and C.L. Broome. (1988). Epidemic listeriosis associated with Mexican-style cheese. *N. Engl. J. Med.* **319:**823-828.

Lissner C.R., Swanson R.N., and A.D. O'Brien. (1983). Genetic control of the innate resistance of mice to *Salmonella typhimurium*: expression of the Ity gene in peritoneal and splenic macrophages isolated *in vitro. J. Immunol.* **131:**3006-3013.

Lorber B. (1990). Clinical listeriosis - implications for pathogenesis. In *Foodborne Listeriosis,* (A.J. Miller, J.L. Smith and G.A. Somkuti, eds.). Elsevier Science Publications Co., Amsterdam, pp. 41-49.

Love P.E., Lyle M.J., and R.E. Yasbin. (1985). DNA-damage-inducible (*din*) loci are transcriptionally activated in competent *Bacillus subtilis. Proc. Natl. Acad. Sci.* USA. **82:**6201-6205.

Lovett J. (1990). Taxonomy and general characteristics of *Listeria* spp. In *Foodborne Listeriosis*, (A.J. Miller, J.L. Smith and G.A. Somkuti, eds.). Elsevier Science Publications Co., Amsterdam, pp. 9-12.

Luchansky J.B., Muriana P.M., and T.R. Klaenhammer. (1988). Application of electroporation for transfer of plasmid DNA to *Lactobacillus, Lactococcus, Leuconostoc, Listeria, Pediococcus, Bacillus, Staphylococcus, Enterococcus* and *Propionobacterium. Mol. Microbiol.* **2:**637-646.

Lucic M.R. (1994). Molecular analysis of two *Listeria monocytogenes* (DRDC8) Tn*917* insertion mutants. Honours Thesis, University of Adelaide, Adelaide, Australia.

Ludwig A., Jarchau T., Benz R., and W. Goebel. (1988). The repeat domain of *Escherichia coli* haemolysin (HlyA) is responsible for its Ca^{2+}-dependent binding to erythrocytes. *Mol. Gen. Genet.* **214:**553-561.

Lugtenberg B., Meijers J., Peters R., Van Der Hoek P., and L. Van Alphen. (1975). Electrophoretic resolution of the "major outer membrane protein" of *Eschericia coli* K-12 into four bands. FEBS *Letts.* **58:**254-258.

Lutsenko S., and J.H. Kaplan. (1995). Organisation of P-type ATPases: significance of structural diversity. *Biochem.* **34:**15607-15613.

Ménard R., Sansonetti P.J., and C. Parsot. (1993). Nonpolar mutagenesis of the *ipa* genes defines IpaB, IpaC, and IpaD as effectors of *Shigella flexneri* entry into epithelial cells. *J. Bacteriol.* **175:**5899-5906.

Ménard R., Prevost M.-C., Gounon P., Sansonetti P.J., and C. Dehio. (1996). The secreted Ipa complex of *Shigella flexneri* promotes entry into mammalian cells. *Proc. Natl. Acad. Sci.* USA **93:**1254-1258.

MacDonald T.T., and P.B. Carter. (1980). Cell-mediated immunity to intestinal infection. *Infect. Immun.* **28:**516-523.

MacGowan A.P., O'Donaghue K., Nicholls S., McLauchlin J., Bennett P.M., and D.S. Reeves. (1993). Typing of *Listeria* spp. by random amplified polymorphic DNA (RAPD) analysis. *J. Med. Microbiol.* **38:**322-327.

Mackaness G.B. (1962). Cellular resistance to infection. *J. Exp. Med.* **116:**381-406.

Mahan M.J., Slauch J.M., and J.J. Mekalanos. (1993). Selection of bacterial virulence genes that are specifically induced in host tissues. *Science* **259:**686-688.

Manning P.A., Heuzenroeder M.W., Yeadon J., Leavesley D.I., Reeves P.R., and D. Rowley. (1986). Molecular cloning and expression in *Eschericia coli* K-12 of the O antigens of the Ogawa and Inaba serotypes of the lipopolysaccharide of *Vibrio cholera* O1 and their potential for vaccine development. *Infect. Immun.* **53:**272-277.

Manoil C., and J. Beckwith. (1985). Tn*phoA*: a transposon probe for protein export signals. *Proc. Natl. Acad. Sci.* USA **82:**8129-8133.

Marget W., and H.P.R. Seeliger. (1988). *Listeria monocytogenes* infections: therapeutic possibilities and problems. *Infection* **16:**S175-S177.

Marquis H., Bouwer H.G.A., Hinrichs D.J., and D.A. Portnoy. (1993). Intracytoplasmic growth and virulence of *Listeria monocytogenes* auxotrophic mutants. *Infect. Immun.* **61:**3756-3760.

Marquis H., Doshi V., and D.A. Portnoy. (1995). The broad-range phospholipase C and a metalloprotease mediate listeriolysin O - independent escape of *Listeria monocytogenes* from a primary vacuole in human epithelial cells. *Infect. Immun.* **63:**4531-4534.

Martin B., Alloing G., Mejean V., and J.-P. Claverys. (1987). Constitutive expression of erythromycin resistance mediated by the *ermAM* determinant of plasmid pAM-β1 results from deletion of 5′ leader peptide sequences. *Plasmid* **18:**250-253.

Matar G.M., Bibb W.F., Helsel L., Dewitt W., and B. Swaminathan. (1992). Immunoaffinity purification, stabilization and comparative characterization of listeriolysin O from *Listeria monocytogenes* serotypes 1/2a and 4b. *Res. Microbiol.* **143:**489-498.

Matousek-de-Abel-de-la-Cruz A.J., Burguera J.L., Burguera M., and N. Anez. (1993). Changes in the total content of iron, copper, and zinc in serum, heart, liver, spleen, and skeletal muscle tissues of rats infected with *Trypanosoma cruzi*. *Biol. Trace. Elem. Res.* **37:**51-70.

Mattingly J.A., Butman B.T., Plank M.C., Durham R.J., and B.J. Robinson. (1988). Rapid monoclonal antibody-based enzyme-linked immunosorbent assay for detection of *Listeria* in food products. *J. Assoc. Off. Anal. Chem.* **71:**679-681.

Maurelli A.T. (1989). Temperature regulation of virulence genes in pathogenic bacteria: a global strategy for human pathogens. *Microb. Pathog.* **7:**1-10.

Maurelli A.T., and P.J. Sansonetti. (1988). Genetic determinants of *Shigella* pathogenicity. *Annu. Rev. Microbiol.* **42:**127-150.

Maurelli A.T., Baudry B., d'Hauteville H., Hale T.L., and P.J. Sansonetti. (1985). Cloning of plasmid DNA sequences involved in invasion of HeLa cells by *Shigella flexneri*. *Infect. Immun.* **49:**164-171.

Mayer L.W. (1988). Use of plasmid profiles in epidemiologic surveillence of disease outbreaks and in tracing the transmission of antibiotic resistance. *Clin. Microbiol. Rev.* **1:**228-243.

McClain M.S., Hurley M.C., Brieland J.K., and N.C. Engleberg. (1996). The *Legionella pnuemophila hel* locus encodes intracellularly induced homologs of heavy-metal ion transporters of *Alcaligenes* spp. *Infect. Immun.* **64:**1532-1540.

McClure P.J., Roberts T.A., and P. Otto-Oguru. (1989). Comparison of the effects of sodium chloride, pH and temperature on the growth of *Listeria monocytogenes* on gradient plates and in liquid medium. *Letts. Appl. Microbiol.* **9:**95-99.

McCord J.M., and I. Fridovich. (1969). Superoxide dismutase - an enzymatic function for erythrocuprein. *J. Biol. Chem.* **244:**6049-6055.

McGowan A.P., Peterson P.K., Keane W., and P.G. Quie. (1983). Human peritoneal macrophage phagocytic killing and chemoluminescent response to opsonized *Listeria monocytogenes*. *Infect. Immun.* **40:**440-443.

McLauchlin J.A. (1987). *Listeria monocytogenes*, recent advances in the taxonomy and epidemiology of listeriosis in humans. *J. Appl. Bacteriol.* **63:**1-11.

McLauchlin J.A. (1995). What is the infective dose for human listeriosis. In *Proceedings of the 12th International Symposium on Problems of Listeriosis*, 2-6 October, Perth, Australia, pp. 365-369.

McLauchlin J.A., and P.N. Pini. (1989). The rapid demonstration and presumptive identification of *Listeria monocytogenes* in food using monoclonal antibodies in a direct immunofluorescent test (DIFT). *Letts. Appl. Microbiol.* **8:**25-27.

McLauchlin J.A., Saunders N.A., Ridley A.M., and A.G. Taylor. (1988). Listeriosis and foodborne transmission. *Lancet.* **i:**177-178.

Mehta P.K., Heringa J., and P. Argos. (1995). A simple and fast approach to prediction of protein secondary structure from multiply aligned sequences with accuracy above 70%. *Prot. Science* **4:**2517-2525.

Mekalanos J.J. (1992) Environmental signals controlling expression of virulence determinants in bacteria. *J. Bacteriol.* **174:**1-7.

Melchers K., Weitzenegger T., Buhmann A., Steinhilber W., Sachs G., and K.P. Schäfer. (1996). Cloning and membrane topology of a P-type ATPase from *Helicobacter pylori. J. Biol. Chem.* **271:**446-457.

Melin L., Magnusson K., and L. Rutberg. (1987). Identification of the promoter of the *Bacillus subtilis sdh* operon. *J. Bacteriol.* **169:**3232-3236

Mencíková E. (1989). Phospholipase C in *Listeria. Acta Microbiol. Hung.* **36:**321-325.

Mengaud J., Chenevert J., Geoffroy C., Gaillard J-L., and P. Cossart. (1987). Identification of the structural gene encoding the SH-activated hemolysin of *Listeria monocytogenes*: listeriolysin O is homologous to streptolysin O and pneumolysin. *Infect. Immun.* **55:**3225-3227.

Mengaud J., Vicente M.-F., Chenevert J., Pereira J.M., Geoffroy C., Gicquel-Sanzey B., Baquero F., Pérez-Díaz J.C., and P. Cossart. (1988). Expression in *Escherichia coli* and sequence analysis of the listeriolysin O determinant of *Listeria monocytogenes. Infect. Immun.* **56:**766-772.

Mengaud J., Vicente M.F., and P. Cossart. (1989). Transcriptional mapping and nucleotide sequence of the *Listeria monocytogenes hlyA* region reveal structural features that may be involved in regulation. *Infect. Immun.* **57:**3695-3701.

Mengaud J., Dramsi S., Gouin E., Vazquez-Boland J.-A., Milon G., and P. Cossart. (1991a). Pleiotropic control of *Listeria monocytogenes* virulence factors by a gene that is autoregulated. *Mol. Microbiol.* **5:**2273-2283.

Mengaud J., Geoffroy C., and P. Cossart. (1991b). Identification of a new operon involved in *Listeria monocytogenes* virulence: its first gene encodes a protein homologous to bacterial metalloproteases. *Infect. Immun.* **59:**1043-1049.

Mengaud J., Ohayon H., Gounon P., Mege R.M., and P. Cossart. (1996). E-cadherin is the receptor for internalin, a surface protein required for entry of *L. monocytogenes* into epithelial cells. *Cell* **84:**923-932.

Mercer J.F.B., Livingston J., Hall B., Paynter J.A., Begy C., Chandrasekharappa S., Lockhart P., Grimes A., Bhave M., Siemieniak D., and T.W. Glover. (1993). Isolation of a partial candidate gene for Menkes disease by positional cloning. *Nature Genet.* **3:**20-25.

Mertz W. (1981). The essential trace elements. *Science* **213:**1332-1336.

Michel E., and P. Cossart. (1992). Physical map of the *Listeria monocytogenes* chromosome. *J. Bacteriol.* **174:**7098-7103.

Michel E., Reich K.A., Favier R., Berche P., and P. Cossart. (1990). Attenuated mutants of the intracellular bacterium *Listeria monocytogenes* obtained by single amino acid substitutions in listeriolysin O. *Mol. Microbiol.* **4:**2167-2178.

Miller J.F., Mekalanos J.J., and S. Falkow. (1989). Coordinate regulation and sensory transduction in the control of bacterial virulence. *Science* **24:**916-922.

Miller J.H. (1972). Experiments in molecular genetics. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, pp. 352-355.

Miller S.I. (1991). PhoP/PhoQ: macrophage-specific modulators of *Salmonella* virulence? *Mol. Microbiol.* **5:**2073-2078.

Miller V.L. (1995). Tissue-culture invasion: fact or artefact? *Trends Microbiol.* **3:**69-71.

Miller V.L., and J.J. Mekalanos. (1988). A novel suicide vector and its use in construction of insertion mutations: osmoregulation of outer membrane proteins and virulence determinants in *Vibrio cholerae* requires *toxR*. *J. Bacteriol.* **170:**2575-2583.

Mills J.A., Buysse J.M., and E.V. Oaks. (1988). *Shigella flexneri* invasion plasmid antigens B and C: epitope location and characterization by monoclonal antibodies. *Infect. Immun.* **56:**2933-2941.

Mingyuan L., Ruifu Y., Zengshan L., and G. Zhaobiao. (1995). Use of multiplex polymerase chain reaction to detect *Listeria monocytogenes* in food. In *Proceedings of the 12th International Symposium on Problems of Listeriosis,* 2-6 October, Perth, Australia, pp. 451-455.

Moore M.A., and A.R. Datta. (1994). DNA fingerprinting of *Listeria monocytogenes* strains by pulse-field gel electrophoresis. *Food Microbiol.* **11:**31-38.

Morange M., Hévin B., and R.M. Fauve. (1993). Differential heat-shock protein synthesis and response to stress in three avirulent and virulent *Listeria* species. *Res. Immunol.* **144:**667-677.

Morgan P.J., Hyman S.C., Byron O., Andrew P.W., Mitchell T.J., and A.J. Rowe. (1994). Modelling the bacterial protein toxin, pneumolysin, in its monomeric and oligomeric form. *J. Biol. Chem.* **269:**25315-25320.

Mounier J., Ryter A., Coquis-Rondon M., and P.J. Sansonetti. (1990). Intracellular and cell-to-cell spread of *Listeria monocytogenes* involves interaction with F-actin in the enterocyte-like cell line Caco-2. *Infect. Immun.* **58:**1048-1058.

Munk M.E., and S.H.E. Kaufmann. (1988). *Listeria monocytogenes* reactive T lymphocytes in healthy individuals. *Microb. Pathog.* **5:**49-54.

Murray E.G.D., Webb R.A., and M.B.R. Swann. (1926). A disease in rabbits characterised by large mononuclear leucocytosis, caused by a hitherto undescribed bacillus *Bacterium monocytogenes* (n. sp.) *J. Pathol. Bacteriol.* **29:**407-439.

Murray P.J., and R.A. Young. (1992). Stress and immunological recognition in host-pathogen interactions. *J. Bacteriol.* **174:**4193-4196.

Myers E.R., Dallmier A.W., and S.E. Martin. (1993). Sodium chloride, potassium chloride, and virulence in *Listeria monocytogenes. Appl. Environ. Microbiol.* **59:**2082-2086.

Nakahama K., Yoshimura K., Marumoto R., Kikuchi M., Lee I.S., Hase T., and H. Matsubara. (1986). Cloning and sequencing of *Serratia* protease gene. *Nucl. Acids Res.* **14:**5843-5855.

Nakamura Y., Wada K., Wada Y., Doi H., Kanaya S., Gojobori T., and T. Ikemura. (1996). Condon usage tabulated from the international DNA sequence databases. *Nucl. Acids Res.* **24:**214-215.

Neilands J.B. (1981). Iron absorption and transport in microorganisms. *Annu. Rev. Nutr.* **1:**27-46.

Ní Bhriain N., Dorman C.J., and C.F. Higgins. (1989). An overlap between osmotic and anaerobic stress responses: a potential role for DNA supercoiling in the coordinate regulation of gene expression. *Mol. Microbiol.* **3:**933-942.

Nichterlein T., and H. Hof. (1991). Effect of various antibiotics on *Listeria monocytogenes* multiplying in L 929 cells. *Infection* **19:**S234-S238.

Niebuhr K., Chakraborty T., Rohde M., Gazlig T., Jansen B., Köllner P., and J. Wehland. (1993). Localization of the ActA polypeptide of *Listeria monocytogenes* in infected tissue culture cell lines: ActA is not associated with actin "comets". *Infect. Immun.* **61:**2793-2802.

Nieman R.E., and B. Lorber. (1980). Listeriosis in adults: a changing pattern. Report of eight cases and review of the literature, 1968-1978. *Rev. Infect. Dis.* **2:**207-227.

Nikaido H., and M. Vaara. (1987). Outer membrane. In Escherichia coli *and* Salmonella typhimurium *cellular and molecular biology* (F.C. Neidhardt, J.L. Ingraham, K.B. Low, B. Magasanik, M. Schaechter, and H.E. Umbarger, eds.), American Society for Microbiology, Washington, DC., pp. 7-22.

Njoku-Obi A.N., Jenkins E.M., Njoku-Obi J.C., Adams J., and V. Covington. (1963). Production and nature of *Listeria monocytogenes* hemolysins. *J. Bacteriol.* **86:**1-8.

Nocera D., Bannerman E., Rocourt J., Jaton-Ogay K., and J. Bille. (1990). Characterization by DNA restriction endonuclease analysis of *Listeria monocytogenes* strains related to the Swiss epidemic of Listeriosis. *J. Clin. Microbiol.* **28:**2259-2263.

Norqvist A., Norrman B., and H. Wolf-Watz. (1990). Identification and characterization of a zinc metalloprotease associated with invasion by the fish pathogen *Vibrio anguillarum. Infect. Immun.* **58:**3731-3736.

Norris V., Chen M., Goldberg M., Voskuil J., McGurk G., and I.B. Holland. (1991). Calcium in bacteria: a solution to which problem. *Mol. Microbiol.* **5:**775-778.

North R.J. (1974). T cell dependence of macrophage activation and mobilisation during infection with *Mycobacterium tuberculosis. Infect. Immun.* **10:**66-71.

Notermans S.H.W., Dufrenne J., Chakraborty T., Steinmeyer S., and G. Terplan. (1991a). The chick embryo test agrees with the mouse bio-assay for asssessment of the pathogenicity of *Listeria* species. *Letts. Appl. Microbiol.* **13:**161-164.

Notermans S.H.W., Dufrenne J., Leimeister-Wächter M., Domman E., and T. Chakraborty. (1991b). Phosphatidylinositol-specific phospholipase C activity as a marker to distinguish between pathogenic and nonpathogenic *Listeria* species. *Appl. Environ. Microbiol.* **57:**2666-2670.

Nucifora G., Chu L., Misra T.K., and S. Silver. (1989). Cadmium resistance from *Staphylococcus aureus* plasmid pI258 *cadA* gene results from a cadmium-efflux ATPase. *Proc. Natl. Acad. Sci.* USA **86:**3544-3548.

Oaks E.V., Wingfield M.E., and S.B. Formal. (1985). Plaque formation by virulent *Shigella flexneri. Infect. Immun.* **48:**124-129.

Odermatt A., Suter H., Krapf R., and M. Solioz. (1993). Primary structure of two P-type ATPases involved in copper homeostasis in *Enterococcus hirae. J. Biol. Chem.* **268:**12775-12779.

Odermatt A., Krapf R., and M. Solioz. (1994). Induction of the putative copper ATPases, CopA and CopB, of *Enterococcus hirae* by Ag^{+} and Cu^{2+}, and Ag^{+} extrusion by CopB. *Biochem. Biophys. Res. Commun.* **202:**44-48.

Ohta T., Nagano K., and M. Yoshida. (1986). The active site structure of Na^{+}/K^{+}-transporting ATPase: location of the 5'-(ρ-fluorosulfonyl) benzoyladenosine binding site and soluble peptides. *Proc. Natl. Acad. Sci.* USA **83:**2071-2075.

Okwumabua O., Swaminathan B., Edmonds P., Wenger J., Hogan J., and M. Alden. (1992). Evaluation of a chemiluminescent DNA probe assay for the rapid confirmation of *Listeria monocytogenes. Res. Microbiol.* **143:**183-189.

Onoda T., and A. Oshima. (1988). Effects of Ca^{2+} and a protonophore on growth of an *Escherichia coli* L-form. *J. Gen. Microbiol.* **134:**3071-3077.

Ordal G.W. (1977). Calcium ion regulates chemotactic behavior in bacteria. *Nature* **270:**66-67.

Otto B.R., Verweij-van Vught A.M.J.J., and D.M. MacLaren. (1992). Transferrins and heme-compounds as sources for pathogenic bacteria. *Crit. Rev. Microbiol.* **18:**217-233.

Pace J., Hayman M.J., and J.E. Galán. (1993). Signal transduction and invasion of epithelial cells by *S. typhimurium. Cell* **72:**505-514.

Pamer E.G. (1993). Cellular immunity to intracellular bacteria. *Curr. Opin. Immunol.* **5:**492-496.

Pamer E.G., Harty J.T., and M.J. Bevan. (1991). Precise prediction of a dominant class I MHC-restricted epitope of *Listeria monocytogenes. Nature* **353:**852-855.

Park S.F., and G.S.A.B. Stewart. (1990). High efficiency transformation of *Listeria monocytogenes* by electroporation of penicillin-treated cells. *Gene* **94:**129-132.

Park S.F., Stewart G.S.A.B., and R.G. Kroll. (1992). The use of bacterial luciferase for monitoring the environmental regulation of expression of genes encoding virulence factors in *Listeria monocytogenes. J. Gen. Microbiol.* **138:**2619-2627.

Parrisius J., Bhakdi S., Roth M., Tranum-Jensen J., Goebel W., and H.P.R. Seeliger. (1986). Production of listeriolysin by beta-hemolytic strains of *Listeria monocytogenes. Infect. Immun.* **51:**314-319.

Passos A.L.S., Andrew P.W., Jones D., and I.S. Roberts. (1995). Studies of the growth of *Listeria monocytogenes* at low temperature. In *Proceedings of the 12th International Symposium on Problems of Listeriosis.* 2-6 October, Perth, Australia, pp. 411-415.

Payne S.M. (1993). Iron acquisition in microbial pathogenesis. *Trends Microbiol.* **1:**66-69.

Pearson W.R., and D.J. Lipman. (1988). Improved tools for biological comparison. *Proc. Natl. Acad. Sci.* USA **85**: 2444-2448.

Pedersen P.L., and E. Carafoli. (1987). Ion motive ATPases. I. Ubiquity, properties, and significance to cell function. *Trends Biochem. Sci.* **12:**146-150.

Peel M., Donachie W., and A. Shaw. (1988a). Temperature-dependent expression of flagella of *Listeria monocytogenes* studied by electron microscopy, SDS-PAGE and Western blotting. *J. Gen. Microbiol.* **134:**2171-2178.

Peel M., Donachie W., and A. Shaw. (1988b). Physical and antigenic heterogeneity in the flagellins of *Listeria monocytogenes* and *L. ivanovii. J. Gen. Microbiol.* **134:**2593-2598.

Pérez-Díaz J.C., Vicente M.F., and F. Baquero. (1982). Plasmids in *Listeria. Plasmid* **8:**112-118.

Persson B., and P. Argos. (1994). Prediction of transmembrane segments in proteins utilising multiple sequence alignments. *J. Mol. Biol.* **237:**182-192.

Phizicky E.M., and S. Fields. (1995). Protein-protein interactions: methods for detection and analysis. *Microbiol. Rev.* **59:**94-123

Phung L.T., Ajlani G., and R. Haselkorn. (1994). P-type ATPase from the cyanobacterium *Synechococcus* 7942 related to the human Menkes and Wilson disease gene products. *Proc. Natl. Acad. Sci.* USA **91:**9651-9654.

Piffaretti J.-C., Kressbuch H., Aeschbacher M., Bille J., Bannerman E., Musser J.M., Selander R.K., and J. Rocourt. (1989). Genetic characterization of clones of the bacterium *Listeria monocytogenes* causing epidemic disease. *Proc. Natl. Acad. Sci.* USA **86:**3818-3822.

Pine L., Kathariou S., Quinn F., George V., Wenger J.D., and R.E. Weaver. (1991). Cytopathogenic effects in enterocytelike Caco-2 cells differentiate virulent from avirulent *Listeria* strains. *J. Clin. Microbiol.* **29:**990-996.

Pinkney M., Beachey E., and M. Kehoe. (1989). The thiol-activated toxin streptolysin O does not require a thiol group for cytolytic activity. *Infect. Immun.* **57:**2553-2558.

Pinto M., Robine-Leon S., Appay M.D., Kedinger M., Triadou N., Dussaulx E., Lacroix B., Simon-Assmann P., Haffen K., Fogh J., and A. Zweibaum. (1983). Enterocyte-like differentiation and polarization of the human colon carcinoma cell line Caco-2 in culture. *Biol. Cell* **47:**323-330.

Pistor S., Chaklraborty T., Niebuhr K., Domann E., and J. Wehland. (1994). The ActA protein of *Listeria monocytogenes* acts as a nucleator inducing reorganization of the actin cytoskeleton. *EMBO J.* **13:**7589-763.

Pistor S., Chakraborty T., Walter U., and J. Wehland. (1995). The bacterial actin nucleator protein ActA of *Listeria monocytogenes* contains multiple binding sites for host microfilament proteins. *Curr. Biol.* **5:**517-525.

Polla B.S. (1991). Heat shock proteins in host-parasite interactions. *Immunoparasitol. Today* A38-A41.

Pollack C., Straley S.C., and M.S. Klempner. (1986). Probing the phagolysosomal environment of human macrophages with a Ca^{2+}-responsive operon fusion in *Yersinia pestis. Nature.* **322:**834-836.

Poole K., Heinrichs D.E., and S. Neshat. (1993). Cloning and sequence analysis of an EnvCD homologue in *Pseudomonas aeruginosa*: regulation by iron and possible involvement in the secretion of the siderophore pyoverdine. *Mol. Microbiol.* **10:**529-544.

Portnoy D.A., Jacks P.S., and D.J. Hinrichs. (1988). Role of hemolysin for the intracellular growth of *Listeria monocytogenes. J. Exp. Med.* **167:**1459-1471.

Portnoy D.A., Chakraborty T., Goebel W., and P. Cossart. (1992a). Molecular determinants of *Listeria monocytogenes* pathogenesis. *Infect. Immun.* **60:**1263-1267.

Portnoy D.A., Tweten R., Kehoe M., and J. Bielecki. (1992b). Capacity of listeriolysin O, streptolysin O, and perfringolysin O to mediate growth of *Bacillus subtilis* within mammalian cells. *Infect. Immun.* **60:**2710-2717.

Postle K. (1990). Aerobic regulation of the *Escherichia coli tonB* gene by changes in iron availability and the *fur* locus. *J. Bacteriol.* **172:**2287-2293.

Poyart C., Abachin E., Razafimanantsoa I., and P. Berche. (1993). The zinc metalloprotease of *Listeria monocytogenes* is required for maturation of phosphatidylcholine phospholipase C: direct evidence by gene complementation. *Infect. Immun.* **61:**1576-1580.

Poyart-Salmeron C., Carlier C., Trieu-Cuot P., Courtieu A.-L., and P. Courvalin. (1990). Transferable plasmid-mediated antibiotic resistance in *Listeria monocytogenes. Lancet* **335:**1422-1426.

Premaratne R.J., Lin W.-L., and E.A. Johnson. (1991). Development of an improved chemically defined minimal medium for *Listeria monocytogenes. Appl. Environ. Microbiol.* **57:**3046-3048.

Püttmann M., Ade N., and H. Hof. (1993). Dependence of fatty acid composition of *Listeria* spp. on growth temperature. *Res Microbiol.* **144:**279-283.

Quinn F., Pine L., White E., George V., Gutekunst K., and B. Swaminathan. (1993). Immunogold labelling of *Listeria monocytogenes* virulence-related factors within Caco-2 cells. *Res. Microbiol.* **144:**597-608.

Rácz P., Tenner K., and E. Mérö. (1972). Experimental *Listeria* enteritis. I. An electron microscopic study of the epithelial phase in experimental *Listeria* infection. *Lab. Invest.* **26:**694-700.

Ralovich B. (1989). Data on the enrichment and selective cultivation of *listeriae. Int. J. Food. Microbiol.* **8:**205-217.

Ratcliff S.W., Luh J., Ganesan A.T., Behrens B., Thompson R., Montenegro M., Morelli G., and T.A. Trautner. (1979). The genome of *B. subtilis* phage SPP1: the arrangement of restriction endonuclease generated fragments. *Mol. Gen. Genet.* **168:**165-172.

Rathjen D.A., and C.I. Geczy. (1986). Conditioned medium from macrophage cell line supports the single-cell growth of hybridomas. *Hybridoma.* **5:**255-261.

Raveneau J., Geoffroy C., Beretti J.-L., Gaillard J.-L., Alouf J.E., and P. Berche. (1992). Reduced virulence of a *Listeria monocytogenes* phospholipase-deficient mutant obtained by transposon insertion into the zinc metalloprotease gene. *Infect. Immun.* **60:**916-921.

Raybourne R.B., and V.K. Bunning. (1994). Bacterium-host cell interactions at the cellular level: fluorescent labelling of bacteria and analysis of short-term bacterium-phagocytic interaction by flow cytometry. *Infect. Immun.* **62:**665-672.

Reed K. (1990). Basic blotting - a quick fix. *Today's Life Sci.* **2:**52-60.

Relman D., Tuomanen E., Falkow S., Golenbock D., Saukkonen K., and S.D. Wright. (1990). Recognition of a bacterial adhesin by an integrin: macrophage CR3 ($\alpha_M\beta_2$, CD11b/CD18) binds filamentous hemagglutinin of *Bordetella pertussis. Cell* **61:**1375-1382.

Rocourt J., and P.A.D. Grimont. (1983). *Listeria welshimeri* sp. nov. and *Listeria seeligeri* sp. nov. *Int. J. Syst. Bacteriol.* **37:**866-869.

Rocourt J., and H.P.R. Seeliger. (1987). Is haemolysis an in vitro marker of the pathogenic strains of the genus *Listeria*. *Ann. Inst. Pasteur Microbiol.* **138:**277-279.

Rocourt J., Schrettenbrunner A., and H.P.R. Seeliger. (1982a). Isolation of bacteriophages from *Listeria monocytogenes* serovar 5 and *Listeria innocua*. *Zentralbl. Bakteriol. Mikrobiol. Hyg.* **251:**505-511.

Rocourt J., Grimont F., Grimont P.A.D., and H.P.R. Seeliger. (1982b). DNA relatedness among serovars of *Listeria monocytogenes sensu lato*. *Curr. Microbiol.* **7:**383-388

Rocourt J., Alonso J.M., and H.P.R. Seeliger. (1983). Virulence comparée des cinq groupes génomiques de *Listeria monocytogenes* (*sensu lato*). *Ann. Inst. Pasteur Microbiol.* **134A:**359-364.

Rocourt J., Audurier A., Courtieu A.L., Durst J., Ortel S., Schrettenbrunner A., and A.G. Taylor. (1985). A multi-centre study on the phage typing of *Listeria monocytogenes*. *Zentralbl. Bakteriol. Parasitenkd. Infektionskr. Hyg.* **259:**489-497.

Rocourt J., Wehmeyer U., and L. Stackebrandt. (1987). Transfer of *Listeria denitrificans* to a new genus, *Jonesia* gen. nov. as *Jonesia denitrificans* comb. nov. *Int. J. System. Bacteriol.* **37:**266-270.

Rocourt J., Boerlin P., Grimont F., Jacquet C., and J.C. Piffaretti. (1992). Assignment of *Listeria grayi* and *Listeria muurayi* to a single species, *Listeria grayi*, with a revised description of *Listeria grayi*. *Int. J. Syst. Bacteriol.* **42:**171-174.

Rogers H.W., Callery M.P., Deck B., and E.R. Unanue. (1996). *Listeria monocytogenes* induces apoptosis of infected hepatocytes. *J. Immunol.* **156:**679-684.

Rohde J.R., Fox J.M., and S.A. Minnich (1994). Thermoregulation in *Yersinia enterocolitica* is coincident with changes in DNA supercoiling. *Mol. Microbiol.* **12:**187-99

Rood J.I., and J.M. Gawthorne. (1984). Apple software for analysis of the size of restriction fragments. *Nucl. Acids Res.* **12:**689-694.

Rosenshine I., Donnenberg M.S., Kaper J.B., and B.B. Finlay. (1992). Signal transduction between enteropathogenic *Escherichia coli* (EPEC) and epethelial cells: EPEC induces tyrosine phosphorylation of host cell proteins to initiate cytoskeletal rearrangement and bacterial uptake. *EMBO J.* **11:**3551-3560.

Rossen L., Holmstrom K., Olsen J.E., and O.F. Rasmussen. (1991). A rapid polymerase chain reaction (PCR) - based assay for the identification of *Listeria monocytogenes* in food samples. *Int. J. Food Microbiol.* **14:**145-151.

Rost B., and C. Sander. (1993). Prediction of protein structure at better than 70% accuracy. *J. Mol. Biol.* **232:**584-599.

Rost B., and C. Sander. (1994). Combining evolutionary information and neural networks to predict protein secondary structure. *Proteins* **19:**55-72.

Rost B., Casadio R., Fariselli P., and C. Sander. (1995). Transmembrane helices predicted at 95% accuracy. *Protein Sci.* **4:**521-533.

Rousset M. (1986). The human colon carcinoma cell lines HT-29 and Caco-2: two *in vitro* models for the study of intestinal differentiation. *Biochimie.* **68:**1035-1040.

Ruhland G.J., Hellwig M., Wanner G., and F. Fiedler. (1993). Cell-surface location of *Listeria*-specific protein p60 - detection of *Listeria* cells by indirect immunofluorescence. *J. Gen. Microbiol.* **139:**609-616.

Ryser E.T., Arimi S.M., Pritchard T.J., Bunduki M., and C.W. Donnelly. (1995). Applications of automated ribotyping for the food industry. In *Proceedings of the 12th International Symposium on Problems of Listeriosis*, 2-6 October, Perth, Australia, pp. 103-116.

Sadoff J.C., Ballou W.R., Baron L.S., Majarian W.R., Brey R.N., Hockmeyer W.T., Young J.F., Cryz S.J., Ou J., Lowell G.H., and J.D. Chulay. (1988). Oral *Salmonella typhimurium* vaccine expressing circumsporozoite protein protects against malaria. *Science* **240**:336-338.

Sahm D.F., and J.A. Washington II. (1991). Antibacterial susceptibility tests: dilution methods. In *Manual of Clinical Microbiology*, (E.H. Lennette, A. Balows, W.J. Hausler Jr, and H.J. Shadomy, eds.), American Society for Microbiology, Washington, DC., pp. 1105-1116.

Saier M.H., Fagan M.J., Hoischen C., and J. Reizer. (1994). Transport mechanisms. In Bacillus subtilis *and other Gram-positive bacteria (Biochemistry, Physiology and molecular genetics)*, (A.L. Sonenshein, J.A. Hoch, and R. Losick, eds.), American Society for Microbiology, Washington, DC., pp. 133-156.

Saint-Joanis B., Garnier T., and S.T. Cole. (1989). Gene cloning shows the alpha-toxin of *Clostridium perfringens* to contain both sphingomyelinase and lecithinase activities. *Mol. Gen. Genet.* **219**:453-460.

Sambrook J., Fritsch E.F., and T. Maniatis. (1989). Molecular cloning: a laboratory manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Sanchez-Campillo M., Dramsi S., Gómez-Gómez J.M., Michel E., Dehoux P., Cossart P., Baquero F., and J.C. Pérez-Díaz. (1995). Modulation of DNA topology by *flaR*, a new gene from *Listeria monocytogenes*. *Mol. Microbiol.* **18**:801-811.

Sanger J.M., Southwick F.S., and J.W. Sanger. (1990). Intracellular propulsion of *Listeria* inside PtK2 cells is coupled to actin polymerisation. *J. Cell. Biol.* **111**:390a.

Sanger J.M., Sanger J.W., and F.S. Southwick. (1992). Host actin assembly is necessary and likely to provide the propulsive force for intracellular movement of *Listeria monocytogenes*. *Infect. Immun.* **60**:3609-3619.

Sansonetti P.J., Kopecko D.J., and S.B. Formal. (1981). *Shigella sonnei* plasmids: evidence that a large plasmid is necessary for virulence. *Infect. Immun.* **34**:75-83.

Sansonetti P.J., Kopecko D.J., and S.B. Formal. (1982). Involvement of a plasmid in the invasive ability of *Shigella flexneri*. *Infect. Immun.* **35**:852-860.

Sansonetti P.J., Ryter A., Clerc P., Maurelli A.T., and J. Mounier. (1986). Multiplication of *Shigella flexneri* within HeLa cells: lysis of the phagocytic vacuole and plasmid-mediated contact hemolysis. *Infect. Immun.* **51**:461-469.

Sarkar B., Lingertat-Walsh K., and J.T. Clarke. (1993). Copper-histidine therapy for Menkes disease. *J. Pediatr.* **123**:828-830.

Saunders F.K., Mitchell T.J., Walker J.A., Andrew P.W., and G.J. Boulnois. (1989). Pneumolysin, the thiol-activated toxin of *Streptococcus pneumoniae*, does not require a thiol group for *in vitro* activity. *Infect. Immun.* **57**:2547-2552.

Schäferkordt S., and T. Chakraborty. (1995). Molecular cloning, sequencing and characterisation of the determinants of the *lmaA*-operon whose gene products induce delayed-type-hypersensitivity in *Listeria monocytogenes* immune mice. In *Proceedings of the 12th International Symposium on Problems of Listeriosis,* 2-6 October, Perth, Australia, pp. 485.

Schafer R., Portnoy D.A., Brassell S.A., and Y. Paterson. (1992). Induction of a cellular immune response to a foreign antigen by a recombinant *Listeria monocytogenes* vaccine. *J. Immunol.* **149:**53-59.

Schindler M., and M.J. Osborn. (1979). Interaction of divalent cations and polymyxin B with lipopolysaccharide. *Biochem.* **18:**4425-4430.

Schlech III W.F. (1990). *Listeria,* animals and man: aspects of virulence. In *Foodborne Listeriosis,* (A.J. Miller, J.L. Smith, and G.A. Somkuti, eds.), Elsevier Science Publications Co., Amsterdam, pp. 51-54.

Schlech III W.F., Lavigne P.M., Bortolussi R.A., Allen A.C., Haldane E.V., Wort A.J., Hightower A.W., Johnson S.E., King S.H., Nicholls E.S., and C.V. Broome. (1983). Epidemic listeriosis - evidence for transmission by food. *N. Engl. J. Med.* **308:**203-206.

Schneewind O., Mihaylova-Petkov D., and P. Model. (1993). Cell wall sorting signals in surface proteins of Gram-positive bacteria. *EMBO J.* **12:**4803-4811.

Schön U., and W. Schumann. (1994). Construction of His_6-tagging vectors allowing single-step purification of GroES and other polypeptides produced in *Bacillus subtilis. Gene* **147:**91-94.

Schuchat A., Swaminathan B., and C.V. Broome. (1991). Epidemiology of human listeriosis. *Clin. Microbiol. Rev.* **4:**169-183.

Schved F., Lalazar A., Henis Y., and B.J. Juven. (1993). Purification, partial characterization and plasmid linkage of pediocin SJ-1, a bacteriocin produced by *Pediococcus acidilactici. J. Appl. Bacteriol.* **74:**67-77.

Schwartz B., Hexter D., Broome C.V., Hightower A.W., Hirschhorn R.B., Porter J.D., Hayes P.S., Bibb W.F., Lorber B., and D.G. Faris. (1989). Investigation of an outbreak of listeriosis: new hypothesis for the etiology of epidemic *Listeria monocytogenes* infections. *J. Infect. Dis.* **159:**680-685.

Seeliger H.P.R. (1981). Apathoge listerien: *Listeria innocua* sp. *Zentralbl. Bakteriol. Mikrobiol. Hyg.* **249:**487-493.

Seeliger H.P.R., and D. Jones. (1986). *Listeria.* In *Bergey's Manual of Systematic Bacteriology,* vol. 2, (P.H.A. Sneath, N.S Mair, M.E. Sharpe, and J.G. Holt, eds.), Williams and Wilkins, Baltimore, Maryland, pp. 1235-1245.

Seeliger H.P.R., Rocourt J., Schrettenbrunner A., Grimont P.A.D., and D. Jones. (1984). Description of *Listeria ivanovii* sp. nov. *Int. J. Syst. Bacteriol.* **34:**336-337.

Selander R.K., Musser J.M., Caugant D.A., Gilmour M.N., and T.S. Whittam. (1987). Population genetics of pathogenic bacteria. *Microb. Pathog* **3:**1-7.

Serrano R. (1988). Structure and function of proton translocating ATPases in plasma membranes of plants and fungi. *Biochim. Biophys. Acta* **947:**1-28.

Serrano R., and F. Portillo. (1990). Catalytic and regulatory sites of yeast plasma membrane H^+-ATPase studied by directed mutagenesis. *Biochim. Biophys. Acta* **1018:**195-199.

Sharma D.P., Stroeher U.H., Thomas C.J., Manning P.A., and S.R. Attridge. (1989). The toxin-coregulated pilus (TCP) of *Vibrio cholerae*: molecular cloning of genes involved in pilus biogenesis and evaluation of TCP as a protective antigen in the infant mouse model. *Microb. Pathog.* **7**:437-438.

Shaw J.H., and D.B. Clewell. (1985). Complete nucleotide sequence of the macrolide-lincosamide-streptogramin B-resistance transposon Tn*917* in *Streptococcus faecalis. J. Bacteriol.* **164**:782-796.

Sheehan B., Kocks C., Dramsi S., Gouin E., Klarsfeld A.D., Mengaud J., and P. Cossart. (1994). Molecular and genetic determinants of the *Listeria monocytogenes* infectious process. *Curr. Top. Microbiol. Immunol.* **192**:187-216.

Sheehan B., Klarsfeld A., Msadek T., and P. Cossart. (1995). Differential activation of virulence gene expression by PrfA, the *Listeria monocytogenes* virulence regulator. *J. Bacteriol.* **177**:6469-6476.

Shen H., Slifka M.K., Matloubian M., Jensen E.R., Ahmed R., and J.F. Miller. (1995). Recombinant *Listeria monocytogenes* as a live vaccine vehicle for the induction of protective ant-viral cell-mediated immunity. *Proc. Natl. Acad. Sci.* USA **92**:3987-3991.

Shull G.E., Schwartz A., and J.B. Lingrel. (1985). Amino acid sequence of the catalytic subunit of the (Na^++K^+) ATPase deduced from a complementary DNA. *Nature* **316**:691-695.

Siddique I.H., Fong Lin I., and R.A. Chung. (1974). Purification and characterization of hemolysin produced by *Listeria monocytogenes. Am. J. Vet. Res.* **35**:289-296.

Sidler W., Niederer E., Suter F., and H. Zuber. (1986). The primary structure of *Bacillus cereus* neutral protease and comparison with thermolysin and *Bacillus subtilis* neutral proteinase. *Biol. Chem.* **367**:643-657.

Signas C., Raucci G., Jönsson K., Lindgren P.-E., Anantharamaiah G.M., Höök M., and M. Lindberg. (1989). Nucleotide sequence of the gene for a fibronectin-binding protein from *Staphylococcus aureus*: use of this peptide sequence in the synthesis of biologically active peptides. *Proc. Natl. Acad. Sci.* USA **86**:699-703.

Silver S. (1978). Transport of cations and anions. In *Bacterial transport*, (B.P. Rosen, ed.), Marcel Dekker, Inc., New York, pp. 221-324.

Silver S., and M. Walderhaug. (1992). Gene regulation of plasmid- and chromosome-determined inorganic ion transport in bacteria. *Microbiol. Rev.* **56**:195-228.

Silver S., Nucifora G., Chu L., and T.K. Misra. (1989) Bacterial resistance ATPases: primary pumps for exporting toxic cations and anions. *Trends Biochem. Sci.* **14**:76-80.

Silver S., Nucifora G., and L.T. Phung. (1993). Human Menkes X-chromosome disease and the staphylococcal cadmium-resistance ATPase: a remarkable similarity in protein sequence. *Mol. Microbiol.* **10**:7-12.

Simon R., Priefer U., and A. Puhler. (1983). A broad host range mobilization system for *in vivo* genetic engineering: transposon mutagenesis in Gram-negative bacteria. *Biotechnology* **1**:784-794.

Sjödahl J. (1977). Repetitive sequences in protein A from *Staphylococcus aureus. Eur. J. Biochem.* 73:343-351.

Smith D.L., Tao T., and M.E. Maguire. (1993). Membrane topology of a P-type ATPase: the MgtB magnesium transport protein of *Salmonella typhimurium. J. Biol. Chem.* **268:**22469-22479.

Smith G.A., and D.A. Portnoy. (1994). The role of two phospholipases in the pathogenicity of *Listeria monocytogenes. Infect. Agents Dis.* **2:**183-185.

Smith G.A., Portnoy D.A., and J.A. Theriot. (1995). Asymmetric distribution of the *Listeria monocytogenes* ActA protein is required and sufficient to direct actin-based motility. *Mol. Microbiol.* **17:**945-951.

Smyth C.J., and J.L. Duncan. (1978). Thiol-activated (oxygen labile) cytolysins. In *Bacterial toxins and cell membranes,* (J. Jeljaszewicz, and T. Wadström, eds.), Academic Press, Inc., New York, pp. 129-183.

Snavely M.D., Florer J.B., Miller C.G., and M.E. Maguire. (1989). Magnesium transport in *Salmonella typhimurium*: $^{28}Mg^{2+}$ transport by the CorA, MgtA, and MgtB systems. *J. Bacteriol.* **171:**4761-4766.

Snavely M.D., Gravina S.A., Cheung T.T., Miller C.G., and M.E. Maguire. (1991). Magnesium transport in *Salmonella typhimurium*: regulation of *mgtA* and *mgtB* expression. *J. Biol. Chem.* **266:**824-829.

Sokolovic Z., and W. Goebel. (1989). Synthesis of listeriolysin in *Listeria monocytogenes* under heat shock conditions. *Infect. Immun.* **57:**295-298.

Sokolovic Z., Fuchs A., and W. Goebel. (1990). Synthesis of species-specific stress proteins by virulent strains of *Listeria monocytogenes. Infect. Immun.* **58:**3582-3587.

Sokolovic Z., Riedel J., Wuenscher M., and W. Goebel. (1993). Surface-associated, PrfA-regulated proteins of *Listeria monocytogenes* synthesized under stress conditions. *Mol. Microbiol.* **8:**219-227.

Solovyev V.V., and A.A. Salamov. (1994). Predicting α-helix and β-strand segments of globular proteins. *Comput. Appli. Biosci.* **10:**661-669.

Southern E.M. (1975). Detection of specific sequences among DNA fragments seperated by gel electrophoresis. *J. Mol. Biol.* **98:**503-517.

Southwick F.S., and D.L. Purich. (1994). Arrest of *Listeria* movement in the host cells by a bacterial ActA analogue: implications for actin-based motility. *Proc. Natl. Acad. Sci.* USA **91:**5168-5172.

Southwick F.S., and D.L. Purich. (1995). Inhibition of *Listeria* locomotion by mosquito oostatic factor, a natural oligoproline peptide uncoupler of profilin action. *Infect. Immun.* **63:**182-190.

Stephens J.C., Roberts I.S., Jones D., and P.W. Andrew. (1991). Effect of growth temperature on virulence of strains of *Listeria monocytogenes* in the mouse: evidence for a dose dependence. *J. Appl. Bacteriol.* **70:**239-244.

Stiles M.E., and J.W. Hastings. (1991). Bacteriocin production by lactic acid bacteria: potential for use in meat preservation. *Trends Food Sci. Technol.* **2:**247-251.

Stoner B.J. (1993). Use of Tn*917-lac* and *lacZ* fusions to investigate listeriolysin O expression in *Listeria monocytogenes* strain DRDC8. Honours Thesis, University of Adelaide, Adelaide, Australia.

Stover C.K., De La Cruz V.F., Fuerst T.R., Buerlein J.E., Benson L.A., Bennett L.T., Bansal G.P., Young J.F., Lee M.H., Hatfull G.F., Snapper S.B., Barletta R.G., Jacobs R.W., Jr, and B.R. Bloom. (1991). New use of BCG for recombinant vaccines. *Nature* **351:**456-460.

Sugiura A., Nakashima K., Tanaka K., and T. Mizuno. (1992). Clarification of the structural and functional features of the osmoregulated *kdp* operon of *Escherichia coli. Mol. Microbiol.* **6:**1769-1776.

Suk K., Das S., Sun W., Jwang B., Barthold S.W., Flavell R.A., and E. Fikrig. (1995). *Borrelia burgdorferi* genes selectively expressed in the infected host. *Proc. Natl. Acad. Sci.* USA **92:**4269-4273.

Sun A.N., Camilli A., and D.A. Portnoy. (1990). Isolation of *Listeria monocytogenes* small-plaque mutants defective for intracellular growth and cell-to-cell spread. *Infect. Immun.* **58:**3770-3778.

Sword C.P. (1966). Mechanisms of pathogenesis in *Listeria monocytogenes* infection. I. Influence of iron. *J. Bacteriol.* **92:**536-542.

Sword C.P., and G.C. Kingdon. (1971). *Listeria monocytogenes* toxin. In *Microbial toxins,* vol 2A, (S. Kadis, T.C. Montie, and S.J. Ajl, eds.), Academic Press, Inc., New York, pp. 357-377.

Tabor S., and C.C. Richardson. (1985). A bacteriophage T7 RNA polymerase/promoter system for controlled exclusive expression of specific genes. *Proc. Natl. Acad. Sci.* USA **82:**1074-1078.

Tagg J.R., Dajani A.S., and L.W. Wannamaker. (1976). Bacteriocins of Gram-positive bacteria. *Bacteriol. Rev.* **40:**722-756.

Tagliabue A. (1989). Immune response to oral *Salmonella* vaccines. *Curr. Top. Microbiol. Immunol.* **146:**225-231.

Takagi M., Imanaka T., and S. Aiba. (1985). Nucleotide sequence and promoter region for the neutral protease gene from *Bacillus stearothermophilus J. Bacteriol.* **163:**824-831.

Takata T., Fujimoto S., and K. Amako. (1992). Isolation of nonchemotactic mutants of *Campylobacter jejuni* and their colonisation of the mouse intestinal tract. *Infect. Immun.* **60:**3596-3600.

Tan A., Li H., Heaton S., and J.R.L. Forsyth. (1995). Probing epidemiological associations of *Listeria monocytogenes* with PFGE techniques. In *Proceedings of the 12th International Symposium on Problems of Listeriosis,* 2-6 October, Perth, Australia, pp. 191-194.

Tanaka M., and H. Shibata. (1985). Poly(L-proline)-binding proteins from chick embryos are a profilin and a profilactin. *Eur. J. Biochem.* **151:**291-297.

Tanycz I., and H.K. Ziegler. (1988). Listeriolysin: characterization and regulation. *FASEB J.* **i:**3434.

Tanzi R.E., Petrukhin K., Chernov I., Pellequer J.L., Wasco W., Ross B., Romano D.M., Parano E., Pavone L., Brzustowicz L.M., Devoto M., Peppercorn J., Bush A.I., Sternleib I., Pirastu M., Gusella J.F., Evgrafov O., Penchaszadeh G.K., Honig B., Edelman I.S., Soares M.B., Scheinberg I.H., and T.C. Gillam. (1993). The Wilson disease gene is a copper transporting ATPase with homology to the Menkes disease gene. *Nature Genet.* **5:**344-350.

Tao Y.-P., and P.S. Vary. (1991). Isolation and characterization of sporulation *lacZ* fusion mutants of *Bacillus megaterium. J. Gen. Microbiol.* **137:**797-806.

Temm-Grove C.J., Jockusch B.M., Rohde M., Niebuhr K., Chakraborty T., and J. Wehland. (1994). Exploitation of microfilament proteins by *Listeria monocytogenes*: microvillus-like composition of the comet tails and vectorial spreading in polarized epithelial sheets. *J. Cell Sci.* **107:**2951-2960.

Theriot J.A. (1995). The cell biology of infection by intracellular bacterial pathogens. *Annu. Rev. Cell Dev. Biol.* **11:**213-239.

Theriot J.A., and T.J. Mitchison. (1992). Actin polymer dynamics in the propulsion of the intracellular bacterial parasite *Listeria monocytogenes*. *Mol. Biol. Cell.* **3:**3a.

Theriot J.A., Mitchison T.J., Tilney L.G., and D.A. Portnoy. (1992). The rate of actin-based motility of intracellular *Listeria monocytogenes* equals the rate of actin polymerization. *Nature.* **357:**257-260.

Theriot J.A., Rosenblatt J., Portnoy D.A., Goldschmidt-Clermont P.J., and J. Mitchison. (1994). Involvement of profilin in the actin-based motility of *L. monocytogenes* in cells and in cell free extracts. *Cell* **76:**505-517.

Thomas C.J. (1995). PCR, RAPD, RFLP and PFGE methods for screening isolates of *Listeria monocytogenes.* In *Proceedings of the 12th International Symposium on Problems of Listeriosis*: Listeria *Methods Workshop Manual*, 2-6 October, Perth, Australia, pp. 84-101.

Thomas E.J., King R.K., Burchak J., V.P. Gannon. (1991). Sensitive and specific detection of *Listeria monocytogenes* in milk and ground beef with the polymerase chain reaction. *Appl. Environ. Microbiol.* **57:**2576-2580.

Thompson J.D., Higgins D.G., and T.J Gibson. (1994). CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position gap penalties and weight matrix choice. *Nucl. Acids Res.* **22:**4673-4680

Tichaczek P.S., Vogel R.F., and W.P. Hammes. (1993). Cloning and sequencing of *curA* encoding curvacin A, the bacteriocin produced by *Lactobacillus curvatus* LTH1174. *Arch. Microbiol.* **160:**279-283.

Tilney L.G., and D.A. Portnoy. (1989). Actin filaments and the growth, movement, and spread of the intracellular bacterial parasite, *Listeria monocytogenes. J. Cell Biol.* **109:**1597-1608.

Tilney L.G., and M.S. Tilney. (1993). The wily ways of a parasite: induction of actin assembly by *Listeria. Trends Microbiol.* **1:**25-31.

Tilney L.G., Connelly P.S., and D.A. Portnoy. (1990). Actin filament nucleation by the bacterial pathogen, *Listeria monocytogenes. J. Cell Biol.* **111:**2979-2988.

Tilney L.G., DeRosier D.J., and M.S. Tilney. (1992a). How *Listeria* exploits host cell actin to form its own cytoskeleton. I. Formation of a tail and how that tail might be involved in movement. *J. Cell Biol.* **118:**71-81.

Tilney L.G., DeRosier D.J., Weber A., and M.S. Tilney. (1992b). How *Listeria* exploits host cell actin to form its own cytoskeleton. II. Nucleation, actin filament polarity, filament assembly, and evidence for a pointed end capper. *J. Cell Biol.* **118:**83-93.

Titani K., Hermodson M.A., Ericsson L.H., Walsh K.A., and H. Neurath. (1972). Amino acid sequence of thermolysin. *Nature* **238:**35-37.

Titball R.W., Hunter S.E.C., Martin K.L., Morris B.C., Shuttleworth A.D., Rubidge T., Anderson D.W., and D.C. Kelly. (1989). Molecular cloning and nucleotide sequence of the alpha-toxin (phospholipase C) of *Clostridium perfringens. Infect. Immun.* **57:**367-376.

Trieu-Cuot P., Carlier C., Martin P., and P. Courvalin. (1987). Plasmid transfer by conjugation from *Escherichia coli* to Gram-positive bacteria. FEMS *Microbiol. Letts.* **48:**289-294.

Trieu-Cuot P., Carlier C., Poyart-Salmeron C., and P. Courvalin. (1990). A pair of mobilizable shuttle vectors conferring resistance to spectinomycin for molecular cloning in *Escherichia coli* and Gram-positive bacteria. *Nucl. Acids Res.* **18:**4296.

Trieu-Cuot P., Carlier C., Poyart-Salmeron C., and P. Courvalin. (1991). Shuttle vectors containing a multiple cloning site and a *lacZα* gene for conjugal transfer of DNA from *Escherichia coli* to Gram-positive bacteria. *Gene* **102:**99-104.

Trieu-Cuot P., Derlot E., and P. Courvalin. (1993). Enhanced conjugative transfer of plasmid DNA from *Escherichia coli* to *Staphylococcus aureus* and *Listeria monocytogenes*. FEMS *Microbiol. Letts.* **109:**19-24.

Tsai K.-J., and A.L. Linet. (1993). Formation of a phosphorylated enzyme intermediate by the CadA Cd^{2+}-ATPase. *Arch. Biochem. Biophys.* **305:**267-270.

Tsai K.-J., Yoon K.P., and A.R. Lynn. (1992). ATP-dependent cadmium transport by the *cadA* cadmium resistance determinant in *Staphylococcus aureus. J. Bacteriol.* **174:**116 121.

Tso J.Y., and C. Siebel. (1989). Cloning and expression of the phospholipase C gene from *Clostridium perfringens* and *Clostridium bifermentans. Infect. Immun.* **57:**468-476.

Tweten R.K. (1988). Nucleotide sequence of the gene for perfringolysin O (theta toxin) from *Clostridium perfringens*: significant homology with the genes for streptolysin and pneumolysin. *Infect. Immun.* **56:**3235-3240.

Uhlén M., Guss B., Nilsson B., Gatenbeck S., Philipson L., and M. Lindberg. (1984). Complete sequence of the staphylococcal gene encoding protein A: a gene evolved through multiple duplications. *J. Biol. Chem.* **259:**1695-1702.

Van Hove B., Staudenmaier H., and V. Braun. (1990). Novel two-component transmembrane transcription control: regulation of iron dicitrate transport in *Escherichia coli* K-12. *J. Bacteriol.* **172:**6749-6758.

Van Leengoed L.A.M.G., and H.W. Dickerson. (1992). Influence of calcium on secretion and activity of the cytolysins of *Actinobacillus pleuropneumoniae. Infect. Immun.* **60:**353-359.

Vasantha N., Thompson L.D., Rhodes C., Banner C., Nagle J., and D. Filpula. (1984). Genes for alkaline protease and neutral protease from *Bacillus amyloliquefaciens* contain a large open reading frame between the regions coding for signal sequence and mature protein. *J. Bacteriol.* **159:**811-819.

Vaudaux P., and F.A. Waldvogel. (1979). Gentamycin antibacterial activity in presence of human polymorphonuclear leukocytes. *Antimicrob. Agents Chemother.* **16:**743-749.

Vazquez-Boland J.-A., Dominguez L., Rodriguez-Ferri E.F., and G Suarez. (1989a). Purification and characterization of two *Listeria ivanovii* cytolysins, a sphingomyelinase C and a thiol-activated toxin (ivanolysin O). *Infect. Immun.* **57:**3928-3935.

Vazquez-Boland J.-A., Dominguez L., Rodriguez-Ferri E.F., Fernandez-Garayzabal J.F., and G. Suarez. (1989b). Preliminary evidence that different domains are involved in cytolytic activity and receptor (cholesterol) binding in listeriolysin O, the *Listeria monocytogenes* thiol-activated toxin. FEMS *Microbiol. Letts.* **65:**95-100.

Vazquez-Boland J.-A., Kocks C., Dramsi S., Ohayon H., Geoffroy C., Mengaud J., and P. Cossart. (1992). Nucleotide sequence of the lecithinase operon of *Listeria monocytogenes* and possible role of lecithinase in cell-to-cell spread. *Infect. Immun.* **60:**219-230.

Verma N.K., Ziegler H.K., Wilson M., Khan M., Safley S., Stocker B.A., and G.K. Schoolnik. (1995). Delivery of class I and class II MHC-restricted T-cell epitopes of listeriolysin of *Listeria monocytogenes* by attenuated *Salmonella. Vaccine* **13:**142-50.

Vesikari T., Bromirska J., and M. Mäki. (1982). Enhancement of invasiveness of *Yersinia enterocolitica* and *Escherichia coli* in HEp-2 cells by centrifugation. *Infect. Immun.* **36:**834-836.

Vicente M.F., Baquero F., and J.C. Pérez-Díaz. (1985). Cloning and expression of the *Listeria monocytogenes* hemolysin in *Escherichia coli.* FEMS *Microbiol. Letts.* **30:**77-79.

Vicente M.F., Baquero F., and J.C. Pérez-Díaz. (1987). A protoplast transformation system for *Listeria* sp. *Plasmid* **18:**89-92.

Vicente M.F., Baquero F., and J.C. Pérez-Díaz. (1988). Conjugative aquistion and expression of antibiotic resistance determinants in *Listeria* spp. *J. Antimicrob. Chemother.* **21:**309-318.

Villani F., Salzano G., Sorrentino E., Pepe O., Marino P., and S. Coppola. (1993). Enterocin 226NWC, a bacteriocin produced by *Enterococcus faecalis* 226, active against *Listeria monocytogenes. J. Appl. Bacteriol.* **74:**380-387.

Visintine A.M., Oleske J.M., and A.J. Nahmias. (1977). Infection in infants and children. *Am. J. Dis. Child.* **131:**393-397.

Von Heijne G. (1992). Membrane protein structure prediction. Hydrophobicity analysis and the positive-inside rule. *J. Mol. Biol.* **225:**487-494.

Vulpe C., Levinson B., Whitney S., Packman S., and J. Gitschier. (1993). Isolation of a candidate gene for Menkes disease and evidence that it encodes a copper-transporting ATPase. *Nature Genet.* **5:**7-13.

Walderhaug M.O., Post R.L., Saccomani G., Leonard R.T., and D.P. Briskin. (1985). Structural relatedness of three ion-transport adenosine triphosphatases around their active sites of phosphorylation. *J. Biol. Chem.* **260:**3852-3859.

Walker J.A., Allen R.L., Falmagne P., Johnson M.K., and G. Boulnois. (1987). Molecular cloning, characterization and complete nucleotide sequence of the gene for pneumolysin, the sulfhydryl-activated toxin of *Streptococcus pneumoniae. Infect Immun.* **55:**1184-1189.

Wang J.C. (1971). Interaction between DNA and an *Escherichia coli* protein. *J. Mol. Biol.* **71:**127-147.

Wang J.C. (1985). DNA topoisomerases. *Annu. Rev. Biochem.* **54:**665-697.

Wang J.-Y., and M. Syvanen. (1992). DNA twist as a transcriptional sensor for environmental changes. *Mol. Microbiol.* **6:**1861-1866.

Wang R.F., Cao W.W., and M.C. Johnson. (1991). Development of a 16S rRNA-based oligomer probe specific for *Listeria monocytogenes. Appl. Environ. Microbiol.* **57:**3666-3670.

Wang R.F., Cao W.W., and M.C. Johnson. (1992). 16S rRNA-based probes and polymerase chain reaction method to detect *Listeria monocytogenes* cells added to foods. *Appl. Environ. Microbiol.* **58:**2827-2831.

Warburton D.W., Farber J.M., Armstrong A., Caldeira R., Hunt T., Messier S., Plante R., Tiwari N.P., and J. Vinet. (1991). A comparative study of the 'FDA' and 'USDA' methods for the detection of *Listeria monocytogenes* in foods. *Int. J. Food. Microbiol.* **13:**105-118.

Watson B.B., and J.C. Lavizzo. (1973). Extracellular antigens from *Listeria monocytogenes.* II. Cytotoxicity of hemolytic and lipolytic antigens of *Listeria* for cultured mouse macrophages. *Infect. Immun.* **7:**753-758.

Watson B.B., and W.C. Eveland. (1965). The application of a phage fluorescent antiphage staining system in specific identification of *Listeria monocytogenes.* I. Species specificity and immunofluorescent sensitivity of *Listeria monocytogenes* phage observed in smear preparations. *J. Infect. Dis.* **115:**363-369.

Watson C., and K. Ott. (1990). *Listeria* outbreak in Western Australia. *Comm. Dis. Intell.* **90:**9-11.

Weagant S.D., Sado P.N., Colburn K.G., Torkleson J.D., Stanley F.A., Krane M.H., Shields S.C., and C.F. Thayer. (1988). The incidence of *Listeria* species in frozen seafood products. *J. Food Protect.* **51:**655-657.

Wehr H.M. (1987). *Listeria monocytogenes* - a current dilemma. *J. Assoc. Off. Anal. Chem.* **70:**769-772.

Weingärtner L., and S. Ortel. (1967). Zur behandlung der listeriose mit ampicillin. *Dtsch. Med. Wochenschr.* **92:**1098-1104.

Welch D.F. (1987). Role of catalase and superoxide dismutase in the virulence of *Listeria monocytogenes. Ann. Inst. Pasteur Microbiol.* **138:**265-268.

Welch D.F., Sword C.P., Brehm S., and D. Dusanic. (1979). Relationship between superoxide dismutase and pathogenic mechanisms of *Listeria monocytogenes. Infect. Immun.* **23:**863-872.

Welshimer H.J. (1981). The genus *Listeria* and related organisms. In *The prokaryotes. A handbook on habitats, isolation and identification of bacteria,* (M.P. Starr, ed.) Springer-Verlag, New York, pp. 1680-1687.

Welshimer H.J., and A.L. Merdith. (1971). *Listeria murrayi* sp.: a nitrate reducing, mannitol fermenting *Listeria. Int. J. System. Bacteriol.* **21:**3-7.

Welshimer H.J., and J. Donker-Voet. (1971). *Listeria monocytogenes* in nature. *Appl. Microbiol.* **21:**516-519.

Wernars K., Heuvelman C.J., Notermans S.H., Domann E., Leimeister-Wächter M., and T. Chakraborty. (1992). Suitability of the *prfA* gene, which encodes a regulator of virulence genes in *Listeria monocytogenes*, in the identification of pathogenic *Listeria* spp. *Appl. Environ. Microbiol.* **58:**765-768.

Wesley I.V., and F. Ashton. (1991). Restriction enzyme analysis of *Listeria monocytogenes* strains associated with foodborne epidemics. *Appl. Environ. Microbiol.* **57:**969-975.

Williams S.G., and P.A. Manning. (1991). Transcription of the *Vibrio cholerae* haemolysin gene, *hlyA*, and cloning of a positive regulatory locus, *hlyU*. *Mol. Microbiol.* **5:**2031-2038.

Winter N., Lagranderie M., Rauzier J., Timm J., Leclerc C., Guy B., Kieny M.P., Gheorghiu M., and B. Gicquel. (1991). Expression of heterologous genes in *Mycobacterium bovis* BCG: induction of a cellular response against HIV-1 Nef protein. *Gene* **109:**47-54.

Wirsing von Koenig C.H., Finger H., and H. Hof. (1982). Failure of killed *Listeria monocytogenes* vaccine to produce protective immunity. *Nature* **297:**233-234.

Wuenscher M.D., Köhler S., Goebel W., and T. Chakraborty. (1991). Gene disruption by plasmid integration in *Listeria monocytogenes*: insertional inactivation of the listeriolysis determinant *lisA*. *Mol. Gen. Genet.* **228:**177-182.

Wuenscher M.D., Köhler S., Bubert A., Gerike U., and W. Goebel. (1993). The *iap* gene of *Listeria monocytogenes* is essential for cell viability, and its gene product, p60, has bacteriolytic activity. *J. Bacteriol.* **175:**3491-3501.

Yamamoto N., and M.L. Droffner. (1985). Mechanisms determining aerobic and anaerobic growth in the facultative anaerobe *Salmonella typhimurium*. *Proc. Natl. Acad. Sci.* USA **82:**2077-2081.

Yang M.Y., Ferrari E., and D. Henner. (1984). Cloning of the neutral protease gene of *Bacillus subtilis* and the use of the cloned gene to create an *in vitro*-derived deletion mutation. *J. Bacteriol.* **160:**15-21.

Yoon K.P., and S. Silver. (1991). A second gene in the *Staphylococcus aureus cadA* cadmium resistance determinant of plasmid pI258. *J. Bacteriol.* **173:**7636-7642.

Yoon K.P., Misra T.K., and S. Silver. (1991). Regulation of the *cadA* cadmium resistance determinant of *Staphylococcus aureus* plasmid pI258. *J. Bacteriol.* **173:**7643-7649.

Yoshikawa H., Kawamura I., Fujita M., Tsukada H., Arakawa M., and M. Mitsuyama. (1993). Membrane damage and interleukin-1 production in murine macrophages exposed to listeriolysin O. *Infect. Immun.* **61:**1334-1339.

Youatt J. (1993). Calcium and microorganisms. *Crit. Rev. Microbiol.* **19:**83-97.

Youngman P. (1987). Plasmid vectors for recovering and exploiting Tn*917* transpositions in *Bacillus* and other Gram-positive bacteria. In *Plasmids: a practical approach.* (K. Hardy, ed.). IRL Press, Oxford, pp. 79-103.

Youngman P., Perkins J.B., and K. Sandman. (1984). New genetic methods, molecular cloning strategies and gene fusion techniques for *Bacillus subtilis* which take advantage of Tn*917* insertional mutagenesis. In *Genetics and biotechnology of Bacilli* (A.T. Ganesan, and J.A. Hoch, eds.), Academic Press, Inc., New York.

Youngman P., Perkins J.B., and K. Sandman. (1985a). Use of Tn*917*-mediated transcriptional fusions to *lacZ* and *cat-86* for the identification and study of regulated genes in the *Bacillus subtilis* chromosome. In *Molecular biology of microbial differentiation.* (J.A. Hoch, and P. Setlow, eds.). American Society for Microbiology, Washington, DC., pp. 47-54.

Youngman P., Zuber P., Perkins J.B., Sandman K., Igo M., and R. Losick. (1985b). New ways to study developmental genes in spore-forming bacteria. *Science* **228:**285-291.

Youngman P., Poth H., Green B., York K., Olmedo G., and K. Smith. (1989). Methods for genetic manipulation, cloning, and functional analysis of sporulation genes in *Bacillus subtilis.* In *Regulation of procaryotic development, structural and functional analysis of bacterial sporulation and germination.* (I. Smith, R.A. Slepecky, and P. Setlow, eds.), American Society for Microbiology, Washington, DC., pp. 65-88.

Zachar Z., and D.C. Savage. (1979). Microbial interference and colonisation of the murine gastrointestinal tract by *Listeria monocytogenes. Infect. Immun.* **23:**168-174.

Zheng W., and S. Kathariou. (1994). Transposon-induced mutants of *Listeria monocytogenes* incapable of growth at low temperature (4°C). FEMS *Microbiol. Letts.* **121:**287-292.

Zhou L., Hui F.M., and D.A. Morrison. (1995). Characterisation of IS*1167*, a new insertion sequence in *Streptococcus pneumoniae. Plasmid* **33:**127-138.

Zuber P., and R. Losick. (1983). Use of a *lacZ* fusion to study the role of the *spoO* genes of *Bacillus subtilis* in developmental regulation. *Cell* **35:**275-283.

M.S. Francis and C.J. Thomas (1997) Mutants in the CtpA copper transporting P-type ATPase reduce virulence ofListeria monocytogenes.
Microbial Pathogenesis, v. 22 (2), pp. 67–78, February 1997

M.S. Francis and C.J. Thomas (1996) Effect of multiplicity of infection on Listeria monocytogenes pathogenicity for HeLa and Caco-2 cell lines.
Journal of Medical Microbiology, v. 45 (5), pp. 323-330, November 1996

M.S. Francis and C.J. Thomas (1997) The Listeria monocytogenes gene ctpA encodes a putative P-type ATPase involved in copper transport.
Molecular and General Genetics, v. 253 (4), pp. 484-491, January 1997