# The effect of mutations in lipopolysaccharide biosynthetic genes on the virulence of *Salmonella typhimurium* for the mouse

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Statement

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L. Vincent Collins

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## SUMMARY

In **Chapter 1** of the work, the literature pertaining to the pathogenesis of *Salmonella* in animals and humans is first reviewed. This section covers the genetic basis of bacterial virulence, as far as it is understood, and describes genetic modifications attenuating for the virulence of *Salmonella* strains. Some such strains have been used as live oral vaccines against salmonellosis of humans and animals. One mutation which is attenuating for the mouse-virulence of *Salmonella typhimurium* is *galE*. Strains with mutations in *galE* are affected in lipopolysaccharide (LPS) biosynthetic ability, among other features .The possibility that other mutations affecting LPS biosynthesis are also attenuating has not been explored for mutations in *rfc* or *pmi*. As this is the work of this thesis, Chapter 1 concludes with a review of the structure of LPS, and the enzymology and genetics of its biosynthesis.

**Chapter 2** covers the Materials and Methods used in the work.

In **Chapter 3**, the *rfc* gene of *S. typhimurium* is cloned, by direct selection in an *rfc* mutant, and confirmed to complement the lesions in both an older point mutant in *rfc*, and a new IS*10* insertion mutant. In **Chapter 4**, the gene is sequenced and the sequence analysed. There is a high frequency of modulating codons in the presumptive gene, explaining why difficulties were experienced in visualisation of the protein product in a variety of systems.

In **Chapter 5**, the *pmi* gene of *S. typhimurium* is cloned and analysed, while **Chapter 6** details the sequence of the gene, which is very like that of *Escherichia coli* K-12. The protein product of this gene was readily visualised in minicells.

In **Chapter 7**, both of the wild-type *rfc* and *pmi* genes are interrupted *in vitro* by the insertion of a Km-resistance cassette, and these insertion mutations recombined (singly) into the genome of virulent *S. typhimurium* C5. Comparisons, in both $LD_{50}$ tests, and in short-term colonisation experiments, of the wild-type C5, and the otherwise isogenic mutants, showed that either mutation greatly reduced the virulence of C5 for the mouse. Both mutants persisted well in the spleen following oral administration, unlike a *galE* mutant of C5. Both mutants were effective as live oral vaccines against C5 infection in the mouse.

In **Chapter 8**, the results of the work are briefly summarised . A conclusion is that an *S. typhimurium* strain which is capable of the biosynthesis of a full LPS core is able to colonise the spleens of animals infected orally with such a strain.

TABLE OF CONTENTS


| | Page |
|---|---|
| CHAPTER 1: INTRODUCTION | 1 |
| 1.1 The pathogenesis of *Salmonella* infection | 2 |
| 1.1.1 Intestinal colonization | 2 |
| 1.1.2 Survival within macrophages | 5 |
| 1.1.3 Host responses to *Salmonella* infection | 6 |
| 1.2 *Salmonella* virulence factors | 8 |
| 1.2.1 *Salmonella* enterotoxin | 8 |
| 1.2.2 Adherence and motility factors | 8 |
| 1.2.3 Lipopolysaccharide | 9 |
| 1.2.4 Virulence plasmids | 11 |
| 1.3 Parenteral vaccines against *Salmonella* infection | 12 |
| 1.4 Peroral vaccination against *Salmonella* infection | 13 |
| 1.4.1 Inactivated oral vaccines | 13 |
| 1.4.2 Live oral vaccines | 14 |
| 1.4.2.1 Spontaneously derived attenuated *Salmonella* strains | 15 |
| 1.4.2.2 Auxotrophic mutants | 15 |
| 1.4.2.3 Mutants defective in global regulation | 18 |
| 1.4.2.4 Virulence plasmid-cured strains | 21 |
| 1.4.2.5 Mutants with defects in virulence genes | 21 |
| 1.5 Bivalent live oral *Salmonella* -based vaccines | 22 |
| 1.6 Core lipopolysaccharide structure and biosynthesis | 23 |
| 1.6.1 LPS structure | 23 |
| 1.6.2 Structure and biosynthesis of lipid A | 23 |
| 1.6.3 Structure and biosynthesis of KDO | 24 |
| 1.6.4 Structure and biosynthesis of the LPS core region | 25 |
| 1.6.4.1 The inner core | 25 |
| 1.6.4.2 The outer core | 26 |
| 1.7 Structure and biosynthesis of O-antigen | 27 |
| 1.7.1 O-repeat unit biosynthesis | 27 |
| 1.7.2 O-repeat unit polymerization | 28 |
| 1.7.3 Modifications to polymerase activity | 29 |
| 1.7.4 Transfer of O-repeat units to the core | 31 |
| 1.8 The topology of lipopolysaccharide biosynthesis | 31 |
| 1.9 The genetics of lipopolysaccharide biosynthesis | 32 |
| 1.9.1 Core LPS | 32 |
| 1.9.2 Genetics of O-repeat unit synthesis | 33 |
| 1.9.3 Genetics of O-repeat unit polymerization | 35 |
| 1.10 Aims of this study | 36 |
| CHAPTER 2: MATERIALS AND METHODS | 38 |
| 2.1. Chemicals and reagents | 39 |
| 2.2 Enzymes | 40 |
| 2.3 Synthesis of oligodeoxynucleotides | 40 |
| 2.4 Growth media | 40 |
| 2.5 Bacterial strains and plasmids, and bacteriophage | 41 |
| 2.5.1 Maintenance of bacterial strains | 41 |
| 2.5.2 Bacteriophage sensitivity tests | 42 |
| 2.6 Phage ES18-mediated transduction | 43 |
| 2.7 Transformation procedure | 43 |
| 2.8 Bacterial conjugation | 43 |

2.9 Transposon mutagenesis 44
2.9.1 Transposition with Tn*5* 44
2.9.2 Transposition with Tn*10* 44
2.9.3 Transposition with Tn*1725* 45
2.10 DNA extraction procedures 45
2.10.1 Plasmid DNA isolation 45
2.10.2 Preparation of genomic DNA 47
2.11 Analysis and manipulation of DNA 48
2.11.1 DNA quantitation 48
2.11.2 Restriction endonuclease digestion of DNA 48
2.11.3 Analytical and preparative separation of restriction fragments 48
2.11.4 Calculation of restriction fragment size 49
2.11.5 Dephosphorylation and ligation of DNA 49
2.11.6 Cosmid bank construction 50
2.11.7 End-filling with Klenow fragment 50
2.11.8 Nick-translation 51
2.11.9 Random primer labelling 51
2.11.10 Southern transfer and hybridization 51
2.12 Autoradiography 52
2.13 Protein and LPS analysis 52
2.13.1 SDS polyacrylamide gel electrophoresis 52
2.13.2 Minicell procedures 53
2.13.3 Maxicell procedures 53
2.13.4 Bacterial cell-free coupled transcription-translation 54
2.13.5 T7 polymerase / promoter system 54
2.13.6 LPS preparation 55
2.13.7 LPS-specific silver staining 55
2.13.8 Cell fractionation 55
2.14 Phosphomannose isomerase enzyme activity assay 56
2.15 M13 cloning and sequencing procedures 56
2.15.1 Preparation of M13 replicative form (RF) DNA 56
2.15.2 Cloning with M13mp18 and M13mp19 57
2.15.3 Transfection of JM101 57
2.15.4 Screening M13 vectors for inserts 57
2.15.5 Purification of single-stranded template DNA 58
2.15.6 Dideoxy sequencing protocol with Sequenase 59
2.15.7 Double stranded sequencing 60
2.15.8 DNA sequencing gels 61
2.15.9 Analysis of DNA sequences 62
2.16 Animal experiments 62
2.16.1 Mice 62
2.16.2 Preparation of bacteria for injection 62
2.16.3 Virulence and protective capacity of *S. typhimurium* derivatives 63
2.16.4 Colonization of mice by strains of *S. typhimurium* 63

CHAPTER 3: Molecular cloning and characterisation of the *rfc* gene for an O-antigen polymerase of *Salmonella typhimurium* 64

3.1 Introduction 65

3.2 Results 66
3.2.1 Construction of a lambda-sensitive *rfc* strain 66
3.2.2 Insertional inactivation of the *rfc* locus of C5 67
3.2.3 Cosmid cloning of the *S. typhimurium rfc* locus 69
3.2.4 Physical mapping of the *rfc*$^+$ cosmid and cutdowns 69
3.2.4.1 *Hin*dIII Cutdowns of pADE201 69
3.2.4.2 Transposon mutagenesis with Tn*5* and Tn*1725* 69
3.2.5 Confirmation of the location of the IS*10* element in J208-B1 and J208-B2 70
3.2.6 LPS phenotype analysis by SDS-PAGE 70
3.2.7 Analysis of plasmid-encoded polypeptides 71
3.2.7.1 Minicell and maxicell analysis of proteins expressed by *rfc*$^+$ plasmids 72
3.2.7.2 *In vitro* transcription-translation analysis of proteins encoded by *rfc*$^+$ plasmids 73
3.2.7.3 T7 polymerase / promoter system analysis of plasmid-encoded proteins 73
3.2.8 Distribution of the *rfc* locus among *Salmonella* strains 74
3.3 Discussion 75

CHAPTER 4: Nucleotide sequence and genetic organisation of the *rfc* gene of *Salmonella typhimurium* 78

4.1 Introduction 79
4.2 Results 79
4.2.1 *rfc* nucleotide sequence determination and analysis 79
4.2.1.1 DNA sequencing strategy 79
4.2.1.2 Nucleotide sequence analysis of *rfc* 80
4.2.1.3 Location of the *rfc*::Tn*1725* insertions, and Tn*1725* i nsertions not inactivating *rfc* 82
4.2.1.4 Transcriptional termination 82
4.2.1.5 Codon and base usage 83
4.2.2 The putative Rfc protein 85
4.2.2.1 Hydropathic profile of the Rfc protein 85
4.2.2.2 Predicted secondary structure of the Rfc protein 85
4.3 Discussion 86

CHAPTER 5: Molecular cloning and characterisation of the phosphomannose isomerase gene of *Salmonella typhimurium* 89

5.1 Introduction 90
5.2 Results 91
5.2.1 Cosmid cloning of $pmi_{stm}$ 91
5.2.2 Transposon mutagenesis of pADE250 92
5.2.3 Sub-cloning the $pmi_{stm}$ gene from pADE250 92
5.2.4 Analysis of the $pmi_{stm}$ -encoded polypeptide 93
5.2.4.1 Minicell analysis 93
5.2.4.2 Determination of the extent of processing of the Pmi polypeptide 93
5.2.5 Cellular localization of the Pmi protein 94
5.2.6 Assays for Pmi enzyme activity 95
5.2.7 Homology of $pmi_{stm}$ with *E. coli*, *Shigella*, and *Salmonella* spp. 95
5.3 Discussion 96

CHAPTER 6: Nucleotide sequence analysis and genetic organisation of the *pmi* stm gene of *Salmonella typhimurium* 99

6.1 Introduction 100
6.2 Results 101
6.2.1 *pmi* stm nucleotide sequence determination and analysis 101
6.2.1.1 DNA sequencing strategy 101
6.2.1.2 Nucleotide sequence analysis of $pmi_{stm}$ 101
6.2.1.3 Location of a $pmi_{stm}$::Tn*1725* insertion 103
6.2.1.4 Codon and base usage 103
6.2.2 The Pmi protein 103
6.2.2.1 Hydropathic profile and secondary structure of the Pmi protein 103
6.2.2.2 Comparison of $pmi_{stm}$ with related sequences 104
6.3 Discussion 105

CHAPTER 7: Construction and characterization of site-directed mutations in the chromosome of *Salmonella typhimurium* , and analysis of the effects of these mutations on the virulence of *S. typhimurium* for mice 107

7.1 Introduction 108
7.2 Results 110
7.2.1 Modifications of pHSG422 110
7.2.2. Construction of a site-specific *rfc* mutation in *S. typhimurium* 110
7.2.2.1 Insertion of a $Km^R$ cartridge into *rfc* 111
7.2.2.2 Sub-cloning *rfc* ::$Km^R$ into pADE262 111
7.2.2.3 Mobilization of pADE264 112
7.2.2.4 Construction and characterization of C5 *rfc*::$Km^R$ 112
7.2.3. Construction of a site-specific *pmi* mutation in *S. typhimurium* 113
7.2.3.1 Insertion of a $Km^R$ cartridge into $pmi_{stm}$ 113
7.2.3.2 Sub-cloning $pmi_{stm}$::$Km^R$ into pADE261 114
7.2.3.3 Mobilization of pADE266 114
7.2.3.4 Construction and characterization of C5 *pmi*::$Km^R$ 114
7.2.4 The virulence and immunogenicity of the *rfc*::Km and the *pmi*::Km constructs in mice 116
7.2.4.1 The virulence and immunogenicity of the strains in mice 116
7.3 Discussion 118

CHAPTER 8: Conclusions 122

Bibliography 126

# CHAPTER 1

# INTRODUCTION

**The pathology of, and immunity to, *Salmonella* infections, with specific reference to the role of lipopolysaccharide in these processes**

Outbreaks of typhoid fever attributable to *Salmonella typhi*, and gastroenteritis due to *Salmonella typhimurium*, are a persistent public health problem in undeveloped countries, and represent a risk to travellers from developed countries visiting such areas. Some knowledge on the mechanisms by which enteric pathogens colonise the gut wall, invade the deeper tissues, and cause symptomatic disease, has been accumulated. Modern techniques of molecular biology have allowed a start to be made on the dissection of the processes of pathogenesis and immunity. An appreciation of the bacterial factors mediating virulence has led to the construction of attenuated strains and the development in recent times of recombinant bacteria, based on such strains, carrying heterologous antigens. Such strains are proposed for use as live oral vaccines.

This Chapter initially describes the current theories on the pathogenesis of *Salmonella* strains and on the host mechanisms of immunity. In particular, the role of lipopolysaccharide (LPS) in these processes is considered. The second part of this review elaborates on existing vaccines, their shortcomings, and the various stratagems employed in the construction of attenuated *Salmonella* strains for use as potential live oral vaccines.

The final part of this Chapter deals with LPS structure and biosynthesis and reviews the current knowledge on the genetics of LPS biosynthesis, particularly as it applies to *S. typhimurium.*

## 1.1 The pathogenesis of *Salmonella* infection

### 1.1.1 Intestinal colonization

Most *Salmonella* infections arise from ingestion of the organism. Invasion of the gastrointestinal mucosa is essential for *Salmonella* pathogenesis and strains unable to

invade epithelial cells are avirulent (Giannella *et al.*, 1973a, 1973b). Infections of *S. typhimurium* and *S. typhi* have distinct clinical manifestations in humans. *S. typhi* readily penetrates the intestinal wall, invades the bloodstream, survives in the mononuclear phagocytic system and produces a secondary bacteremia (Hornick *et al.*, 1970a; Rout *et al.*, 1974). Human infection by *S. typhimurium*, on the other hand, is characterized by intestinal alterations, with occasional invasion of the bloodstream, and secondary infection of different organs is usually seen only in patients with impaired host defences (Edelman and Levine, 1986).

Invasion of the gastrointestinal mucosa occurs *via* surface epithelial penetration (Takeuchi *et al.*, 1967). Multiplication of *Salmonella* strains, internalized in vacuoles within epithelial cells, occurs after a lag period of several hours (Finlay *et al.*, 1988). Cytotoxic damage, manifested in loss of epithelial microvilli, is often an associated feature of epithelial invasion. The primary site of invasion is thought to be the ileal M cells in Peyers's patches, although some invasion of columnar epithelial cells also occurs (Hohmann *et al.*, 1978; Kohbata *et al.*, 1986). Invasion is followed by transcytosis to the opposite surface of the epithelial cell (Finlay *et al.*, 1988). Invading bacteria may be released from the eukaryotic cell by the actions of cytotoxins. During infection *Salmonella* strains may be internalized by macrophages, within which they can survive (Fields *et al.*, 1986). Dissemination through the host within phagocytic cells, via the lymph nodes to the blood, is therefore possible. The pathogenesis of *Salmonella* has been studied mainly through the use of the experimental mouse model of typhoid caused by *S. typhimurium* or *Salmonella enteritidis* (Collins, 1969a, 1969b, 1974). Peroral infection of mice with virulent *S. typhimurium* leads to the establishment of foci of infection in the gut wall, with subsequent dissemination of the bacteria to the liver and spleen. Eventual death is due to the toxic effects of LPS (Collins and Carter, 1972).

*Salmonella* has generally been regarded as a facultative intracellular parasite, based on its observed abilities to invade non-phagocytic cells, to withstand the bactericidal agents within phagolysosomes, and to survive and proliferate within macrophages (Finlay and Falkow, 1989a, 1989b). A great deal of research has therefore focussed on identifying the factors which equip *Salmonella* strains for entry into cells, especially macrophages, and for survival in the intracellular environment.

Finlay and colleagues (1988) identified six classes of Tn*phoA* mutants of *Salmonella choleraesuis* which were unable to enter epithelial cells. Two of these classes were defective in either LPS core or O-antigen synthesis. A direct correlation was drawn between the virulence of *S. typhimurium* and the ability to adhere to and invade epithelial cells (Finlay *et al.*, 1989). This correlation has been used recently to assay potential live oral typhoid vaccines (*S. typhi* -based) for residual virulence in human monocyte-macrophage lines (Dragunsky *et al.*, 1990). Mutants of *S. typhimurium* which could adhere to cultured cells, but which had decreased invasion levels, have also been described, and the mutation assigned to an *inv* locus (Galán and Curtiss, 1989a). Recently, a DNA region of *S. typhi* , inferred to carry four *inv* genes, was shown to confer on an *Escherichia coli* K-12 strain the ability to enter, but not to replicate within, cultured human intestinal cells (Elsinghorst *et al.*, 1989). Interaction of bacteria with epithelial cells stimulated production of several new polypeptides required for adherence and invasion in *S. choleraesuis* and *S. typhimurium* (Finlay *et al.*, 1989). A Tn*10* mutant of *S. typhimurium*, defective in adherence and invasion, was found to be defective in synthesis of proteins normally induced by epithelial cells (Finlay *et al.*, 1989). Curing of the virulence plasmid resident in many *Salmonella* spp.(see below) does not affect entry of *S. typhimurium* into epithelial cells (Hackett *et al.*, 1986).

### 1.1.2 Survival within macrophages

*Salmonella* strains may enter macrophages by two mechanisms: by classical phagocytosis or by a specific invasion pathway (Finlay and Falkow, 1989a, 1989b; Gahring *et al.*, 1990). Survival within macrophages has been analysed using Tn*10* mutagenesis of *S. typhimurium* to produce avirulent strains, many of which penetrated and replicated normally within epithelial cells but no longer survived or proliferated within macrophages (Fields *et al.*; 1986; Gahring *et al.*, 1990). Of the eighty-three Tn*10* insertions isolated, twelve were auxotrophs affected in purine, pyrimidine, aromatic acid, histidine or methionine biosynthesis, three had altered responses to oxidative stress, one had an insertion in the virulence plasmid, eight were non-motile, and one had altered LPS (although the parent strain was rough) (Fields *et al.*, 1986). The macrophage type with which the invading organism came into contact was critical to its survival. Peritoneal macrophages killed complement-coated *Salmonella* bacteria, whereas liver and spleen macrophages did not (Saxen, 1984; Saxen *et al.*, 1984). Smooth *Salmonella* strains, and mutants with complete core, survived and multiplied within macrophages, whereas core-defective mutants lacked this ability (Saxen *et al.*, 1984).

It is appropriate here to mention that the view that survival within macrophages is critical to the virulence of *Salmonella* strains does not have universal acceptance. An alternative view on the early stages of *Salmonella* pathogenesis has been proposed by Hsu (1989). He argued that the evidence for regarding *Salmonella* as a facultative intracellular pathogen, proliferating within macrophages, was not conclusive. In support of this, he pointed to the evidence for macrophage killing of *Salmonella* (Briles *et al.*, 1986; van Zwet *et al.*, 1975), and the direct observation by electron microscopy of bacterial destruction within phagocytes (Hsu, 1989). In an alternative model of *Salmonella* infection, Hsu (1989) proposed that the primary site of bacterial

proliferation is extracellular, with those bacteria which escape macrophage killing disseminating into host tissues and causing systemic infection. In the susceptible mouse, according to this model, invasion of host tissues (eg. hepatocytes) occurs, acute inflammation ensues and death is due to the the lethal effects of endotoxin (Hsu, 1989). In the resistant mouse, the extracellular *Salmonella* bacteria are opsonized, or agglutinated, by antibodies directed primarily against O-antigens, and are subsequently phagocytosed (Hsu, 1989). Proponents of these two theories of *Salmonella* pathogenesis disagree mainly on the role played by macrophages in the proliferation and/or killing of the invading organism.

### 1.1.3 Host responses to *Salmonella* infection

The adhesion of *Salmonella* species to the intestinal epithelium may be inhibited by the action of secretory IgA (sIgA) which probably works by increasing the surface hydrophilicity of the pathogen and thus enhancing its entrapment in the mucous layer (Magnusson *et al.*, 1979; Magnusson and Stjernstrom, 1982). The antitoxic activity of sIgA has been established in experiments on the protection of mice against experimental cholera infection by oral immunization with cholera toxin (CT) (Svennerholm *et al*, 1978). Induction of specific sIgA was also observed following oral immunization with the heat-labile toxin (LT) of enterotoxigenic *Escherichia coli* (ETEC), or with the B subunit of LT (Klipstein *et al.*, 1982a) and these antitoxin antibodies were protective (Klipstein *et al.*, 1982a, 1982b, 1983). The relative protection provided by immunization with whole*Vibrio cholerae* or CT, was assessed by Neoh and Rowley (1972), who found that anti-bacterial antibody was 10-fold more protective, on a weight basis, than antitoxin antibody. Whether or not a protective antitoxin sIgA response occurs following *Salmonella* infection is not known. It has been suggested by Svennerholm and Holmgren(1976) that the highest levels of protection against enteric bacterial pathogens may be afforded by the synergistic actions of antibacterial and antitoxin antibodies.

Both macrophages and T-cells may be involved in cell-mediated immunity (CMI) to *Salmonella* infection, while antibody also plays a role (Cooper *et al.*, 1983). Mice infected intravenously with sub-lethal doses of *S. typhimurium* were protected against virulent challenge; this resistance was proposed to be due to the development, in the infected mice, of activated macrophages with enhanced antibacterial activity (Blanden *et al.*, 1966; Mackaness *et al.*, 1966; Collins, 1969a, 1969b). Antigen-specific T-cells have been suggested to play a major role in development of immunity to intracellular parasites (Mackaness, 1971, Lefford, 1975, North, 1981). The activity and number of tissue macrophages was shown to be due to the action of lymphokines released by T-cells (Collins, 1979, North, 1981). Immune T-cells were also implicated in immunologic memory (Collins, 1979, North, 1981).

The view that acquired immunity to salmonellosis is primarily cell-mediated has recently been disputed (Hsu, 1989), partly on the basis that non-viable vaccines, which induce only humoral immunity, have been observed to protect against subsequent virulent challenge (Ornellas *et al.*, 1970; Germanier, 1972; Herzberg *et al.*, 1972 ). An alternative theory of immunity is proposed in which suppression of early propagation and of systemic dissemination allows time for the development of cell-mediated immunity (Hsu, 1989). In this model, *Salmonella* is regarded as an obligate extracellular pathogen and acquired immunity is due to the synergistic actions of circulating antibodies involved in bacterial agglutination and opsonisation, macrophages (with cytophilic antibodies) involved in bacterial phagocytosis and killing, and delayed-type hypersensitivity acting to accelerate the influx of cellular and humoral components into the site of infection (Hsu, 1989).

## 1.2 *Salmonella* virulence factors

Interactions between *Salmonella* strains and the host organism involve an array of bacterial virulence factors which have evolved either to actively facilitate the infectious process, or to sidestep host defences. In this section a review of the components of *Salmonella* strains required for invasion, bacterial survival, and bacterial intracellular replication, is presented.

### 1.2.1 *Salmonella* enterotoxin

The intestinal inflammation and diarrhea associated with *Salmonella* -mediated gastroenteritis is thought to be due, in part, to a *Salmonella* enterotoxin (Koupal and Deibel, 1975). Differences in enterotoxin production among various strains could be correlated with differences in LPS production and associated differences in invasiveness, although the reasons for this are not clear (Mintz and Deibel, 1983).

### 1.2.2 Adherence and motility factors

Two adherence factors have been described for *Salmonella* spp.: type 1 pili (fimbriae) and mannose-resistant haemagglutinin (MRHA). Type 1 pili are not required by *S. typhimurium* for entry into eukaryotic cells (Jones and Richardson, 1981). Non-fimbriated strains of *S. typhimurium* are as virulent as fimbriated strains when fed orally to mice (Duguid *et al.*, 1976). MRHA is secreted by *S. typhimurium* and *S. enteritidis* but not by *S. typhi*, *S. choleraesuis* , or *Salmonella dublin* ; its possible role in pathogenesis has not been clarified (Halula and Stocker, 1987).

Hackett and colleagues (1988) have reported that non-flagellated *S. typhimurium* fed to mice colonized the intestine with an efficiency equal to that of flagellated *S.*

*typhimurium*.. However, flagellated strains provided superior protection to mice against virulent challenge (Hackett *et al.*, 1988). Fields *et al.* (1986) isolated non-motile mutants of *S. typhimurium* that were less able to invade and survive within macrophages *in vitro*. Likewise, Liu *et al.* (1988) isolated *fla* (defective in flagellar synthesis) and *mot* (non-motile) mutants of *S. typhi* which displayed decreased invasiveness for cultured HeLa cells. Carsiotis *et al.* (1984) and Weinstein *et al.* (1984), studied (wild-type) motile and (*fla, mot* ) non-motile derivatives of *S. typhimurium*, and found that the presence of flagella enhanced bacterial invasiveness and survival within macrophages, and also influenced *S. typhimurium* virulence ($LD_{50}$ values) in mice. However, it subsequently appeared that the *fla* mutants used in these studies were defective not only in *fla* gene function, but carried, in addition, deletion mutation(s) affecting an undescribed virulence gene which was located close to, but was not part of, the recognized *fla* gene cluster (Carsiotis *et al.*, 1987). Clarification of the role played by flagella in *S. typhimurium* virulence has been provided recently by Lockman and Curtiss (1990), who demonstrated that flagellated and non-flagellated *S. typhimurium* mutants had wild-type virulence for mice when administered either orally or intraperitoneally. Taken together, the studies outlined above indicated that while flagella of *S. typhimurium* may be important for invasion of tissue culture monolayers, their absence does not appear to affect the virulence of *Salmonella* strains in mice.

### 1.2.3 Lipopolysaccharide

The LPS of *Salmonella* can be divided into three structurally distinct regions (Lüderitz *et al.*, 1971, Stocker and Mäkelä, 1971). The outermost region consists of O- antigenic side chains which are composed of repeating oligosaccharide units containing specific sugars in specific linkages. The O-antigenic polysaccharide is linked to the core LPS region which is itself linked, via a ketosidic linkage, to lipid A. The relationship of LPS structure to virulence has been extensively studied (Mäkelä *et al.*, 1973; Roantree, 1979). Rough *Salmonella* forms that have lost the O-antigenic side chains are less

virulent than smooth forms (Lyman *et al.*, 1967; Nakano and Saito, 1969; Roantree, 1971). In addition, subtle differences in the structure of the O-repeat units have significant effects on virulence (Valtonen, 1970; Mäkelä *et al.*, 1973; Valtonen *et al.*, 1975).

Several factors contribute to the avirulence of rough strains. LPS has been implicated in adhesion and colonization of the colon by enteric bacteria (Izhar *et al.*, 1982; Cohen *et al.*, 1983, 1985; Myhal et al, 1983). In *Salmonella*, LPS may be involved in attachment and invasion of gut epithelial cells (Finlay *et al.*, 1988; Mintz and Deibel, 1983). In support of this is the finding that adhesion and penetration of HeLa cell monolayers by *S. typhi* requires complete LPS (Mroczenski-Widley *et al.*, 1989). An examination of the relationship between the degree of LPS defect and the ability of *S. typhimurium* to colonise mouse intestine revealed that as LPS structure became less complete in a series of strains, the colonising ability of the strains decreased, indicating that *in vivo* also, LPS may be involved in bacterial adhesion to the epithelial or mucosal layers of the gut (Nevola *et al.*, 1985).

A requirement for smooth LPS has also been proposed for invasion and lysis of HeLa cell monolayers by *S. typhi* Ty2 (Mroczenski-Wildey *et al.*, 1989). Similarly, in *S. choleraesuis*, LPS is required for cell invasion because many mutants that do not invade MDCK cells are LPS-defective (Finlay *et al.*, 1988). However, in apparent contradiction of these results, other investigators have found that rough strains of *S. typhimurium* invaded HeLa cells more efficiently than smooth strains (Kihlström, 1977; Kihlström and Latkovic, 1978).

Rough mutants are more sensitive than smooth strains to complement-mediated killing (Grossman *et al.*, 1986; Joiner, 1988) but become resistant upon restoration of their ability to make O-antigen (Taylor and Robinson, 1980). Resistance to this type of killing by complement is thought to be due to the inability of the C5b-9 complement

complex to insert in the bacterial membrane (Joiner *et al.*, 1982, 1983). Rough LPS activates the classical pathway resulting in phagocytosis, while long O-antigenic polysaccharides activate the alternative complement pathway (Valtonen, 1970; Morrison and Kline, 1977; Grossman and Leive, 1984) to various degrees, the degree of activation being inversely proportional to virulence (Valtonen 1970, 1977; Liang-Takasaki *et al.*, 1982, 1983; Saxen *et al.*, 1984.). Thus, modified *S. typhimurium* strains with O-6, 7 side chains, which activate the alternative pathway and are phagocytosed, are less virulent than *S. typhimurium* strains with O-4, 12 side chains which do not activate it and are not readily phagocytosed. Similarly, an *S. typhimurium* strain expressing O-9,12 was less virulent in mice after intra-peritoneal injection than a transductant expressing O-4, 12, and an O-9, 12 *S. enteritidis* strain made O-4,12 was more virulent in mice than its parent (Valtonen, 1970; Mäkelä *et al.*, 1973; Valtonen *et al.*, 1975). By activating the alternative pathway of complement, smooth LPS thus influences phagocytosis by macrophages (Liang-Takasaki *et al.*, 1982, 1983), which is thought by some (see Section 1.1.2 above) to be an essential step in progress towards a successful infection.

### 1.2.4 Virulence plasmids

The presence of a large (60-100 kb) plasmid appears to be essential for the mouse-virulence of *S. typhimurium* and other *Salmonella* species (Jones *et al.*, 1982; Helmuth *et al.*, 1985; Hackett *et al.*, 1986; Pardon *et al.*, 1986; Gulig and Curtiss, 1987; Kawahara *et al.*, 1988). Strains of *S. typhimurium*, cured of the virulence plasmid, are able to invade the intestinal epithelium but are incapable of multiplying within macrophages (Hackett *et al.*, 1986). A gene on the virulence plasmid encodes an 11 kDa protein essential for serum resistance (Hackett *et al.*, 1987). Although the presence of this gene conferred serum resistance on both a plasmid-cured *S. typhimurium* , and an *E. coli* K-12 strain, it was not sufficient to promote resistance to macrophage killing (Hackett *et al.*, 1987). An additional 28 kDa protein, encoded by

the *S. typhimurium* virulence plasmid, has recently been identified, and appears to play a role in facilitating bacterial colonization of the liver and spleen (Gulig and Curtiss, 1988).

Hackett and colleagues (1987) found that a strain of *S. typhimurium* cured of the resident virulence plasmid was serum-sensitive and had lost most of the parental O-antigenic polysaccharide, but that serum resistance was restored by introduction of a region of the virulence plasmid encoding an 11-kilodalton outer membrane protein. In contrast, other workers have found that the presence of the virulence plasmid was not related to LPS production (Jones *et al.*, 1982; Vandenbosch *et al.*, 1987; Gulig and Curtiss, 1987). Kawahara *et al.* (1989), however, found that the presence of the virulence plasmid was necessary for the synthesis of normal LPS by *S. dublin.*

## 1.3 Parenteral vaccines against *Salmonella* infection

Parenteral inoculation of mice with inactivated *S. typhimurium* or *S. enteritidis* failed to elicit significant cell mediated immunity (CMI) (Mackaness *et al.*, 1966; Collins, 1969a, 1969b; Collins and Carter, 1972). Killed *S. typhi* bacteria administered parenterally have been used for effective vaccination of humans but side effects are common and often severe (Ashcroft *et al.*, 1964; Polish Typhoid Committee, 1965). In humans, parenteral vaccination may be most effective in persons with prior exposure to the pathogen (Svennerholm *et al*, 1980; Levine *et al.*, 1983). Except when used in conjunction with adjuvants, or where inactivation is produced by a method which preserves antigen integrity (Robbins and Robbins, 1984; Tacket *et al.*, 1986), inactivated parenteral vaccines appear to be of limited use.

Parenteral vaccination of mice with sub-lethal doses of live virulent *S. typhimurium* provided good protection against subsequent homologous challenge (Germanier, 1972). Also, immunization of mice via the same route with an avirulent live *S.*

*typhimurium aro* $^{-}$ mutant, deficient in aromatic amino acid biosynthesis, provided immunity, probably as a result of macrophage activation, aginst challenge doses up to 9000 times the $LD_{50}$ of *S. typhimurium* (Eisenstein *et al.*, 1984) (see below).

## 1.4 Peroral vaccination against *Salmonella* infection

Attention has been focussed on the stimulation of the local intestinal secretory response, and CMI, using oral vaccines. Oral vaccine candidates which have been administered as either inactivated preparations or preparations of attenuated live organisms are described below.

### 1.4.1 Inactivated oral vaccines

Inactivated oral vaccines in general elicit relatively poor immune responses and large doses of antigen are required for effective immunization (DuPont *et al.*, 1971a, 1971b; Waldman *et al.*, 1972; Giannella, 1973; Cash *et al.*, 1974a; Stokes *et al*, 1979). In human volunteers, an inactivated oral cholera vaccine did not provide protection as high as that observed after natural *V.cholerae* infection (Cash *et al.*, 1974a).

An oral cholera vaccine (B-WCV) composed of the B subunit of cholera toxin in combination with whole cells, induced intestinal and serum antibody responses in volunteers, and the immune response resembled that seen in patients with naturally acquired cholera disease (Svennerholm *et al*, 1984). Vaccination of volunteers with B-WCV followed by challenge with virulent *V.cholerae* demonstrated 64% protection against the disease (Black *et al.*, 1987a). In subsequent field trials the estimated protective efficacy of B-WCV was 53% (Clements *et al.*, 1986b).

In contrast to these promising results with B-WCV, acetone-inactivated preparations of *S. typhi* Ty2, given in tablet form at 1 X $10^{11}$ organisms per tablet, had low protective efficacy (Hornick *et al.*, 1970b; DuPont *et al.*, 1971a, 1971b).

Several factors may have contributed to the failure of inactivated oral vaccines to induce immunity. Adherence to the gut wall may be an important initial step in inducing an immune response, and inert materials may have been inefficient in effecting such attachment. The attachment of macromolecules to the gut wall is affected by the intestinal mucous layer (Walker and Bloch, 1983), intestinal proteolysis (Udall *et al.*, 1984), the indigenous microflora, and peristalsis (Udall *et al.*, 1981). In addition, passage through the stomach may affect antigen immunogenicity (Levine *et al.*, 1986).

### 1.4.2 Live oral vaccines

Prior exposure to *S. typhi* infection affords significant protection against subsequent *S. typhi* infection (Ashcroft, 1964), although secondary infection by *S. typhi* serovar variants has been reported (Marmion *et al.*, 1953). Immunity induced after infection with *V.cholerae* provided protection against homologous challenge (Cash *et al.*, 1974a) and this immunity lasted for up to three years (Levine *et al.*, 1984).

Live oral attenuated organisms may afford higher levels of immunity than normally seen with the virulent organisms. The development of efficacious and attenuated *Salmonella* vaccines is based on three main criteria. Firstly, the attenuation must be sufficient to prevent symptomatic disease. Secondly, the attenuation must not prevent effective colonization of the intestine and interaction with the gut-associated lymphoid tissue since these factors are thought to be important in the determination of vaccine efficacy (Mackaness *et al.*, 1966; Germanier and Furer, 1971; Carter and Collins, 1974; Hohman *et al.*, 1978). Finally, the frequency of reversion of the vaccine strain to

virulence must be minimized by the use of two or more independently attenuating deletion mutations.

Many different approaches have been taken in the construction of live oral *Salmonella* vaccines for use in humans or animals (Dougan, 1989; Curtiss, 1990; Hackett, 1990). A description and evaluation of these approaches is given below.

### 1.4.2.1 Spontaneously derived attenuated *Salmonella* strains

A streptomycin-dependent (SmD) mutant of *S. typhi* was found to be avirulent compared to its parent *S. typhi* 19V in mice when injected intrperitoneally with hog gastric mucin (Reitman, 1967). This mutant afforded good protection (78% efficacy) when repeated doses of freshly-grown bacteria were administered orally in conjunction with streptomycin to humans, but minimal protection (28% efficacy) was afforded when the the same strain was administered as a lyophilised preparation (Levine *et al.*, 1986). An additional disadvantage of SmD mutants as potential vaccine strains was their tendency to revert to wild-type (and hence to virulence) at high frequencies (Vladoianou *et al.*, 1975).

### 1.4.2.2 Auxotrophic mutants

The introduction of mutations in genes for key metabolites causes the inhibition of bacterial growth and survival *in vivo* and leads to attenuation. One of the earliest reports of auxotrophic *S. typhi* strains which were avirulent in mice described mutants which were unable to synthesize the aromatic *p* - aminobenzoic acid (*p* ABA) or purines (Bacon *et al.*, 1950a, 1950b, 1951). Later, an *aroA* mutant of *S. typhimurium* , which was dependent on tryptophan, tyrosine, phenylalanine, *p* ABA and 2, 3-dihydroxybenzoic acid (DHB) for growth, was derived by Tn*10* mutagenesis (Hoiseth and Stocker, 1981; Stocker *et al.*, 1983). Stable deletion mutants in *aroA* were non-

virulent for mice and immunization by intraperitoneal injection of live organisms of the strain protected mice against challenge with virulent *S. typhimurium* (Hoiseth and Stocker, 1981; Stocker *et al.*, 1983). These results encouraged investigators to demonstrate that *Salmonella aroA* mutants were highly attenuated and effective oral vaccines when administered to a variety of mammalian species (Robertsson *et al.*, 1983; Dougan *et al.*, 1987b; Nnalue and Stocker, 1987; O'Callaghan *et al.*, 1988). The introduction of two stable, independent, attenuating mutations decreases the probability of reversion to virulence. *S. typhimurium aroA, aroC* double mutants were as highly attenuated in mice as an *S. typhimurium aroA* mutant and were protective against challenge with virulent *S. typhimurium* (Dougan *et al.*, 1988). Attenuation of *aro*$^-$ strains was partly attributed to the non-availability of *p* ABA and DHB in intestinal fluid or within cells of vertebrate hosts (Hoiseth and Stocker, 1981; Stocker *et al.*, 1983). A possible serious drawback to the use of *aro*$^-$ vaccine strains is the finding that feeding *p* ABA to mice infected with mutants unable to synthesize this compound, led to wild-type virulence (Bacon *et al.*, 1951).

The results with the *Salmonella aroA* vaccines have encouraged the development of similar mutants of other pathogenic bacterial species. Roberts *et al.* (1990) recently constructed an *aroA* mutant of *Bordetella pertussis* and discovered that aerosol-mediated immunization of mice with live organisms of this mutant provided effective protection against aerosol challenge with virulent *B. pertussis*.

Purine-requiring *Salmonella* strains have also attracted attention as potential vaccine strains. *S. typhimurium* mutants having a requirement for adenine (or adenosine) include *purA* (defective in adenylosuccinate synthetase) and *purB* (defective in adenylosuccinate lyase) mutants; both were highly attenuated in mouse virulence tests (McFarland and Stocker, 1987). Mutants with defects in the proximal end of the *de novo* purine biosynthetic pathway, for example *purE* mutants, were not as severely attenuated as *purA* or *purB* mutants (McFarland and Stocker, 1987; O'Callaghan *et*

*al.*, 1988). The failure of *purA* mutants to induce protective immunity indicated that they were hyperattenuated and of little value as vaccines (McFarland and Stocker, 1987; O'Callaghan *et al.*, 1988; Sigwart *et al.*, 1989). *Salmonella* strains carrying mutations in both *aroA* and *purA* were even more attenuated than strains carrying *purA* alone (O'Callaghan *et al.*, 1988; Sigwart *et al*, 1989).

*S. typhimurium galE* mutants, defective in the synthesis of UDP-galactose epimerase, are phenotypically rough and thus are avirulent when grown without galactose but can be rendered smooth in the presence of low amounts of galactose (Nikaido, 1961). High concentrations of exogenous galactose cause the accumulation of phosphorylated galactose within *galE* mutants, which is thought to result in cell lysis (Fukasawa and Nikaido, 1961a, 1961b). *galE* mutants of *S. typhimurium* were avirulent and immunogenic in mice (Germanier and Furer, 1971). The avirulence *in vivo* of *galE* mutants could be due to either sensitivity to galactose or the inability to produce smooth LPS. A *galE* mutant of *S. typhi* Ty2, Ty21a, was derived by extensive undirected mutagenesis and was shown to be sensitive to galactose-induced lysis, and both avirulent and immunogenic in mice (Germanier and Furer, 1975). This strain also carried a mutation at *via*, rendering it incapable of the synthesis of the Vi, or virulence, antigen. Hone and colleagues (1988) demonstrated that a *galE* deletion mutant (also *via* ) of *S. typhi* retained virulence in half (2 of 4) of the human volunteers immunized. Introduction of an identical *galE* mutation into *S. typhimurium* strains rendered those strains completely avirulent for mice, yet immunogenicity was retained (Hone *et al.*, 1987). These results highlighted, firstly, the dangers in extrapolating data from the mouse typhoid model to humans, and, secondly, the likelihood that the use of non-specific mutagenesis in the construction of Ty21a had introduced attenuating mutations which were not *galE* or *via* .

The *S. typhi* Ty21a strain has been tested widely, as a typhoid vaccine, in field trials. Although it has been shown to be safe, the levels of protection observed varied (Gilman

*et al.*, 1977; Wahdan *et al.*, 1980; Wahdan *et al.*, 1982; Germanier and Levine, 1986). A large scale field trial to test the efficacy of Ty21a in a typhoid-endemic area of Egypt, for example, demonstrated that the vaccine had 95% efficacy (Wahdan *et al.*, 1982; Woodward and Woodward, 1982). In contrast, Hirschell *et al.* (1985) studied a group of Ty21a-immunized travellers who had visited a typhoid-endemic area and could not demonstrate that the vaccine produced any protection against the disease. A second field trial, in Chile, highlighted that protection afforded by Ty21a was overly sensitive to the system of delivery (i.e. the type of capsule used), and the immunization schedule (Black *et al.*, 1983; Levine *et al.*, 1987 ).

The avirulence and immunogenicity of *Salmonella galE* strains has been correlated with the level of galactose required to produce smooth LPS. *S. typhimurium galE* mutants require relatively high amounts of galactose to become smooth, and were thus avirulent and immunogenic in mice. On the other hand, *galE* mutants of *S. choleraesuis* require low concentrations of galactose to make smooth LPS and are relatively virulent and only weakly immunogenic (Nnalue and Stocker, 1987). The paucity of non-phosphorylated galactose, the substrate for the galactose permease enzymes of *Salmonella* strains, in and around mammalian tissues, may mean that highly avirulent *galE* strains are unable to synthesize complete LPS (Curtiss, 1990).

### 1.4.2.3 Mutants defective in global regulation

The development of sensory and regulatory networks has enabled bacteria to adapt rapidly to different environments both within and outside the host. A response to changed conditions ouside the bacterial cell may involve the coordinate alteration of expression of sets of genes. Several two-component systems, involving perception of environmental stimuli, and transduction of the signal to a regulatory element, are known (Miller *et al.*, 1989a). For example, in *E. coli* the *envZ /ompR* system senses changes in extracellular osmolarity and responds by controlling porin gene expression (Clark

and Parker, 1984; Le Redulier *et al.*, 1984). Coordinate regulation of virulence factors is used by pathogens to respond to environmental stimuli encountered during infection. Expression of the cholera toxin (*ctx* ) operon in *V.cholerae* is dependent on the *toxR* gene product (Miller and Mekalanos, 1984), which responds to signals such as extracellular osmolarity changes. ToxR also controls the expression of the *tcpA* gene which encodes the major subunit of a *V.cholerae* pilus essential for intestinal colonization (Taylor *et al.*, 1987). In addition to *ctx* and *tcpA*, ToxR regulates at least fourteen other *V.cholerae* genes (Taylor *et al.*, 1987, 1988; Peterson and Mekalanos, 1988). A second regulatory locus, *toxS*, lies downstream from *toxR* and enhances the activity of *toxR* under certain conditions (Miller *et al.*, 1989b).

A regulatory locus, *vir*, which controls the expression of pertussis toxin at the transcriptional level in *Bordetella pertussis* , has also been described (Nicosia and Rappuoli, 1987). The *vir* locus regulates at least two other groups of genes, one of which encodes virulence factors (Knapp and Mekalanos, 1988). A gene called *mod* has been identified which apparently modulates *vir* gene expression in response to environmental signals (Knapp and Mekalanos, 1988).

Two major regulators of gene expression involved in virulence of *Salmonella* have been identified: the *cya*, *crp* and the *phoP* genes. The cAMP (cyclic AMP)-CRP (cAMP receptor protein) global regulon encompasses operons which are regulated at the transcriptional level in response to intracellular levels of cAMP and CRP. Mutations in the *cya* gene result in an inability of the cell to grow on most carbon sources other than glucose, due to defective production of adenylate cyclase, the enzyme involved in conversion of ATP to cAMP. Mutations in the *crp* gene result in an inability to produce the CRP. The cAMP-CRP complex is essential for initiation of transcription at several metabolic operons; for example, the *gal*, *lac*, *ara* and *mal* operons (Magasanik and Neidhardt, 1987). In this system cAMP is the metabolic signal and CRP is the regulatory element. The mechanism by which the cell senses the environmental levels

of carbon and energy sources is not understood. A group of *Salmonella* mutants defective in expression of the cAMP-CRP transcriptional regulation system have been investigated for vaccine potential. Mutants of this type were derived by Tn*10* mutagenesis in the *cya* and *crp* genes; deletion mutations were subsequently constructed (Curtiss and Kelly, 1987). Strains of *S. typhimurium* carrying *cya*, *crp* double deletions were avirulent when fed orally to mice and induced a high level of protective immunity to a virulent *S. typhimurium* challenge (Curtiss and Kelly, 1987; Curtiss *et al.*, 1988).

The *phoP* gene product is postulated to modulate the expression of several genes, including *phoN* , at the transcriptional level. The *phoN* gene is involved in the expression of a non-specific acid phosphatase. Other affected gene(s) determine resistance to defensins (microbicidal peptides present in granules of host phagocytic cells)(Groisman *et al.*, 1989). Phosphatase-defective (*phoP* ) mutants of *S. typhimurium* were avirulent by oral and intraperitoneal routes and induced immunity to virulent challenge in mice (Galán and Curtiss, 1989b). *Salmonella phoP* mutants are extremely sensitive to defensins, and this may account for their inability to survive in macrophages (Fields *et al.*, 1989). A second gene called *phoO* lies immediately downstream of *phoP* and encodes a protein which has homology to known environment-sensory proteins. Although the precise mode of attenuation of *phoP* mutants is yet to be clarified, it has been suggested that *phoP* and *phoO* are components of a regulatory system that controls *Salmonella* virulence (Miller *et al.*, 1989a).

Genetic manipulation of coordinately-regulated virulence expression systems in *Salmonella* will lead to the identification of additional genes involved in virulence. Modifications in the regulatory genes, resulting in pleiotropic phenotypic changes, may be an efficient method of creating attenuated vaccine strains.

### 1.4.2.4 Virulence plasmid-cured strains

Strains of *Salmonella* virulent for mice usually contain a large (60 - 100 kb) plasmid, the removal of which is attenuating for infection via the oral route (Jones *et al.*, 1982). When plasmid-free *S. typhimurium* and *S. dublin* strains were fed to mice , they were capable of attaching to and persisting in the gut-associated lymphoid tissue but infection did not spread to the liver or spleen (Hackett *et al.*, 1986; Pardon *et al.*, 1986; Gulig and Curtiss, 1987; Heffernan *et al.*, 1987; Hoertt *et al.*, 1989). Mice fed plasmid-free *S. typhimurium* were protected against oral challenge (Hackett *et al.*, 1986). However, the safety of plasmid-free *S. typhimurium* strains has been called into question by the findings that high doses of a plasmid-cured *S. typhimurium* killed mice and the $LD_{50}$ values for plasmid-cured and plasmid-containing strains, injected intraperitoneally, were similar (Gulig and Curtiss, 1987). A gene on the virulence plasmid of *S. typhimurium* has been implicated as being required for the expression, by the bacterium, of the ability to colonise the liver and spleen (Gulig and Curtiss, 1987). The virulence plasmid has also been shown to encode a serum-resistance factor (Hackett *et al.*, 1987; Terakado *et al.*, 1988).

### 1.4.2.5 Mutants with defects in virulence genes

Strains which can adhere to the intestinal epithelium, but which are non-invasive, may stimulate sufficient local immunity to be useful vaccine strains. *Salmonella* mutants defective in invasion of epithelial cells have been identified by transposon mutagenesis and assayed for penetration of tissue-culture cells (Finlay *et al.*, 1988; Galán and Curtiss, 1989a). The *S. typhimurium invA* mutants were able to attach, but not to invade, cultured epithelial cells and were attenuated for virulence when given orally to mice. The strains retained virulence when given intraperitoneally (Galán and Curtiss, 1989a).

## 1.5 Bivalent live oral *Salmonella* -based vaccines

The feasibility of using attenuated bacterial strains to carry the gene(s) for a protective antigen from another enteric species, and thus to effect immunization against both the carrier and the pathogen from which the gene was cloned, has been investigated by several groups (Formal *et al.*, 1981; Clements and El-Morshidy, 1984; Stevenson and Manning, 1985; Yamamoto *et al.*, 1985; Brown *et al.*, 1987; Maskell *et al.*, 1987). The capacity of attenuated *Salmonella* strains to colonise the gut-associated lymphoid tissue and to evoke both local and systemic immune responses (Moser *et al.*, 1980) made these strains attractive as potential carriers. An *aroA* mutant of *S. enteritidis*, expressing the enterotoxin B subunit (LT-B) of ETEC induced local intestinal sIgA and serum IgG antitoxin responses (Clements *et al.*, 1986). *S. typhimurium aroA* mutants carrying LT-B produced similar immune responses (Clements and El-Morshidy, 1984; Maskell *et al.*, 1987). An *S. typhimurium aroA* deletion mutant has also been used to express the M protein from *Streptococcus pyogenes* DNA, and this recombinant protected mice from challenge with *S. pyogenes* (Poirer *et al.*, 1988). Localization of the cloned foreign antigen on the surface of the *Salmonella* carrier is not absolutely necessary for immunogenicity, as demonstrated by the induction of an immune response to a cloned *E. coli* K-12 β-galactosidase expressed in an *aroA* mutant of *S. typhimurium* (Brown *et al.*, 1987).

A *galE* strain of *S. typhimurium* expressing cloned fimbrial antigens of ETEC was safe and immunogenic when fed to pregnant sows, and protection against challenge with ETEC carrying homologous fimbriae was afforded to suckling piglets (Attridge *et al.*, 1988). Strain Ty21a has been used to deliver the cloned Form 1 antigen of *Shigella sonnei* to human volunteers(Formal *et al.*, 1981; Seid *et al.*, 1984). When this hybrid was administered only moderate levels of protection (47% efficacy) were afforded against virulent *S. sonnei* challenge (Black *et al.*, 1987b). A hybrid of Ty21a which

expressed the O-antigen genes of *V.cholerae* from recombinant DNA, was shown to be safe and immunogenic in humans (Forrest *et al.*, 1987).

Another attenuated strain, the *S. typhimurium del cya, del crp* double mutant ,has been used successfully in the expression of antigens of *Streptococcus mutans* (Curtiss, 1986; Curtiss *et al.*, 1987; Curtiss and Kelly, 1987).

## 1.6 Core lipopolysaccharide structure and biosynthesis

### 1.6.1 LPS structure

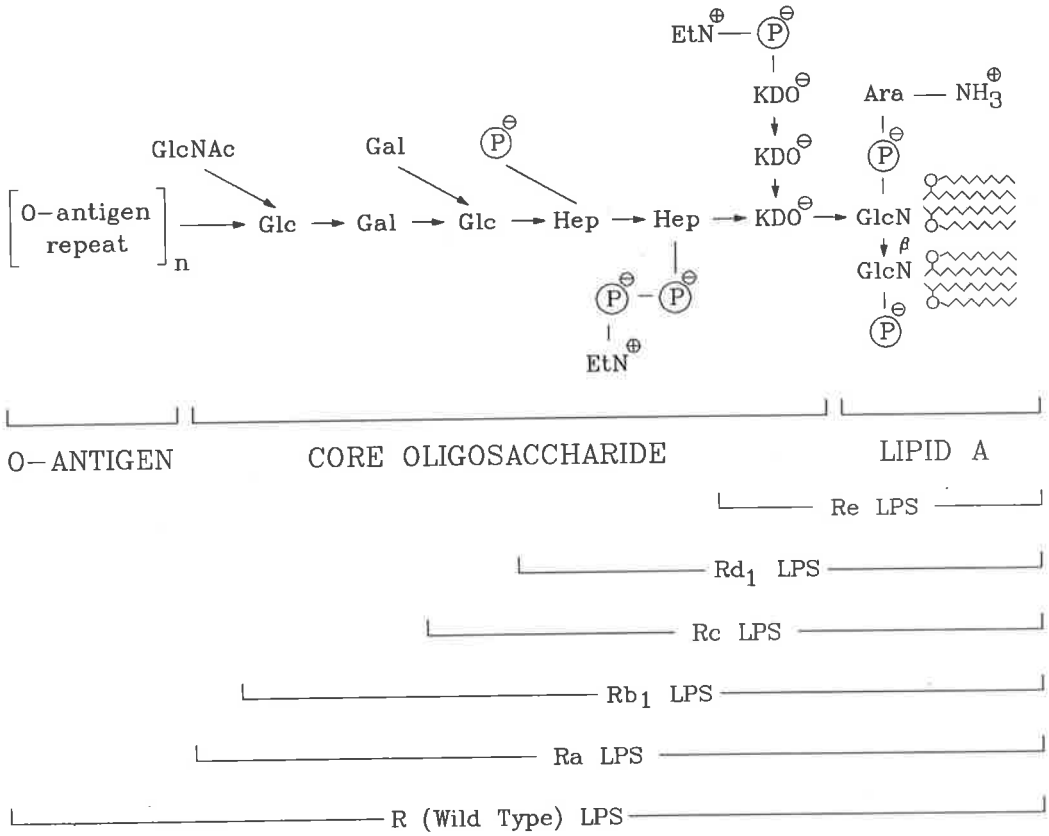
LPS consists of a complex heteropolysaccharide covalently linked to lipid A, and constitutes a major component of the outer membrane of Gram-negative bacteria (Inouye, 1979). The polysaccharide component of LPS consists of two main regions (Fig 1.1), the core polysaccharide, and the O-somatic (O-antigenic) side-chains. Wild-type strains capable of synthesizing complete LPS are called smooth (S) while strains with mutations in LPS biosynthetic genes are either rough (R) or semi-rough (SR).

### 1.6.2 Structure and biosynthesis of lipid A

Lipid A plays a prominent role in the stabilization of the cell membrane and the anchoring of LPS. The endotoxic effects mediated by lipid A in infected mammals include toxic shock and fever induction in addition to the activation of lymphocytes and macrophages (Galanos *et al.*, 1977; Rietschel, 1984). Lipid A of *E. coli* consists of a β -1-6 interlinked disaccharide of glucosamine, which is phosphorylated at positions 1 and 4'. The disaccharide is acylated with four β-hydroxymyristoyl residues and substituted with short chain fatty acids (Rietschel, 1984). Two 3-deoxy-D-*manno* -octulosonic acid (KDO) residues are attached at position 6' (Strain *et al*, 1983a ). UDP-N-acetylglucosamine (UDP-GlcNAc) is a key precursor in lipid A and peptidoglycan

**Figure 1.1: The structure of the LPS of *Salmonella* strains**

The lipid A, core region, and the O-antigen are shown. The truncation of the LPS in various mutants (chemotypic designations also given) appear on the bottom. Ara: arabinose; EtN: ethanolamine; Gal: galactose; Glc: glucose; GlcNAc: N-acetylglucosamine; Hep: heptose; P: phosphate.


EtN
P
KDO
KDO
KDO
Ara
NH3
GlcNAc
Gal
P
O-antigen repeat
n
Glc
Gal
Glc
Hep
Hep
GlcN
β
GlcN
P
P
P
EtN
O-ANTIGEN
CORE OLIGOSACCHARIDE
LIPID A
Re LPS
Rd1 LPS
Rc LPS
Rb1 LPS
Ra LPS
R (Wild Type) LPS

biosynthesis (Anderson *et al.*, 1985). Two genes involved in *E. coli* lipid A synthesis have been cloned and characterized: *lpxA* encodes UDP-GlcNAc acyltransferase, and *lpxB* encodes the disaccharide synthetase (Crowell *et al.*, 1986).

### 1.6.3 Structure and biosynthesis of KDO

KDO serves as a structural bridge between the lipid A and polysaccharide components of the core oligosaccharide. It was originally proposed that the KDO residues occurred as a branched trisaccharide with two KDO residues in the main chain and the third in the side chain (Dröge *et al.*, 1970; Prehm *et al.*, 1976). Subsequently, however, it was discovered that only two KDO residues were present in the KDO-lipid A region of core defectve, Re mutants of *E. coli* (Brade *et al.*, 1983; Strain *et al.*, 1983a, 1983b; Brade and Rietschel, 1984), indicating that synthesis of part or complete core was required for incorporation of the third KDO residue. The occurence of a linear KDO trisaccharide was subsequently detected in an Rb2 mutant of *Salmonella* (Brade *et al.*, 1983; Brade and Rietschel, 1984).

The mechanism of KDO synthesis has been elucidated by the use of conditionally lethal mutants defective in KDO synthesis (Lehmann *et al.*, 1977; Rick and Osborn, 1977). Cell growth is inhibited, at the restrictive temperature, in mutants temperature-sensitive for KDO synthesis, due either to outer membrane instability/non-functionality as a result of an incomplete KDO-lipid A molecule, or the accumulation of lipid A precursors in the cytoplasmic membrane (Rick and Osborn, 1977). The KDO synthesis pathway involves the enzymes D-ribulose-5-phosphate isomerase, KDO-8-phosphate synthetase, and KDO-8-phosphate phosphatase. Studies with temperature-sensitive KDO mutants suggest that a KDO disaccharide is synthesized initially, and that this is later transferred to nascent lipid A, prior to the addition of saturated fatty acid residues and the further addition of core sugars (Heath *et al.*, 1966; Walenga and Osborn,

1980a, 1980b). The transfer of the third KDO residue to the inner core region may occur after conversion of free KDO to CMP-KDO (Ghalambor and Heath, 1966).

### 1.6.4 Structure and biosynthesis of the LPS core region

Six different types of core polysaccharides have been identified in the *Enterobacteriaceae* (Jansson *et al.*, 1981). Qualitative analysis of LPS revealed that all *Salmonella* species had the same five basal sugars in the core region: KDO, L-glycero D-*manno* -heptose, D-glucose, D-galactose, and N-acetylglucosamine (Lüderitz *et al.*, 1971; Hellerqvist and Lindberg, 1971). The sugars of the core LPS are added sequentially, from high energy UDP precursors in the case of glucose, galactose and N-acetylglucosamine (Ginsburg, 1964). The core LPS structure in *Salmonella* strains is highly conserved (Hellerqvist and Lindberg, 1971; Osborn, 1979). In contrast, at least three different core types exist in *E. coli* (Schmidt *et al.*, 1969, 1970; Orskov *et al.*, 1977; Jansson *et al.*, 1981 ). Mutants in which particular steps in LPS synthesis are blocked have proven invaluable in investgations into LPS structure and assembly. LPS mutants have been mainly studied in *Salmonella* (Wilkinson and Stocker, 1968; Wilkinson *et al.*, 1972; Roantree *et al.*, 1977; Mäkelä and Stocker; 1984) and *E. coli* K-12 ( Eriksson-Greenberg *et al.*, 1971; Monner *et al.*, 1971; Rapin and Kalckar, 1971; Watson and Paigen, 1971; Reiner, 1974; Tamaki *et al.*, 1974; Hancock and Reeves, 1976; Havekes *et al.*, 1976; Coleman and Leive, 1979; Sandulache *et al.*, 1984). The genetics of LPS biosynthesis in *Salmonella* will be reviewed below.

#### 1.6.4.1 The inner core

The L-glycero-D-*manno* -heptose of the inner core is synthesized via D-glycero-D-*manno* -heptose-7-phosphate from sedoheptulose-7-phosphate (Eidels and Osborn, 1971). It is thought that ADP-L-glycero-D-*manno* -heptose acts as the donor of heptose residues to the inner core. In some mutants of *E. coli* the epimerase

neccessary for conversion of D-glycero-D- *manno* -heptose to L-glycero-D- *manno* -heptose is inactive(Coleman, 1983; Coleman and Leive, 1979). In these mutants, the precursor form is accumulated and partially incorporated into the core LPS, leading to poor completion of core polysaccharides (Lehmann *et al.*, 1973). Additional mutants defective in the heptose region of the inner core have been described, which are defective either in synthesis of heptosyltransferase or some earlier step in heptose synthesis (Mäkelä and Stocker, 1984).

Incorporation of phosphate into the heptose region follows the addition of the first glucose residue to the outer core (Mühlradt, 1969; Hämmerling *et al.*, 1973). Substitution of the heptose-KDO region by ethanolamine has also been reported (Hasin and Kennedy, 1982).

### 1.6.4.2 The outer core

The outer core region consists usually of a branched pentasaccharide with little structural variation among *Salmonella* strains (Jansson *et al.*, 1981). In a model for core LPS synthesis (Rothfield and Romeo, 1971), membrane bound glycosyltransferases catalyse the sequential transfer of sugars from nucleotide-sugar donors to the non-reducing terminus of the growing polysaccharide chain. The LPS molecules, according to the model, are attached to the cytoplasmic membrane by lipid A, and during synthesis move laterally along the inner surface of the membrane, coming in contact with relatively immobile glycosyltransferases which transfer each sugar from its UDP precursor (Rothfield and Romeo, 1971). A mutation affecting addition of a particular sugar at any point in the core will prevent addition of all sugars distal to it. Mutants, therefore, with defects in individual glycosyltransferases, can be identified by differences in LPS lengths. The incomplete LPS of *Salmonella* mutants were used as acceptors in experiments to identify individual activities of glucosyltransferase I (Rothfield *et al.*, 1964), $\alpha$ - 1,3 - galactosyltransferase (Osborn *et*

*al.*, 1962; Rosen *et al.*, 1964), glucosyltransferase II and N-acetylglucosaminyltransferase (Osborn and D'Ari, 1964; Osborn and Rothfield, 1971).

## 1.7 Structure and biosynthesis of O-antigen

### 1.7.1 O-repeat unit biosynthesis

The O-antigenic region of LPS consists of oligosaccharide repeat units (O-repeat unit) which may be either linear trisaccharides, pentasaccharides, or branched oligosaccharides with four to six sugars. The structure of the *Salmonella* O-repeat unit determines the O-serogroup specificity (Kauffmann, 1966).

In *S. typhimurium* the tetrasaccharide O-repeat unit consists of two hexoses (D-galactose, D-mannose), one deoxyhexose (L-rhamnose) and one dideoxyhexose (abequose). Four distinct steps are implicated in the synthesis and assembly of the O-specific polysaccharide. In the first step, the O-repeat unit is synthesized on an a carrier lipid (antigen carrier lipid- ACL). The second step involves polymerization of individual ACL-bound O-repeat units to form a chain. This complex is transfered to the core LPS, with recycling of the ACL, in the third step. Finally, the core-O-antigen oligosaccharide is translocated to the outside of the cell. The O-repeat unit synthesis is mediated by a multienzyme system that utilises the membrane-bound polyisoprenoid, ACL, as an intermediate carrier for the growing O-repeat unit (Weiner *et al.*, 1965; Wright *et al.*, 1965). In *Salmonella* O serogroups A, D, E (Robbins and Wright, 1971; Wright and Kanegasaki, 1971) and $C_2$, $C_3$ (Shibaev *et al.*, 1979), synthesis is initiated with the reversible transfer of galactose-1-phosphate from UDP-galactose to the phosphorylated ACL. In the case of *S. typhimurium*, sequential transfer of L-rhamnose, D-mannose, and abequose to this lipid-linked intermediate is catalysed by membrane-bound transferases using TDP-rhamnose, GDP-mannose and CDP-abequose, respectively, as donors; O-repeat unit formation results. The specificity of

the transferases with respect to acceptor requirement is not rigorous. In the case of rhamnosyltransferase, for example, and using a chemically synthesized monosaccharide diphosphate linked to ACL, it was discovered that fucosyl or talosyl residues could substitute for the galactosyl acceptor residue (Shibaev, 1978; Shibaev *et al.*, 1982; Shibaev, 1986).

### 1.7.2 O-repeat unit polymerization

Polymerization of O-repeat units occurs in the cytoplasmic membrane, using membrane-bound enzymes and intermediates. Completed O-repeat units are required for polymerization to occur *in vivo* (Weiner *et al.*, 1965, Yuasa *et al.*, 1969). The linkage formed between units by the polymerase partially determines O-antigenic specificity (Staub and Bagdian, 1966).

The activity of the polymerase of *Salmonella anatum* on exogenous, enzymatically synthesized polyprenol trisaccharide diphosphate was demonstrated by repeated freeze-thawing of a mixture of the *S. anatum* cell fraction which contained the polymerase, and the lipid-intermediate mixture (Kanegasaki and Wright, 1970). The *in vivo* mechanism by which individual O-repeat units are polymerized to complete O-antigen has been described (Bray and Robbins 1967a; Robbins *et al.*, 1967). In *Salmonella newington*, elongation of the lipid-linked O-antigen occurs by growth at the reducing end, i.e. a glycosidic linkage is formed between the reducing terminal galactose of the growing chain and the non-reducing mannose of an O-repeat unit. The substrate specificity of the polymerase is not rigid and polymerase of either *Salmonella* groups B or D could polymerise the O-repeat units of the other (Mäkelä, 1965; Nurminen *et al.*, 1971; Valtonen *et al.*, 1975). However, when the *rfb* genes, for O-repeat unit synthesis, of *Salmonella* group B were transferred into *Salmonella* groups $C_1$ or $C_2$ (Naide *et al.*, 1965; Mäkelä, 1966) or into *E. coli* (Hämmerling *et al.*, 1971), the donor type O-repeat units were not polymerised and the recombinants were semi-rough. This

indicated that a different system of O-repeat unit polymerization was operative in these strains.

In *Salmonella* groups $C_1$ and L (Mäkelä and Stocker, 1984) and in *E. coli* 08 and 09 (Schmidt *et al.*, 1976; Jann *et al.*, 1979), the synthesis of O-antigen occurs by a mechanism different from that described above. The O-antigen of *E. coli* 09 is a D-mannan, composed of pentasaccharide repeat units (Prehm *et al.*, 1976). Synthesis of the O-repeat unit of *E. coli* 09 occurs by initial linkage of a glucose residue to the ACL, a reaction analogous to attachment of galactose to ACL in *Salmonella* groups B and E. However, construction of the polymannan does not involve the synthesis and polymerization of individual ACL-linked repeat units, but is synthesized by the sequential transfer of mannose residues from GDP-mannose to the non-reducing terminus of the growing chain (Jann *et al.*, 1982). This monomeric polymerization (known as *rfe* -dependent) results in a polysaccharide of O repeat units. Since only one ACL molecule is required per O-antigen chain, this type of synthesis is less sensitive to inhibition by bacitracin than the *rfe* -independent system (Siewert and Strominger, 1967; Stone and Strominger, 1971). Ligation of *E. coli* 09 O-antigen to the core LPS involves formation of a linkage between the terminal reducing glucose of the polymerised O-antigen and an incomplete R1 core lacking one glucose residue (Weisgerber *et al.*, 1984)

### 1.7.3 Modifications to polymerase activity

The temperate bacteriophage P27 has the ability ,upon lysogeny of *Salmonella bredeney* and *S. typhimurium*, to modify the polymerase-determined linkages (Lindberg *et al.*, 1978). Thus, the linkage between the O-repeat units is changed from (1 - 2) to (1 - 6), and the new O-antigenic factor 27 is expressed (Lindberg *et al.*, 1978).

In the lysogenic transformation of *S. anatum* with bacteriophage $e^{15}$, a change of LPS structure occurs which involves three bacteriophage-encoded proteins and results in the expression of O-antigenic factor 15 (Bray and Robbins, 1967b; Losick and Robbins, 1967; Losick, 1969). These proteins include a repressor of an enzyme involved in LPS acetylation, an inhibitor of the host α-(1 - 6) glycosidic polymerase, and a novel polymerase that catalyses the formation of β-(1 - 6) linkages.

Considerable heterogeneity of O-antigenic side-chain length has been observed in purified LPS preparations (Goldman and Leive, 1980; Munford *et al.*, 1980; Palva and Mäkelä, 1980). The factors which determine this heterogeneity remain unclear. The degree of O-antigen polymerization has been found to be dependent on growth temperature (McConnell and Wright, 1979) and carbon source (Taylor *et al.*, 1981). The coordinate regulation of the enzymes responsible for O-antigen polymerization, ligation of O-antigen to core LPS, and translocation of completed LPS to the outer surface of the cell, appears to be poor, based on two lines of evidence. Firstly, core-defective mutants synthesize polymeric O-antigen linked to ACL although there is no core for attachment (Kent and Osborn, 1968a, 1968b). Secondly, strains which fail to synthesize O-repeat units (due to defects in *rfb* genes), transport normal amounts of core LPS to the cell surface (Smit *et al.*, 1975).

LPS structure is influenced by plasmids in *Shigella sonnei* (Kopecko *et al.*, 1980), *Shigella dysenteriae* (Watanabe and Timmis, 1984), *E. coli* (Riley *et al.*, 1987) and *Salmonella* (Popoff and LeMinor, 1985). Recently, Kawahara and co-workers (1989) discovered that curing of the virulence plasmid of *S. dublin* produced semi-rough LPS and postulated that a regulatory gene for O-antigen synthesis, possibly acting at the level of polymerization, was present on the plasmid.

### 1.7.4 Transfer of O-repeat units to the core

In *Salmonella* groups B, D and E, following polymerization, the completed O-antigen chain is transferred from ACL to the completed core LPS with the release of ACL-pyrophosphate. A 1- 4 linkage is formed, by the action of an O-antigen ligase, between the terminal non-reducing glucose of the core and the reducing galactose of the O-antigenic chain. A single, incomplete O-repeat unit (rhamnosylgalactose) can be transferred to the core in a cell-free system (Nikaido, 1965). However, the presence of the abequosyl side-chain is essential for the biosynthesis of O-polysaccharide *in vivo*, since a mutant blocked in synthesis of CDP-abequose does not produce long chain LPS (Yuasa *et al.*, 1969). Individual O-repeat units may be transferred to the core as seen in polymerase defective mutants of *Salmonella* (Naide *et al.*, 1965). The possibility that O-repeat units are added individually to the core rather than after polymerization seems unlikely, in view of the evidence for the existence of O-hapten as a precursor to O-antigen (Kent and Osborn, 1968a, 1968b) and the demonstration that growth of the lipid-linked polymer occured at its reducing end (Robbins *et al.*, 1967).

## 1.8 The topology of lipopolysaccharide biosynthesis

Definition of the mechanism by which LPS is translocated and the proteins and structures mediating this activity, remains elusive. It has been demonstrated by localization of biosynthetic enzymes in isolated membrane fractions (Osborn *et al.*, 1972a), and by pulse-chase experiments *in vivo* (Osborn *et al.*, 1972b), that core and O-antigen synthesis, as well as attachment of O-antigen to core, occured in the cytoplasmic membrane, from where export to the outer membrane took place.

Mulford and Osborn (1983) showed by immunoelectron microscopy that in core-defective mutants, the newly made ACL-linked O-side-chain was located on the outer

surface of the inner membrane. These results suggest that polymerization of the O-antigen repeat units is coupled to the translocation of the polysaccharide from the inner to the outer surface of the cytoplasmic membrane.

In contrast to the translocation of phospholipids (Jones and Osborn, 1977), the process of LPS translocation appears to be non-reversible (Osborn *et al.*, 1972a, 1972b). A model for LPS translocation proposes that LPS translocation occurs at sites of contact, or zones of adhesion, of the inner and outer membranes (Mühlradt *et al.*, 1974; Bayer, 1975). Once inserted in the outer membrane, LPS molecules are postulated to diffuse laterally from the insertion location to cover the entire cell surface (Mühlradt *et al.*, 1974).

## 1.9 The genetics of lipopolysaccharide biosynthesis

### 1.9.1 Core LPS

The isolation of temperature-sensitive mutants defective in KDO synthesis (Rick and Osborn, 1977) facilitated the identification of the genes *kdsA*, which encodes KDO-8-P-synthetase, and *kdsB*, which encodes KDO-CMP synthetase (Rick and Young, 1982) (see Section 1.6.3).

The glycosyltransferases required for core polysaccharide elongation, and an O-antigen ligase which is required for attachment of the polymerised O side-chain to a completed core, are encoded by the *rfa* group of genes (Mäkelä and Stocker, 1981). Most of the *rfa* genes map between *cysE* at 77 min. and *pyrE* at 79 min. (Kuo and Stocker, 1972; Sanderson and Hurley, 1987).

Four *S. typhimurium* genes, involved in the synthesis or transfer of the heptose residues to the core, have been identified. Mutants producing Rd2 type LPS had a

mutation in the *rfaF* gene, which is postulated to control the heptosyltransferase II enzyme (Kuo and Stocker, 1972; Sanderson and Saeed, 1972a, 1972b; Sanderson *et al.*, 1974). Three different mutations, in either *rfaC* ,*rfaD* ,or *rfaE*, resulted in the synthesis of heptoseless (Re) LPS (Sanderson *et al.*, 1974). The *rfaC* gene encodes either the heptosyltransferase I enzyme or an enzyme involved in an earlier step of heptose biosynthesis (Mäkelä and Stocker, 1984). The *rfaD* gene is postulated to encode the epimerase which converts ADP-D-glycero-D-*manno* -heptose to ADP-L-glycero-D-*manno* -heptose (Coleman, 1983; Coleman and Deshpande, 1985). The *rfaE* gene maps near *metC*, outside the *cysE -pyrE* region (Kuo and Stocker, 1972; Sanderson *et al.*, 1974). The *rfaE* gene may be the structural gene for a heptosyltransferase (Mäkelä and Stocker, 1984). Phosphorylation of the heptose region in *Salmonella minnesota* is determined by the *rfaP* locus (Mühlradt, 1970; Jousimies and Mäkelä, 1974).

The following genes, with their putative products in brackets, have been implicated in the synthesis of outer core LPS: *rfaG* (glucosyltransferase I); *rfaB* (galactosyltransferase II); *rfaI* (galactosyltransferase I); *rfaJ* (glucosyltransferase II); *rfaK* (N-acetylglucosamine transferase) and *rfaL* (a component of the O-antigen translocase / ligase system) (Creeger and Rothfield, 1979; Mäkelä and Stocker, 1984; Kadam *et al.*, 1985). A gene, *rfaH*, has also been identified, which positively regulates the activities of at least two of the glycosyltransferases encoded in core polysaccharide synthesis (Creeger *et al.*, 1984).

### 1.9.2 Genetics of O-repeat unit synthesis

The genes required for synthesis of the O-repeat unit of *S. typhimurium* are clustered between the *his* (44 min.) and *metG* (46 min.) loci (Sanderson and Hurley, 1987). This region of the chromosome encodes at least nine enzymes involved in the synthesis of GDP-mannose, TDP-rhamnose and CDP-abequose, and four glycosyltransferases

(Nikaido, 1967; Brahmbhatt *et al.*, 1986, 1988). The *galU* and *galE* genes responsible for synthesis of UDP-galactose are located outside the *rfb* cluster and also have general housekeeping functions. However, a gene within the *rfb* group, *rfbF*, works to modify the *galU* gene product (UDP-glucose pyrophosphorylase) in a step required for incorporation of galactose into the O-repeat units (Nakae, 1971).

All of the genes (namely, *rfbA*, *B* and *D* ) associated with the TDP-rhamnose synthesis pathway are found within the *rfb* cluster (Nikaido *et al.*, 1967). Mutants with defects in monosaccharide transferase genes have proven difficult to isolate due to their instability. Nevertheless, the gene *rfbN* has been identified as responsible for a rhamnosyltransferase which transfers rhamnose from dTDP-rhamnose to ACL-polysaccharide precursor (Levinthal and Nikaido, 1969).

Except for the *pmi* gene, all of the genes for GDP-mannose synthesis and incorporation are located within the *rfb* cluster. The *rfbL* gene encodes phosphomannomutase, which may be modified by the action of the *rfbK* gene product (Mäkelä and Stocker, 1984).

The O-antigenic side-chains of *S. typhimurium*, *S. typhi* and *Salmonella paratyphi* A, representative of groups B, A and D, respectively, have identical O-repeat unit trisaccharide backbone subunits. Each of these strains, however, has a different 3,6-dideoxyhexose attached to the mannosyl residue as a side chain. The side branch sugar is abequose in *S. typhimurium*, tyvelose in *S. typhi*, and paratose in *S. paratyphi* A (Jann and Jann, 1984; Mäkelä and Stocker, 1984). This variation in substitution of the O-repeat unit backbone results in antigenic variation among these *Salmonella* species and is reflected by DNA sequence differences in their *rfb* clusters (Verma *et al.*, 1988). Five of the genes (*rfbF*, *G*, *H*, *I* and *J* ) in the *rfb* cluster have been associated with the synthesis of CDP-abequose (Nikaido *et al.*, 1967; Yuasa *et al.*, 1969; Wyk and Reeves, 1989). In *Salmonella* groups A and D, abequose synthase is replaced by paratose

synthetase and an additional epimerase in group D *Salmonella* strains converts CDP-paratose to CDP tyvelose. The DNA sequences of *rfbS* for paratose synthetase, *rfbE* for CDP-tyvelose-2-epimerase (Verma and Reeves, 1989), and *rfbJ* for abequose synthetase (Wyk and Reeves, 1989), have been determined.

In *Salmonella montevideo* (group $C_1$) and *S. minnesota* (group L), a mutation in the *ilv* -linked, *rfe* locus resulted in a rough phenotype resembling that of *rfb* -defective mutants (Mäkelä *et al.*, 1970). The *rfe* -encoded function was required for synthesis of O-antigen in *E. coli* 08 and 09 by a mechanism different from that mediated by the *rfc* gene product in *Salmonella* strains of group B (see Section 1.7.2). Although *S. typhimurium* (group B) also has *ilv* - linked *rfe* genes, which can direct the production of O-6,7-specific polysaccharide, these genes are not required for O-4,12 polysaccharide synthesis (Mäkelä and Mayer, 1974). The *rfe* locus has also been implicated in the synthesis of enterobacterial common antigen (Mäkelä and Mayer, 1974; Schmidt *et al.*, 1976).

### 1.9.3 Genetics of O-repeat unit polymerization

Mutants defective in the polymerisation of O-antigen units, described as semi-rough (SR), of *S. typhimurium* , have been characterised as having only a single O-repeat unit attached to any particular LPS core molecule (Naide *et al.*, 1965; Yuasa *et al.*, 1970). The gene postulated to be responsible for polymerization, called *rfc*, was mapped between *gal* at 18 min., and *trp* at 34 min. (Naide *et al.*, 1965; Sanderson and Hurley, 1987). Conservation of the *rfc* allele among strains of *Salmonella* O serogroups A, B and D has been proposed, based on the discovery that hybrids between groups B and D involving transfer of *rfb* genes were always smooth (Mäkelä, 1965; Nurminen *et al.*, 1971; Valtonen *et al.*, 1975). In contrast, hybrids in which group B *rfb* genes were transferred to a group E strain were found to be SR, implying differences between the O-antigen subunit polymerases of groups B and E (Nyman *et*

*al.*, 1979). Similarly, hybrids in which the group B *rfb* region was transferred into *Salmonella* strains of group $C_1$ or $C_2$, produced SR type LPS (Naide *et al.*, 1965; Mäkelä, 1966).

A locus analogous to the *rfc* gene may be present in other bacterial species. The O-antigen of *Shigella flexneri* is assembled by polymerization of ACL-linked O-repeat units. The identification of *S. flexneri* SR-type LPS mutants, the formation of solvent-extractable, multiple repeating units during *in vitro* synthesis, and the inhibition of O-antigen synthesis by bacitracin, all suggested that O-antigen biosynthesis in *S. flexneri* resembled that observed for *S. typhimurium* and the involvement of an *rfc* - like polymerase activity was postulated (Simmons and Romanowska, 1987).

The recent observation of predominantly SR-type LPS in some strains of *B. pertussis* has led to the hypothesis that the kinetics of polymerization of O-repeat units in this species is regulated (Caroff *et al.*, 1990). It remains to be determined whether an *rfc* - like gene is involved in O-repeat unit polymerization in *B. pertussis*.

The capsular polysaccharide of some *E. coli* strains is synthesized in an O-repeat unit structure similar to that of the O-repeat unit assembly in *Salmonella*. A gene for a putative polymerase of capsular polysaccharide in *E. coli* K27 was mapped to a *trp* - linked locus, in an analogous position to that of the *rfc* gene in *S. typhimurium* (Schmidt *et al.*, 1977).

### 1.10 Aims of this study

Some insight has been gained into the virulence factors of *Salmonella* , and their role in the process of infection. The structure of the LPS, in particular whether the material is wild-type or mutant in phenotype, is thought to be important in *Salmonella* pathogenesis, yet the genetics and molecular biology of LPS assembly and translocation

are not fully understood. The relative virulence of smooth and rough *Salmonella* strains has been investigated with strains not necessarily isogenic at all loci other than the locus affecting LPS biosynthesis in any particular mutant.

The objectives of this study were therefore three-fold. The first objective was to characterize, by cloning and sequencing, two genes, *rfc* and *pmi* , which are involved in LPS biosynthesis. The second objective was the construction of defined mutations in the chromosomal loci of *rfc* and *pmi* by replacement of the wild-type genes with mutations constructed *in vitro* in the cloned genes. The final objective was to compare the intestinal invasion and virulence characteristics of these two constructs, in mice, with those of the parent *Salmonella* strain.

# CHAPTER 2

## Materials and Methods

## 2.1. Chemicals and reagents

Chemicals were Analar grade. Phenol, polyethylene glycol 6000 (PEG), sodium dodecyl sulphate (SDS), and sucrose, were from BDH Chemicals. Adenosine-5'-triphosphate, sodium salt (ATP), dithiothreitol (DTT), herring sperm DNA, ethylene-diamine-tetra-acetic-acid, disodium salt (EDTA), ethidium bromide, glucose-6-phosphate (G6P) dehydrogenase, D-mannose-6-phosphate, nicotinamide adenine dinucleotide phosphate (NADP), phosphoglucose isomerase (PGI), and Trizma base were from Sigma (St. Louis, MO). Caesium chloride, citric acid, calcium chloride, magnesium chloride and sodium hydroxide were obtained from Ajax Chemicals, NSW, Australia.

Antibiotics were purchased from Sigma (ampicillin, kanamycin sulphate, rifampicin), and Calbiochem (tetracycline, chloramphenicol).

The following electrophoresis grade reagents were obtained from the sources indicated: acrylamide and ammonium persulphate (Bio-Rad), ultra pure N,N'-methylene bis-acrylamide and urea (BRL).

The four deoxyribonucleotide triphosphates (dATP, dCTP, dGTP and dTTP), X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) and IPTG (isopropyl-β-D-thiogalactopyranoside) were purchased from Boehringer Mannheim. M13 sequencing primer and [$^{32}$P]-dCTP, at a specific activity of 1,700 Ci/mM, were obtained from BRESA (Adelaide, Australia). The -35 sequencing primer was obtained from New England Biolabs. [$^{35}$S]-methionine (1,270 Ci/mM), [$^{35}$S]-dATP (>1000 Ci/mM) and Sequenase, were purchased from Amersham.

## 2.2 Enzymes

Lysozyme was obtained from Sigma. Pronase was from Boehringer Mannheim. Restriction endonucleases were purchased from either Boehringer Mannheim, New England Biolabs, Pharmacia or Amersham. DNA-modifying enzymes were purchased from New England Biolabs (T4 DNA ligase), Amersham (T4 DNA polymerase) and Boehringer Mannheim (Klenow fragment of DNA polymerase I; DNAse I).

## 2.3 Synthesis of oligodeoxynucleotides

Oligodeoxynucleotides were synthesized using reagents purchased from Applied Biosystems or Ajax Chemicals (acetonitrile). Synthesis was performed on an Applied Biosystems 381A DNA synthesizer. Oligonucleotides were routinely of a purity such that no further purification was required.

## 2.4 Growth media

The following nutrient media were used for bacterial cultivation. Nutrient broth (NB) (Difco, Detroit, MI), prepared at double strength (16 g/l) with added sodium chloride (NaCl) (5 g/l) was the general growth medium for *Salmonella* and *E.coli* K-12 strains. NBG was NB with glucose (0.5% (w/v)) added; NBGal was NB with galactose (0.2% (w/v)) added; NBGG was NBG with galactose (0.2% (w/v)) added, and NBGM was NBG supplemented with mannose (0.5% w/v)). These liquid media were solidified, when required, by the addition of agar (1.6% (w/v)). Luria broth (LB), Luria agar (L agar), and 2 x TY medium were prepared as described by Miller (1972). Minimal M9 medium, and A medium (M13 minimal medium) were also prepared as described by Miller (1972) and supplemented prior to use with $MgSO_4$ (0.2 mg/ml), glucose (2 mg/ml) and thiamine-HCl (50 μg/ml). Methionine assay medium (Difco) was reconstituted according to the manufacturer's instructions.

NA is nutrient agar, which is blood base agar (Difco) prepared without the addition of blood. Soft agar contained equal volumes of NB and NA. Mannose-tetrazolium (MTZ) agar contained Bacto-antibiotic medium 2 (Difco), supplemented with mannose (10 g/l) and 2,3,5 - triphenyltetrazolium (50 mg/l). H agar consisted of bacto-tryptone (16 g/l) (Difco), NaCl (8 g/l) and Bacto-agar (12 g/l) (Difco). H top agar was as H agar but with 8 g/l Bacto-agar.

Antibiotics were added to broth and solid media at the following final concentrations: ampicillin (Ap) 50 µg/ml; chloramphenicol (Cm) 25 µg/ml; kanamycin (Km) 50 µg/ml; tetracycline (Tc) 4 µg/ml.

Bacterial growth was at 37°C unless otherwise specified. Normally, liquid cultures were grown in 20 ml McCartney bottles or 125 ml side-arm flasks. Optical densities (OD) were measured at 650 nm using a Unicam Instruments spectrophotometer which had been adapted to read side-arm flasks.

## 2.5 Bacterial strains and plasmids, and bacteriophage

Table 2.1 describes the *E. coli* K-12 and *Salmonella* strains used in this study. The plasmids and phage cloning vectors which were used in this study are listed in Table 2.2. The bacteriophages used here (9NA, P22, FO and P22I) have been described in Wilkinson *et al.* (1972). Bacteriophage propagation and storage methods were those described by Miller (1972).

### 2.5.1 Maintenance of bacterial strains

For long-term storage, all strains were maintained as lyophilized cultures, stored *in vacuo* in sealed glass ampoules. When required, an ampoule was opened and its contents suspended in several drops of the appropriate sterile broth. Half the contents were then transferred to NB (10 ml) and grown with shaking overnight at the appropriate temperature. The other half was streaked onto two NA plates and incubated overnight at the appropriate growth temperature. Antibiotics were added to the media when appropriate. If the colony form was uniform, single

**Table 2.1: The bacterial strains used in this work**

| STRAIN | GENOTYPE | RELEVANT PHENOTYPE | SOURCE/REFERENCE |
|---|---|---|---|
| *Escherichia coli* K-12 | | | |
| DH1 | *gyrA*96, *recA*1, *relA*1, *endA*, *thi*-1, *hsdR*17, *supE*44, lambda$^-$ | | Maniatis *et al.*,1982 |
| DS410 | *minA*, *minB*, *rpsL* | Minicell producer | Dougan and Sherratt,1977 |
| K38 | HfrC (lambda) | Host for T7 polymerase/promoter system | Russell and Model,1984 |
| S17-1 | RP4-2-Tc::Mu-Km::Tn7/*pro*, *hsdR* | Promotes transfer of *mob*$^+$ plasmids | Simon *et al.*,1983 |
| JM101 | *supE*, (del)[*lac-proA*, *B*], *thi*-1/ F'[*traD*36, *proA*, *B*, *lacI*$^q$, *lacZ*, (del)M15] | Host for M13 propagation | Sanger *et al.*,1980 |
| RU2901 | $R_{ts}$1::Tn*1725* | Donor of Tn*1725*, (Cm resistant) | Ubben and Schmitt,1986 |
| WR6016 | *thr*, *leu*, *thi*, *recA*/F'$_{ts}$114*lac*::Tn*5* | Donor of Tn*5* | Sansonetti *et al.*,1981 |
| AC80 | *thr*, *leu*, *met*, *hsdR*, *hsdM* | ManA$^+$ | Darzins and Chakrabarty,1984 |
| CD1 | *thr*, *leu*, *met*, *hsdR*, *hsdM*, *manA* | ManA$^-$ | Darzins *et al.*,1985 |
| CD5 | *thr*, *leu*, *met*, *hsdR*, *hsdM*, *cap-1*, *manA* | ManA$^-$, makes capsule if made Man$^+$ | Darzins *et al.*,1985 |
| CSR603 | *uvrA*, *uvrB*, *recA* | Maxicell strain | Sancar *et al.*, 1979 |
| *Shigella flexneri* | | | |
| PE523 | Type 2a | | IMVS* |
| *Salmonella typhimurium* | | | |
| C5 | wild-type | Virulent for mice | IMVS |
| SL901 | *rfc-497* , *metA22*, *trpC*, *flaA66*, Hi-b, H2-e,n,x, Fels2$^-$ | SR | Wilkinson *et al.*,1972 |
| SL1101 | As SL901, *pmi-408* | SR in mannose, Man$^-$ | Dr. B. Stocker** |
| TV119 | *rfb* | R | Subbaiah and |

| | | | |
|---|---|---|---|
| | | | Stocker,1964 |
| LV56 | As SL901, *galE*, | SR, galactose-resistant | This study |
| LV203 | As LV56, *recA*, | As LV56, but recombination-defective | This study |
| LV242 | LV203/pADE200 *lamB*$^{+}$, Km$^{R}$ | As LV203, but lambda-sensitive and cryptic plasmid-free | This study |
| TT521 | *recA*1, *rpsL*, *srl*-202::Tn*10* | Donor of *recA* allele | Kleckner *et al.*,1977 |
| TT628 | *pyrC*7, *str*A1/F'$_{ts}$114*lac*, *zzf*-21::Tn*10* | Donor of Tn*10* | Kleckner *et al.*,1977 |
| J208 | *rfc*::IS*10*, F'*lac*, *zzf*-21::Tn*10* | Rfc | This study |
| J208-B1 | *rfc*::IS*10* | Rfc | This study |
| J208-B2 | *rfc*::IS*10* | Rfc | This study |
| LV374 | *pmi*::Km | Pmi | This study |
| LV386 | *rfc*::Km | Rfc | This study |
| G30 | *galE* | Gal$^{-}$ | This laboratory |
| Other *Salmonella* strains | | | |
| *S.paratyphi* A | | | IMVS |
| *S.typhi* | | | IMVS |
| *S.derby* | | | IMVS |
| *S.dublin* | | | IMVS |
| *S.chester* | | | IMVS |
| *S.strasbourg* | | | IMVS |
| *S.newington* | | | IMVS |

*:IMVS: Institute of Medical and Veterinary Science, Adelaide, Australia. **: Dr. Stocker is at Stanford University, Stanford, CA.
R: rough lipopolysaccharide produced; SR: semi-rough lipopolysaccharide produced. Km: resistant to kanamycin.

**Table 2.2: The bacterial plasmids used in this work**

| Plasmid | Relevant Characteristics | Reference |
|---|---|---|
| pHC79 | Carries Ap, Tc resistances | Hohn and Collins, 1981 |
| pBR322 | Carries Ap, Tc resistances | Vieira and Messing, 1982 |
| pUC18 | Carries Ap resistance | Simon *et al.*, 1983 |
| pSUP401 | Carries Cm, Km resistances | Simon *et al.*, 1983 |
| pSUP201-1 | Carries Cm, Tc resistances | Stoker *et al.*, 1982 |
| pLG339 | Source of Km-resistance DNA | Tabor and Richardson, 1985 |
| pGP1-2 | Can express T7 polymerase | Stratagene Inc. |
| pBLUESCRIPT KS$^+$ | Carries T7 promoter; Ap resistance | Hashimoto-Gotoh *et al.*, 1981 |
| pHSG422 | Replication is ts | Messing and Vieira, 1982 |
| M13mp18 | M13 cloning vector | Messing and Vieira, 1982 |
| M13mp19 | M13 cloning vector | Morona and Reeves, 1981 |
| pPR11 | *tolC*::Tn*10*; source of IS*10* DNA | Harkki *et al.*, 1987 |
| pAMH70 | *lamB*$^+$, carries Ap resistance | This study |
| pADE200 | *lamB*$^+$, carries Km resistance | This study |
| pADE201 | pHC79/*rfc*$^+$ cosmid, carries Ap resistance | This study |
| pADE206 | pUC18/*rfc*$^+$, carries Ap resistance | This study |
| pADE207 | pSUP401/*rfc*$^+$, carries Cm resistance | This study |
| pADE218 | pBLUESCRIPT KS+/(*rfc*$^+$); insert of pADE219 inverted | This study |
| pADE219 | pBLUESCRIPT KS+/*rfc*$^+$, *rfc* after T7 promoter | This study |
| pADE249 | $pmi_{stm}^+$ cosmid, carries Ap resistance | This study |
| pADE250 | $pmi_{stm}^+$, cutdown of pADE249 | This study |
| pADE253 | pBR322/ $pmi_{stm}^+$ | This study |
| pADE254 | $pmi_{stm}^-$ deletion of pADE253 | This study |

| | | |
|---|---|---|
| pADE261 | pHSG422:: *mob*$^{+}$, carries Cm, Ap resistances | This study |
| pADE262 | *Hinc*II cutdown of pADE261, carries Cm resistance | This study |
| pADE263 | pUC18/*rfc*::Km | This study |
| pADE264 | pADE262/*rfc*::Km | This study |
| pADE265 | pHC79/$pmi_{stm}$::Km | This study |
| pADE266 | pADE261/$pmi_{stm}$:Km | This study |

colonies were selected and picked off plates for subsequent storage or use. Short-term storage of strains in routine use was as a suspension of freshly grown bacteria in glycerol (32% v/v) and peptone (0.6% w/v) at -70°C. Fresh cultures from glycerols were prepared by streaking a loopful of the glycerol suspension onto a NA plate (with or without antibiotic as appropriate) followed by incubation overnight just prior to use.

Bacterial strains were prepared for long-term storage by suspension of several colonies in a small volume of sterile skimmed milk. Approximately 0.2 ml aliquots of this thick bacterial suspension were dispensed into sterile 0.25 x 4 in. freeze-drying ampoules and the end of each ampoule was plugged with cotton wool. The samples were then lyophilized. After the vacuum was released, 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 and then sealed at the constriction without releasing the vacuum. Finally ,the ampoules were labelled and stored at 4°C.

### 2.5.2 Bacteriophage sensitivity tests

Bacteria were grown to stationary phase in either NB, NBG, NBGG or NBGM, and swabbed across an NA plate supplemented with the same sugar(s) as incorporated in the liquid culture. Bacteriophages were spotted (ca. $10^6$ plaque-forming units in 5 µl NB) onto the bacterial swabs and the plates were incubated at 37 °C for 16 h. Strains were scored bacteriophage-sensitive if a clear zone of lysis appeared.

The efficiency of plaquing of bacteriophage on given strains was effected by mixing of 0.1ml amounts of phage stock dilutions (in NB) with 0.1ml amounts of bacteria, prepared as above. After 10 min at 37 °C, the mixture was spread on NA plates supplemented with the same sugar(s) as incorporated in the liquid culture. Incubation at 37 °C for 16 h followed; plaques were then counted.

## 2.6 Phage ES18-mediated transduction

Bacteriophage ES18 was propagated on TT521 and the lysate used to cotransduce the *recA* allele with *srl-202*::Tn*10* into LV56 (grown in NBG). Tetracycline-resistant, recombination-deficient transductants were identified by their increased sensitivity to killing by ultraviolet light irradiation in comparison with wild-type *Salmonella* strains. A derivative of a *recA* strain, which was $Tc^S$ due to loss of the Tn*10* element, was selected on fusaric acid-containing medium (Bochner *et al.*, 1980).

## 2.7 Transformation procedure

Transformation was performed essentially according to the method described by Brown *et al.* (1979). *E.coli* K-12 strains were made competent for transformation with plasmid DNA as follows: an overnight shaken culture (in NB) was diluted 1:20 into NB and incubated with shaking until the culture reached an OD of 0.6 ($4 \times 10^8$ cells/ml). The cells were chilled on ice for 20 min, pelleted at 4°C in a bench centrifuge, resuspended in a half-volume of cold 100 mM $MgCl_2$, centrifuged again, and resuspended in a tenth of the original volume of cold 100 mM $CaCl_2$. The suspension was allowed to stand for 60 min on ice before addition of DNA. Competent cells (0.2 ml) were mixed with DNA (volume made to 0.1 ml with TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0)) and left on ice for a further 30 min. The cell / DNA mixture was heated at 42°C for 2 min and then 3 ml NB was added followed by incubation with shaking at 37°C for 1-2 h. The culture was then plated onto selection plates directly or concentrated by centrifugation and plated. Cells with sterile buffer were included as a control.

## 2.8 Bacterial conjugation

Overnight broth cultures grown in LB were diluted 1 in 20 in the same medium and grown to early exponential phase with slow agitation. Donor and recipient bacteria were mixed at a ratio of 1:10 and the cells pelleted by centrifugation (5000 rpm, 5 min, bench centrifuge). The

pellet was gently resuspended in 200 µl of broth and spread onto a nitrocellulose membrane filter (0.45 µm, type HA, Millipore Corp.) on a NA plate. This plate was incubated for 6-16 h at 37°C. The cells were then resuspended in 10 ml NB and samples plated onto selective agar and incubated overnight at 37°C.

## 2.9 Transposon mutagenesis

### 2.9.1 Transposition with Tn*5*

The donor strain, WR6016, carried the temperature-sensitive F'$_{ts}$ 114*lac*::Tn*5*. Transfer of this plasmid into *S. typhimurium* strains carrying target plasmids was effected by mating of a mixture of 0.1 ml of donor and 0.1 ml of recipient stationary phase cultures for 3 h at 30°C. Selection for transconjugants was made by plating the mating mixture on NA containing Ap and Km, and incubating the plates overnight at 30°C. To select simultaneously for loss of the F'*lac* plasmid and transposition of Tn*5*, the transconjugants were cultured at 42°C in NB containing Ap and Km. The Lac$^-$ phenotype of transconjugants which had lost the F'*lac* plasmid, was confirmed by plating on MacConkey lactose agar (Difco) containing Km. Plasmids with Tn*5* insertions were purified and transformed into *Salmonella* mutants to test for complementation.

### 2.9.2 Transposition with Tn*10*

Insertions of Tn*10* in the chromosome of *S. typhimurium* C5 were sought by the mating of stationary phase cultures of TT628, carrying F'$_{ts}$ 114*lac*$^+$ *zzf*-21::Tn*10*, and C5, in a donor to recipient ratio of 1:10, followed by incubation for 3 h at 30°C. Transconjugants were selected on M9 medium containing Tc and purified on the same medium at 30°C. Individual transconjugants were then grown at 42°C in LB containing Tc, and plated for single colonies on L agar containing Tc at 42°C. Individual Tc$^R$ colonies were screened for resistance to phage 9NA and for sensitivity to phage FO at 37°C.

### 2.9.3 Transposition with Tn*1725*

Transposition to plasmid DNA of Tn*1725* ($Cm^R$) was performed as follows. The plasmid $R_{ts}1$::Tn*1725* was transferred into the *S. typhimurium* strains containing the target plasmids by mating for 3 h at 30°C in standing culture of 0.1 ml of a stationary phase culture of the donor strain RU2901, and 0.9 ml of a stationary phase culture of the recipient strain. The mating mixture was plated on NA with selection for Cm- and Ap- resistance. Independent transconjugants were purified, and single colonies grown for 16 h at 42°C with selection for Cm- and Ap- resistance. Purified DNA of plasmids containing independent Tn*1725* insertions were used to transform either *rfc* or *pmi* mutants of *S. typhimurium* to test for the complementing ability of the transposon-bearing plasmids.

## 2.10 DNA extraction procedures

### 2.10.1 Plasmid DNA isolation

Plasmid DNA was isolated by one of the four following procedures:

**Method 1:** Rapid plasmid preparation by the boiling method of Holmes and Quigley (1981) was performed as follows: Cells from 1 ml of a 10 ml shaken overnight culture were pelleted (this yielded about $10^9$ cells) in an Eppendorf 5414 centrifuge for 30 sec, resuspended in 50 µl STET buffer (5% (w/v) sucrose, 5% (v/v) Triton X-100, 50 mM EDTA, 50 mM Tris-HCl, pH 8.0). Lysozyme (5 mg/ml; 5 µl) was added and the suspension left at room temperature for several min. Samples were then placed in boiling water for 35 sec and immediately spun for 10 min in an Eppendorf centrifuge. The chromosomal pellet was removed and plasmid DNA in the supernatant precipitated with 0.6 volumes of propan-2-ol at -20°C for 10 min. DNA was pelleted by centrifugation in an Eppendorf centrifuge for 10 min, washed once with 1 ml 70% (w/v) ethanol, dried *in vacuo* and dissolved in 20 µl TE buffer. This method was scaled up (X10) for use with 10 ml cultures.

**Method 2:** Triton X-100 cleared lysates were prepared from 10 ml overnight cultures by a modification of the procedure of Clewell and Helinski (1969, 1970). Cells were resuspended in 0.4 ml 25% (w/v) sucrose in 50 mM Tris-HCl, pH 8.0. Lysozyme (50 µl, 10 mg/ml freshly prepared in $H_2O$) and 50 µl of 0.25M EDTA, pH 8.0 were added to cells in Eppendorf tubes and left to stand on ice for 15 min. Some 0.5 ml of TET buffer (50 mM Tris-HCl, 66 mM EDTA, 0.4% (w/v) Triton X-100,pH 8.0) was added, followed by a brief mixing by inversion of the tubes. The chromosomal DNA was then pelleted by centrifugation (15,000 rpm, 20 min, 4°C, SS34, Sorvall). The supernatant was extracted twice with TE-saturated phenol (pH 7.5) and twice with diethyl ether. Plasmid DNA was precipitated by the addition of an equal weight of propan-2-ol; the precipitate formed at -70°C for 30 min. The precipitate was collected (10 min, Eppendorf 5414), washed once with 1 ml 70% (v/v) ethanol, dried, and resuspended in 50 µl TE buffer.

**Method 3:** Large scale plasmid purification was performed by the three-step alkaline lysis method (Garger *et al.*, 1983). Cells from a 1l culture were harvested (6,000 rpm, 15 min, 4°C, GS-3, Sorvall) and resuspended in 24 ml solution 1 (50 mM glucose, 25 mM Tris-HCl, 10 mM EDTA, pH 8.0). Freshly prepared lysozyme (4 ml of 20 mg/ml in solution 1) was mixed with the cell suspension and the mixture incubated at room temperature for 10 min. Addition of 55 ml of solution 2 (0.2 M NaOH, 1% (w/v) SDS), followed by 5 min incubation on ice, resulted in total lysis of the cells. After the addition of 28 ml solution 3 (5M potassium acetate, pH 4.8) and incubation on ice for 15 min, protein, chromosomal DNA, and high molecular weight RNA were removed by centrifugation (8,000 rpm, 20 min, 4°C, GSA, Sorvall). The supernatant was then extracted with an equal volume of a TE-saturated phenol, chloroform, isoamyl alcohol mixture (25:24:1,v/v/v). Plasmid DNA from the aqueous phase was precipitated with 0.6 volume of propan-2-ol at room temperature for 10 min and collected by centrifugation (10,000 rpm at 4°C, 35 min, GSA, Sorvall). After washing in 70% (v/v) ethanol, the pellet was dried *in vacuo* and resuspended in 4.6 ml TE. Plasmid DNA was purified from contaminating protein and RNA by centrifugation on a two step CsCl ethidium bromide gradient according to Garger *et al.* (1983). The DNA band was removed by side puncture of the tube with a 19 gauge needle attached to a 1 ml syringe. The ethidium bromide

was extracted using isoamyl alcohol. CsCl was then removed by dialysis overnight against three changes of TE (2l/change) at 4°C. DNA was stored at 4°C.

**Method 4:** Small scale plasmid purification was performed by the three-step alkaline lysis method using a modification of Garger *et al.* (1983). Overnight bacterial cultures (1.5 ml) were transferred to a microfuge tube and harvested by centrifugation (45 sec, Eppendorf 5414), and resuspended in 0.1 ml solution 1 (above). The addition of 0.2 ml solution 2 (above) followed by a 5 min incubation on ice, resulted in cell lysis. After the addition of 0.15 ml solution 3 (above) and a 5 min incubation on ice, protein, chromosomal DNA and high molecular weight RNA were collected by centrifugation (90 sec, Eppendorf 5414). The supernatant was transferred to a fresh tube and extracted once with TE-saturated phenol and once with diethyl ether. Plasmid DNA was precipitated by the addition of 2 volumes of ethanol, followed by a 2 min incubation at room temperature. The DNA was collected by centrifugation (15 min, Eppendorf 5414), washed with 70% (v/v) ethanol ,and dried *in vacuo*. The pellet was resuspended in 40 µl TE.

### 2.10.2 Preparation of genomic DNA

Whole genomic DNA was prepared as follows. Cells from a 20 ml shaken overnight culture were pelleted in a bench centrifuge for 10 min and washed once with TES buffer (50 mM Tris-HCl, 5 mM EDTA, 50 mM NaCl, pH 8.0). The pellet was then resuspended in 2 ml 25%(w/v) sucrose, 50 mM Tris-HCl (pH 8.0) and 1 ml lysozyme (10 mg/ml in 0.25 M EDTA, pH 8.0) was added and the mixture incubated on ice for 20 min. TE buffer (0.75 ml) and 0.25 ml lysis solution (5% (w/v) sarkosyl, 50 mM Tris-HCl, 0.25M EDTA, pH 8.0) were added, together with 10 mg pronase. The mixture was gently mixed, transferred to a 50 ml Ehrlenmeyer flask and incubated at 56°C for 60 min. This was followed by three extractions with TE-saturated phenol, and two with diethyl ether. The genomic DNA was then transferred to dialysis tubing and dialysed against 5 l TE for 16 h with one change.

## 2.11 Analysis and manipulation of DNA

### 2.11.1 DNA quantitation

The concentration of DNA in solutions was determined by measurement of absorption at 260 nm, with the assumption that an $A_{260}$ of 1.0 was equal to 50 μg DNA/ml (Miller, 1972).

### 2.11.2 Restriction endonuclease digestion of DNA

All restriction enzyme digestions of DNA were performed as described by Davis *et al.* (1980). Some 0.1-0.5 μg of DNA or purified restriction fragments were incubated with 2 units of each restriction enzyme in a final volume of 20 μl, at 37°C, for 1-2 h. The reaction buffers were as recommended by the manufacturers of the enzymes. The reactions were terminated by heating at 65°C for 10 min. Prior to loading onto a gel, a one-tenth volume of tracking dye (15% (w/v) Ficoll, 0.1% (w/v) bromophenol blue, 0.1 mg/ml RNase A) was added.

### 2.11.3 Analytical and preparative separation of restriction fragments

Electrophoresis of digested DNA was carried out at room temperature on horizontal, 0.6%, 0.8% or 1% (w/v) agarose gels (Seakem HGT), 13 cm long, 13 cm wide and 0.7 cm thick. Gels were run at 100V for 4-5 h in TBE buffer (67 mM Tris base, 22 mM boric acid and 2 mM EDTA, final pH 8.8). After electrophoresis the gels were stained in distilled water containing 2 μg/ml ethidium bromide. DNA bands were visualized by trans-illumination with ultraviolet light and photographed using either Polaroid 667 positive film or 665 negative film.

For preparative gels, Sea Plaque (Seakem) low-gelling-temperature agarose at a concentration of 0.6% (w/v) was used for the separation of restriction fragments, which were recovered by the following method. DNA bands were excised and the agarose melted at 65°C. Five volumes of 20 mM Tris-HCl, 1 mM EDTA, pH 8.0 buffer was added, and the agarose

extracted with water-saturated phenol (1ml/g) and then phenol:chloroform (1:1, v/v) (1 ml/g). Residual phenol was removed with chloroform and the DNA precipitated with two volumes of ethanol and one- tenth volume of 3M sodium acetate, pH 5.0. DNA was collected by centrifugation (15 min, Eppendorf 5414), washed once with 70% (v/v) ethanol, and dried *in vacuo* before being resuspended in TE buffer.

### 2.11.4 Calculation of restriction fragment size

The size of restriction enzyme fragments were calculated by comparing their relative mobility with that of *Eco* RI-digested bacteriophage SPP1 (of *Bacillus subtilis* ) DNA. The calculated sizes of the SPP1 *Eco* RI standard fragments used differ from those published (Ratcliff *et al.*, 1979) and were calculated with the program DNAFRAG (Rood and Gawthorne, 1984) using bacteriophage lambda and plasmid pBR322 as standards. The sizes (in kb) used were: 8.37; 7.2; 6.05; 4.90; 3.55; 2.63; 1.73; 1.61; 1.29; 1.19; 0.99; 0.86; 0.63; 0.48; 0.38.

### 2.11.5 Dephosphorylation and ligation of DNA

Some 0.1-0.5 µg of digested plasmid DNA was incubated with 1 unit of (molecular biology grade) alkaline phosphatase (Boehringer-Mannheim) for 30 min at 37°C. The reaction was terminated by the addition of EDTA, pH 8.0 to a final concentration of 3 mM, followed by heating at 65°C for 10 min. The reaction mix was then extracted twice with hot (56°C) TE-saturated phenol, and twice with diethyl ether. DNA was precipitated overnight at -20°C with two volumes of absolute ethanol, and one-tenth volume of 3M sodium acetate, pH 5.0. The precipitate was collected by centrifugation (15 min, Eppendorf 5414), washed once with 1 ml 70% (v/v) ethanol, dried *in vacuo* , and dissolved in TE buffer.

Ligation reactions, with T4 DNA ligase, were performed in 1 X ligase buffer (20 mM Tris - HCl, 10 mM $MgCl_2$, 0.6 mM ATP, 10 mM DTT and bovine serum albumin (100 µg/ml)), and incubated at 10°C for 16 h. Restriction enzymes were heat-inactivated at 65°C prior to DNA ligation.

### 2.11.6 Cosmid bank construction

High molecular-weight chromosomal DNA of *S. typhimurium* C5 was partially digested with *Sau* 3A1 to generate fragments in the size range 30-50 kb. Digests were terminated by heating at 65°C for 10 min, followed by extraction with phenol, and precipitation of the DNA with two volumes of ethanol and one-tenth volume of 3M sodium acetate, pH5.0. The fragments were mixed with *Bam*HI-cut pHC79 (Hohn and Collins, 1980), and ligated overnight using T4 DNA ligase. The ligation mixture was packaged using the Packagene lambda DNA packaging extracts (Promega, WI, USA). In order to obtain a cosmid clone containing *pmi*, the packaged mixture was adsorbed for 20 min to *E. coli* CD1, grown to late logarithmic phase in LB containing 0.4% (w/v) maltose, and concentrated 10-fold in 0.01M $MgSO_4$. In the case of the cosmid cloning of *rfc*, the packaged mixture was adsorbed to LV242, which had been grown to late logarithmic phase in LB containing glucose (0.5% (w/v)) and Km (50 µg/ml), and concentrated 10-fold in 0.01M $MgSO_4$. Cells and packaged mxtures were incubated for a further 60 min and plated on NA containing Ap (50 µg/ml). Recombinants were screened for DNA inserted in pHC79 by patch plating of the $Ap^R$ colonies on duplicate NA plates containing either Ap (50 µg/ml) or Tc (4 µg/ml). MTZ plates containing Ap (50 µg/ml) were used to distinguish $Man^-$ and $Man^+$ recombinants in the cosmid-containing recombinants of CD1.

### 2.11.7 End-filling with Klenow fragment

Protruding ends created by cleavage with restriction endonucleases were filled in using the Klenow fragment of *E.coli* DNA polymerase I. Typically, 1 µg of digested DNA, 2 µl of 10x nick-translation buffer (500 mM Tris-HCl, pH 7.2, 100 mM $MgCl_2$, 1 mM DTT, 500 µg/ml bovine serum albumin)(Maniatis *et al.*, 1982), 1 µl of each dNTP (2 mM) and 1 unit Klenow fragment were mixed and incubated for 30 min. The reaction was stopped by heating at 65°C for 10 min, followed by removal of unincorporated dNTPs and enzyme by centrifugation through a Sepharose CL-6B column.

### 2.11.8 Nick-translation

Nick-translation reactions with DNA polymerase I were modified from Maniatis *et al.* (1982) and carried out as follows: 25 µCi α-[$^{32}$P]-dCTP (1,700 Ci/mmole in ethanol) was dried *in vacuo* in an Eppendorf tube, and resuspended with 80 µl water, 10 µl of 10x nick-translation buffer , and 1 µl of each of 2 mM dATP, dGTP and dTTP. DNA (1 µg) and DNAse I (10 mg/ml; 1 µl) were added, and the mixture was incubated at 37°C for 10 min. DNA polymerase I (5 units) was added to the mix, and incubation continued at 16°C for 2 h. [$^{32}$P]-labelled DNA was separated from unincorporated label by centrifugation through a mini-column of Sepharose CL-6B.

### 2.11.9 Random primer labelling

DNA fragments were labelled with α-[$^{32}$P]-dCTP using a random oligonucleotide primer kit purchased from Bresatec (Adelaide).

### 2.11.10 Southern transfer and hybridization

Bidirectional transfers of DNA from agarose gels to nitrocellulose paper (Schleicher and Schuell, Dassel, FRG) were performed as described by Southern (1975) and modified by Maniatis *et al.* (1982).

Prior to hybridization with radiolabelled probe, filters were incubated for 4 h at 44°C in a pre-hybridization solution containing 50% (v/v) formamide, 50 mM sodium phosphate buffer, pH 6.4, 5x SSC (0.34 M NaCl, 75 mM sodium citrate, pH 7.0), 5x Denhardt's reagent (0.1%(w/v) Ficoll, 0.1% (w/v) polyvinylpyrrolidone, 0.1%(w/v) fraction V bovine serum albumin) and 83 µg/ml single stranded herring sperm DNA (Maniatis *et al.*, 1982). Pre-hybridization fluid was discarded and replaced with fresh hybridization buffer (as for pre-

hybridization solution, with the exclusion of herring sperm DNA). Denatured probe (specific activity ca. $10^6$ cpm/μg) was added and hybridization allowed to occur for 16-24 h at 44°C.

Filters were washed twice with shaking at 37°C for 30 min in 2x SSC, containing 0.1% (w/v) SDS. This was followed by two further washes in 0.1x SSC plus 0.1% (w/v) SDS at 65°C. After drying in air (15 min, room temperature), the filters were covered in plastic wrap and placed on film, with intensifying screens, for autoradiography at -70°C .

## 2.12 Autoradiography

SDS-PAGE gels were dried on Whatman 3MM chromatography paper at 60°C for 2 h on a Bio-Rad gel drier. [$^{35}$S]-methionine autoradiography was performed at room temperature for 1-7 days without intensifying screens using Kodak XR-100 film. For autoradiography with [$^{32}$P]-phosphate labelled DNA, the gels were exposed to film for 6-72 h at -70°C, using intensifying screens.

## 2.13 Protein and LPS analysis

### 2.13.1. SDS polyacrylamide gel electrophoresis

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was usually performed on 11-20%(w/v) polyacrylamide gradients using a modification of the procedure of Lugtenberg *et al.* (1975) as described previously by Achtman *et al.* (1978). Samples were heated at 100°C for 3 min prior to loading. Gels were generally electrophoresed at 100 V for 5 h. Proteins were stained with gentle agitation overnight at room temperature in 0.06% (w/v) Coomassie Brilliant Blue G250 (dissolved in 5% (v/v) perchloric acid). Destaining was accomplished with several changes of 5% (v/v) acetic acid, with gentle agitation for 24 h.

Protein size markers (Pharmacia) were phosphorylase B (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20.1 kDa) and α - lactalbumin (14.4 kDa).

### 2.13.2 Minicell procedures

Minicells were purified and the plasmid-encoded proteins labelled with [$^{35}$S]-methionine as described by Kennedy *et al.* (1977) and modified by Achtman *et al.* (1979). This involved separation of minicells from whole cells (500 ml overnight culture in LB medium) by centrifugation through two successive sucrose gradients, pre-incubating in minimal medium to degrade long-lived mRNAs corresponding to chromosomally encoded genes, then pulse labelling with [$^{35}$S]-methionine in the presence of methionine assay medium. Minicells were subsequently solubilized by heating at 100°C in 100 µl of an SDS/2-mercaptoethanol buffer (solubilisation buffer) (Lugtenberg *et al.*, 1975) and analysed by SDS-PAGE.

### 2.13.3 Maxicell procedures

Maxicell-encoded proteins were studied using the methods of Sancar *et al* (1979). Plasmids were transformed into the maxicell strain CSR603 and grown in M9 medium containing 1% (w/v) Casamino acids (Difco) to 2 X $10^8$ cells/ml. Aliquots of 5 ml of each strain were irradiated with a ultraviolet light fluence of 50 J $m^{-2}$ from a germicidal lamp at a fluence rate of 0.5 J $m^{-2}$ $s^{-1}$, and incubated at 37°C for 16 h. Cells were washed with M9 medium and suspended in 5 ml of M9 medium supplemented with 1 mM $MgSO_4$, 0.1 mM $CaCl_2$, 2 mg glucose, 20 µg thiamine and 0.1 mg of each of 18 amino acids (excluding methionine and cysteine). After 90 min shaking incubation at 37°C, [$^{35}$S]-methionine was added to a final concentration of 5 µCi/ml and incubation was continued for an additional 1h. Cells were then collected by centrifugation, suspended in solubilisation buffer and boiled for 2 min prior to analysis by SDS-PAGE. Labelled proteins were visualized by autoradiography.

### 2.13.4 Bacterial cell-free coupled transcription-translation

The prokaryotic DNA-directed translation kit was obtained from Amersham; this system was essentially as described by Zubay (1973), and modified by Collins (1979). Reactions were carried out according to the manufacturer's instructions.

### 2.13.5 T7 polymerase / promoter system

Plasmid-encoded polypeptides were analysed by using the bacteriophage T7 RNA polymerase -containing plasmid pGP1-2 (Tabor and Richardson, 1985) and the pBluescript KS+ cloning vector (Stratagene Inc., CA, USA). DNA fragments to be analysed were ligated into the multiple cloning site of pBluescript KS+ and transformed into *E. coli* K38, with selection on NA containing Km and Ap, incubated at 30°C. Recombinants were identified which contained the required fragment cloned in pBluescript KS+, and pGP1-2. Recombinants were grown overnight in LB containing Ap and Km, at 30°C. Cultures were diluted 20-fold in fresh LB and incubated with shaking at 30°C for 3 h. Some 0.2 ml volumes of cells were centrifuged, washed twice in 5 ml M9 medium, and resuspended in 1 ml of M9 medium, which was 1 mM in $MgSO_4$, 0.1 mM in $CaCl_2$,and which contained 2 mg glucose, 20 µg thiamine and 0.1 mg of each of 18 amino acids (excluding methionine and cysteine). The cells were then incubated for 1 h with shaking at 30°C, followed by 15 min with shaking at 42°C (to induce synthesis of bacteriophage T7 RNA polymerase). Rifampicin was added to a final concentration of 200 µg/ml, and incubation at 42 °C was continued with shaking for 10 min. The cells were incubated at 30°C for a further 45 min, and 10 µl of [$^{35}$S]-methionine was added. Labelling was effected with shaking at 30°C for 30 min. The cells were pelletted by centrifugation. The cell pellet was resuspended in 200 µl of solubilisation buffer and boiled for 3 min. Proteins were analyzed by SDS-PAGE and gels were subjected to autoradiography after drying.

### 2.13.6 LPS preparation

LPS for analysis by SDS-PAGE followed by silver staining was prepared as follows. A volume of 1 ml of cells grown in liquid culture to stationary phase (ca. 5 X $10^9$ cells / ml) was pelleted in an Eppendorf tube. The pellet was suspended in 50 µl of lysing buffer (2% (w/v) SDS, 4% (v/v) β-mercaptoethanol, 10% (v/v) glycerol, 1M Tris-HCl , 0.1% (w/v) bromophenol blue, pH7.6), and boiled for 5 min. Proteinase K (10 µl of a 2.5 mg/ml solution in lysing buffer) was added and the mixture was incubated at 55°C for 2 h. Samples (10-20 µl) were boiled for 3 min prior to SDS-PAGE.

### 2.13.7 LPS-specific silver staining

Silver staining of LPS was performed using a modification of the method described by Tsai and Frasch (1982). The following procedure was used: SDS-PAGE gels were fixed for 16 h in 40% (v/v) ethanol, 10% (v/v) acetic acid, then oxidised for 5 min with 0.7% (v/v) periodic acid in 40% (v/v) ethanol, 10% (v/v) acetic acid. After oxidation the gels were washed 4 times (30 min each) with water and then stained for 10 min in a solution composed of 28 ml sodium hydroxide (0.1 M), 2 ml ammonium hydroxide (30% (w/v)) and 5 ml of silver nitrate (20% (w/v)). The stained gels were developed in a solution of 50 µg/ml citric acid and 0.05% (w/v) formaldehyde.

### 2.13.8 Cell fractionation

The cell fractionation procedure was a modification of that described by Osborn *et al.* (1972a). Cells were grown in NB to mid exponential phase at 37°C (50 ml, $OD_{650}$ of 0.6). Cells were pelleted in a Sorvall SS-34 rotor, (10,000 rpm, 10 min, 4°C) and resuspended in 1 ml of 20%(w/v) sucrose, 30 mM Tris-HCl, pH 8.1, transferred to SM-24 tubes, and chilled on ice. Cells were converted to sphaeroplasts with 0.1 ml of 1 mg/ml lysozyme in 0.1 M EDTA pH 7.3 for 30 min on ice. Cells were centrifuged as above and the supernatant collected

(periplasmic fraction). The cell pellet was frozen in an ethanol-dry ice bath for 30 min, thawed and dispersed vigorously in 3 ml 3 mM EDTA pH 7.3. Cells were lysed by 60 x 1 sec. bursts with a Branson Ultrasonifier. Unlysed cells and large cell debris were removed by low speed centrifugation (5,000 rpm, 5 min, 4°C). The supernatant containing the membranes and the cytoplasm was centrifuged at 35,000 rpm in a 50Ti rotor for 60 min at 4°C in a Beckman L8-80 ultracentrifuge. The supernatant (cytoplasmic fraction) was collected and the membrane pellet was resuspended in 25% (w/v) sucrose, 10 mM Tris-HCl, 1 mM EDTA, pH 7.8. The separation of the outer and inner membrane fractions was performed by extraction with Sarkosyl according to Achtman *et al.* (1979).

## 2.14 Phosphomannose isomerase enzyme activity assay

Cell extracts were prepared from washed cells by sonication in 50mM potassium phosphate (pH 7.4) containing 0.5 mM EDTA, followed by centrifugation at 15,000*g* for 10 min. The Pmi enzyme activity was measured using the method of Kang and Markovitz (1967). Reaction mixtures contained 34 mM Tris (pH 7.6), 6 mM $MgCl_2$, 0.2 mM NADP, and 0.01 to 0.1 ml of supernatant enzyme in phosphate buffer (5mM, pH 7.6). To this was added D-mannose-6 phosphate to 4mM, 0.48 units of G6P dehydrogenase/ml, and 0.65 units of PGI /ml. The rate of production of $NADPH_2$ at 340 nm was monitored using a spectrophotometer. The protein concentration was measured by the method of Lowry *et al.* (1951) with bovine serum albumin as the standard. The Pmi units of activity are expressed as nanomoles of mannose-6-phosphate converted to fructose-6-phosphate per min per mg of protein.

## 2.15 M13 cloning and sequencing procedures

### 2.15.1 Preparation of M13 replicative form (RF) DNA.

Fresh 2 x TY broth (10 ml) was inoculated with 10 µl of an overnight culture of JM101 (in M13 minimal medium). A single plaque of M13mp18 or M13mp19 picked from a TY agar plate with a sterile toothpick was added to this bottle. The culture was grown at 37°C with

vigorous shaking for 6 h. Bacterial cells were removed by centrifugation (5,000 rpm, 10 min, bench centrifuge) and the supernatant added to 1 l NB containing 10 ml of a shaken overnight culture of JM101. Following incubation for 14 h at 37°C with shaking, replicative form (RF) DNA was prepared as described above for plasmid DNA purification.

### 2.15.2 Cloning with M13mp18 and M13mp19

The M13 vectors, M13mp18 and M13mp19 (Messing and Vieira, 1982; Vieira and Messing, 1982) were used for selective cloning of restriction enzyme-generated DNA fragments. Stocks of M13 vectors cleaved with various enzyme combinations were stored at 4°C, after heat-inactivation of enzymes. Plasmid DNA was cut with the appropriate enzyme combinations for subcloning into the M13 vectors. The ligation conditions used for blunt ends and cohesive ends were identical. The reaction mixtures consisted of the DNA to be cloned (100 ng) and the DNA vector (20 ng) in a final volume of 10 µl of ligation buffer. Ligation with T4 DNA ligase was carried out overnight at 4°C.

### 2.15.3 Transfection of JM101

Strain JM101 was made competent for transformation/transfection as described in section 2.7. Competent cells (0.2 ml) were added directly to the ligation mixes and incubated on ice for 30 min. This was followed by a 2 min heat shock at 42°C. Cells were then transferred to sterile test tubes to which was added a mixture of JM101 indicator cells (200 µl), 100 mM IPTG (40 µl), 2% (w/v) X-gal in N, N'- dimethyl formamide (40 µl) and, finally, H top agar (4 ml). The mixture was poured as an overlay onto an H-agar plate and incubated overnight at 37°C.

### 2.15.4 Screening M13 vectors for inserts

White plaques were picked from X-gal, IPTG plates with sterile toothpicks and added to 1 ml 2 x TY broth in microfuge tubes containing a 1:100 dilution of an overnight culture of JM101. These tubes were incubated for 5 h at 37°C. The cells were pelleted by centrifugation (30 sec,

Eppendorf 5414) and 0.1 ml of supernatant was used to inoculate 10 ml NB containing 0.1 ml JM101 (overnight culture). This mixture was incubated with shaking at 37°C overnight. RF DNA, suitable for restriction analysis, was prepared by the Triton X-100 cleared lysate or the miniprep method (section 2.10.1). After restriction enzyme digestion, DNA was examined on 1% (w/v) agarose gels.

### 2.15.5 Purification of single-stranded template DNA

M13 RF DNA containing appropriate inserts were reintroduced into JM101 and single white plaques from this transfection picked with sterile toothpicks to inoculate 2 ml 2x TY broth containing 20 µl of an overnight culture of JM101. After vigorous shaking at 37°C for 6 h, the culture was transferred to Eppendorf tubes and centrifuged for 10 min. The supernatant was transferred to clean tubes and recentrifuged for 5 min. The method of lysis of the phage, and collection of single-stranded phage DNA was as follows. A 1 ml aliquot of the supernatant from each tube was withdrawn and mixed in a fresh tube with 0.27 ml 20% (w/v) PEG, 2.5 M NaCl. These tubes were then incubated at room temperature for 15 min. The phage were pelleted by centrifugation for 5 min in an Eppendorf 5414 centrifuge and the supernatant discarded. Following another short spin (10 sec), the remainder of the PEG / NaCl supernatant was removed with a drawn out Pasteur pipette. The pellets were resuspended in 0.2 ml TE buffer. TE-saturated phenol (0.1 ml) was then added to the phage suspension and the tubes were briefly vortexed. After standing for 15 min at room temperature, the tubes were centrifuged for 2 min and 0.15 ml of the top phase transferred to clean tubes. To this aqueous phase, 6 µl of 3M sodium acetate, pH 5.0, and 400 µl ethanol were added. Single-stranded DNA was precipitated at -20°C overnight, and collected by centrifugation for 15 min in an Eppendorf centrifuge. DNA pellets were washed once with 1 ml 70% (v/v) ethanol, followed by centrifugation. After drying *in vacuo* , the pellets were resuspended in 25 µl TE buffer and stored at -20°C until required.

### 2.15.6 Dideoxy sequencing protocol with Sequenase

The dideoxy chain-termination preocedure of Sanger *et al.* (1977) was modified to encompass the use of Sequenase (modified T7 DNA polymerase) in place of the Klenow enzyme (Tabor and Richardson, 1987). All reagents were stored at -20°C. Two types of labelling and termination mixes were used, namely the dGTP mixes and the dITP mixes. The contents of the dGTP mixes are as follows :

| | |
|---|---|
| **Labelling Mix (dGTP):** | 7.5 µM dGTP, dCTP and dTTP |
| **ddG Termination Mix (dGTP):** | 80 µM dNTP, 8 µM ddGTP, 50 mM NaCl |
| **ddA Termination Mix (dGTP):** | 80 µM dNTP, 8 µM ddATP, 50 mM NaCl |
| **ddC Termination Mix (dGTP):** | 80 µM dNTP, 8 µM ddCTP, 50 mM NaCl |
| **ddT Termination Mix (dGTP):** | 80 µM dNTP, 8 µM ddTTP, 50 mM NaCl |

The dITP mixes were used to reduce gel artifacts due to secondary structures in DNA synthesized in the sequencing reaction (Barnes *et al.*, 1983; Gough and Murray, 1983). The dITP mixes were as follows :

| | |
|---|---|
| **Labelling Mix (dITP):** | 15 µM dITP, 7.5 µM dCTP, 7.05 µM dTTP |
| **ddG Termination Mix (dITP):** | 160 µM dITP, 80 µM dATP, dCTP dTTP, 1.6 µM ddGTP, 50 mM NaCl |
| **ddA Termination Mix (dITP):** | 160 µM dITP, 80 µM dATP, dCTP dTTP, 8 µM ddATP, 50 mM NaCl |

**ddC Termination Mix (dITP):** 160 µM dITP, 80 µM dATP, dCTP dTTP, 8 µM ddCTP, 50 mM NaCl

**ddT Termination Mix (dITP):** 160 µM dITP, 80 µM dATP, dCTP dTTP, 8 µM ddTTP, 50 mM NaCl

Normally the labelling mix was diluted 1:5 with water to obtain the working concentration; however, to read long sequences in a single reaction, a dilution of 4:5 was used. The synthetic primer was annealed to the template as follows. Some 7 µl template (5-10 nmoles), 1 µl primer (500 nmoles) and 2 µl 5x Sequenase buffer (200 mM Tris-HCl, 100 mM $MgCl_2$, 250 mM NaCl; pH 7.5) were mixed,and the mixture was heated in a metal block at 65°C for 3 min. The block was allowed to cool to room temperature. To the annealed mixture, 2 µl of the appropriately diluted labelling mix, 1 µl DTT (0.1 M), 0.5 µl [$\alpha$-$^{35}$S]-dATP (1000 Ci/mmol) and 2 µl of diluted Sequenase (1:8 dilution in 1x TE buffer) was added. The mixture was spun, mixed, resuspended and then incubated for 5 min at room temperature. Four microfuge tubes were prepared, each containing 2.5 µl of the A,C,G, or T termination mix. The tubes were warmed to 37°C, and each tube received 3.5 µl of the mixture prepared above. The tubes were then briefly spun to start the termination reaction. After 5 minutes at 37°C, 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 of the reactions. Reaction mixes were heated to 100°C for 2 min and immediately loaded (1.2 µl) onto the sequencing gel. These samples could be held at -20°C for up to 2 weeks and heated to 100°C for 3 min prior to loading.

### 2.15.7 Double stranded sequencing

Plasmid DNA (2-4 µg) was diluted to a volume of 18 µl with water. The DNA was denatured by the addition of 2 µl of 2 M NaOH, and incubation for 5 min at room temperature. To this mix, 8 µl of 5M ammonium acetate (pH 7.5) was added, followed by 100 µl of ethanol. After centrifugation (Eppendorf, 15 min at full speed), the supernatant was removed and the DNA

pellet washed in 70%(v/v) ethanol. The pellet was dried *in vacuo* and dissolved in 7 µl of water. Some 2 µl of sequencing buffer (1x TM) and 1 µl of primer (0.5 pmol) was added to the DNA, and the mixture heated to 37°C for 20 min. The labelling and termination reactions were run exactly as described above for M13 single-stranded template DNA.

### 2.15.8 DNA sequencing gels

Polyacrylamide gels for DNA sequencing were prepared using glass plates of 33 x 39.4 cm and 33 x 42 cm. Spacers and combs were high density polystyrene (0.25 mm thick). The gel mix contained 70 ml acrylamide stock (5.7% (w/v) acrylamide, 0.3% (w/v) bis-acrylamide, 8M urea in 1x TBE buffer (89 mM Tris base, 89 mM boric acid, 2.5 mM EDTA, pH 8.3)), 420 µl 25% (w/v) ammonium persulphate and 110 µl TEMED (N,N,N',N'-tetramethyl-ethylene-diamine, Sigma). After thorough mixing this gel mix was poured into a clean gel sandwich and the comb inserted. Polymerization took place for 60 min, with the gel in a horizontal position. The gel was mounted onto the sequencing apparatus and a waterjacket was attached to the outside plate of the gel. This consisted of a plastic bag wedged between two 0.3 cm thick spacers and a third plate. The plastic bag was filled with 1x TBE buffer and this was sufficient to evenly distribute heat throughout the gel. Gels were pre-electrophoresed at 700 V for 30 min. After the samples had been loaded the gel was electrophoresed at a constant voltage (700 V) for 15 min, which was then increased to 1200 V, which caused a current flow of ca.33 mA. After 4 h the samples were reloaded into a second set of wells on the same gel. The gel was further electrophoresed, initially at 700 V, then at 1200 V for 2.5 h, by which time the bromophenol blue dye front from the second loading had reached the bottom of the gel. Plates were separated and tissue paper packed along the borders of the gel to hold it to the plate during the fixation procedure. This involved slow washing of the gel using 2.0 l of 10% (v/v) acetic acid, 20% (v/v) ethanol in a 60 ml syringe. The gel was then dried at 100°C for 20 min. Plastic wrap was used to cover the gel before placing on film for autoradiography. Autoradiography was performed at room temperature, without the use of intensifying screens, for 16-24 h.

### 2.15.9 Analysis of DNA sequences

Sequencing data was analysed using LKB DNA and protein analysis computer programs, DNASIS and PROSIS.

## 2.16 Animal experiments

### 2.16.1 Mice

Female BALB/c mice, of average weight 25 g, were used in this study.The mice were bred under specific-pathogen-free conditions at the Waite Agricultural Institute, Adelaide. Mice were allowed to acclimatize in a conventional animal house for 2-4 d with free access to food and water.

### 2.16.2 Preparation of bacteria for injection

Live bacteria used for immunization and challenge of mice were prepared by inoculation of 10 ml NB with 1.0 ml of a stationary phase broth culture of the strain required. Inoculated cultures were incubated with aeration at 37°C for 3 h, to ca. 2 X $10^9$ bacteria / ml. Bacteria were harvested by centrifugation, washed, and resuspended in saline. The bacterial concentration was estimated by making a dilution of the culture in 1% (v/v) formalin-saline followed by microscopic enumeration. Dilutions of the suspension were also plated for estimation of viable counts.

Mice received 25 µl bacterial suspension via a disposable plastic tip attached to a Gilson P100 micopipette, the tip being placed behind the incisors before expulsion of the dose. No attempt was made to neutralize gastric acidity by prior or simultaneous administration of sodium bicarbonate, nor were the animals starved before dosing. Preliminary experiments had

indicated that neither of these procedures significantly altered the extent of Peyer's patch colonization by strain C5.

### 2.16.3 Virulence and protective capacity of *S. typhimurium* derivatives

The virulence of *S. typhimurium* mutants was assessed by determining 28 d $LD_{50}$ doses. Groups of 5-7 mice received graded doses of the test strain, and survival data at day 28 was used to calculate $LD_{50}$ values by the method of Reed and Muench (1938).

The protective efficacy of *rfc* and *pmi* mutants of *S. typhimurium* C5 was assessed as follows. Mice received (on day 0) various oral doses of the test strain, and were challenged orally 28-30 d later with ca. 25 $LD_{50}$ doses (ca. 1.5 X $10^6$ bacteria) of the highly virulent *S. typhimurium* C5 strain. Survival was followed for a further 28 d and compared with that of a control group which had received saline on day 0.

### 2.16.4 Colonization of mice by strains of *S. typhimurium*

Mice were used to assess the capacities of *S. typhimurium* strains to colonize the gut and spleen after oral administration. Mice were fed ca. 1 X $10^8$ bacteria of the strain of interest, and at various times thereafter, 5 animals were sacrificed and their spleens and Peyer's patches removed. To collect the latter, the small intestine was removed and flushed through with 10 ml of cold sterile saline, before excision of the lymphoid aggregates with scissors. Spleens and Peyer's patches were homogenized separately in 5 ml saline, using an Ultra-Turrax homogenizer, before enumeration of persisting bacteria by dilution and plating onto NA (supplemented with antibiotic where appropriate).

# CHAPTER 3

## Molecular cloning and characterization of the *rfc* gene for an O-antigen polymerase of *Salmonella typhimurium*

## 3.1 Introduction

The structure and biosynthesis of bacterial lipopolysaccharide has been described in Chapter 1. Three major genetic regions (apart from those involved in general housekeeping functions) have been implicated in the biosynthesis of the polysaccharide component of the LPS of *S. typhimurium*. The *rfa* cluster of genes is involved in core synthesis, and the *rfb* region in the synthesis of the O-antigenic tetrasaccharide units (Mäkelä and Stocker, 1984). In addition, it is thought that genes of both the *rfa* and *rfb* clusters are involved in the transport and ligation of core-O-antigen complexes (Mäkelä and Stocker, 1984). The *rfc* region is thought to encode a polymerase responsible for the linking of the O-antigen tetrasaccharide units into long chains, giving rise to typical "smooth" LPS (Mäkelä and Stocker, 1984). Mutants of *S. typhimurium* which are defective in the O-antigen polymerase due to a mutation at the *rfc* locus produce LPS structures termed semi-rough (SR), which have at most one O-antigenic tetrasaccharide unit attached to any given core unit (Naide *et al.*, 1965, Yuasa *et al.*, 1970).

A bacteriophage sensitivity pattern characteristic of SR mutants has been described. Such strains of *S. typhimurium* were resistant to the smooth-specific phages 9NA and P22, sensitive to phage FO, and resistant to all phages specific for rough LPS except P22I (Wilkinson *et al.*, 1972).

Mutants of the SR LPS phenotype have been identified in *Salmonella* groups B and E (Naide *et al.*, 1965; Losick and Robbins, 1967). In *S. typhimurium* (Group B), the *rfc* locus has been located between *gal* and *trp* (Naide *et al.*, 1965; Stocker *et al.*, 1966), corresponding to a map position between 18 and 34 minutes. An analogous locus has been postulated in some *Salmonella* strains of group D, based on the observation that in hybrids between *Salmonella* groups B (O-4, 5, 12) and D (O-9, 12), involving transfer of *rfb* genes, the polymerase of one group could polymerize the O units of the other (Mäkelä, 1965; Nurminen *et al.*, 1971; Valtonen *et al.* 1975).

This chapter describes the cloning of the *rfc* gene of *S. typhimurium* by complementation of *rfc* defects in spontaneous and IS*10* -derived SR mutants. A physical map of the DNA region encoding the *rfc* gene is presented and attempts to visualise the gene product(s) are described. The distribution of *rfc* -homologous DNA in other *Salmonella* strains is also determined.

## 3.2 Results

### 3.2.1 Construction of a lambda-sensitive *rfc* strain

Plasmid pAMH70 (*lamB*$^+$; Harkki *et al.*, 1987) was cut with *Sca* I and ligated with the end-filled 1.4 kb *Mlu* I fragment (which encodes Km$^R$) of pLG339 (Stoker *et al.*, 1982) to give pADE200. Maintenance of pADE200 and the cosmid vector pHC79 (Ap$^R$) (Hohn and Collins, 1980) in the same cell was possible since the Ap$^R$ determinant of pAMH70 had been inactivated during the cloning step.

The *rfc* strain SL901 was subjected to genetic manipulation in order to facilitate its use as a recipient in cosmid bank construction, and to render it more receptive as a recipient in transformation than the unmodified strain. Initially, a *galE* mutation was introduced into SL901. The *S. typhimurium* strain rendered lambda-sensitive by Harkki *et al.* (1987), by introduction of a *lamB*$^+$ plasmid, was a *galE* mutant. A *galE* mutant of *S. typhimurium*, grown without added galactose, does not synthesise O-antigen. Membrane receptor structures, such as the *lamB* protein, may therefore be more accessible to phage. Also, *S. typhimurium galE* mutants were more efficient recipients in transformation than wild-type smooth strains. A *galE* mutant of SL901 was isolated by initial selection for resistance to FO, followed by screening for susceptibility to galactose-induced lysis, which is a characteristic of *galE* mutants (Fukasawa and Nikaido, 1961a, 1961b). Subsequently, a galactose-resistant derivative of the *galE* strain was obtained by selection on NAGal. This galactose-resistant strain (LV56) retained the ability to make SR LPS in the presence of galactose, and accordingly did not completely lack *galT* or *galK* activities (Fukasawa and Nikaido, 1961a, 1961b). Strain LV56 then received, by phage ES18-mediated cotransduction with *srl*-

*202*::Tn*10*, the *recA* allele of strain TT521. A $Tc^S$ derivative, which had apparently lost the Tn*10* element but remained *recA*, was selected on fusaric acid-containing medium, to give strain LV203.

Strain LV203 contained a large plasmid characteristic of *S. typhimurium* (Jones *et al.*, 1982). In early experiments with this strain, problems were encountered with recombination between the resident plasmid and transformed plasmids, and with purification of cloned DNA from the strain. Accordingly, strain LV203 was cured of the resident plasmid (Cerin and Hackett, 1989), and then received, by transformation, pADE200 (*lamB*$^+$), to give the final strain, LV242. Strain LV242 was lambda-sensitive, while SL901/pADE200 was lambda-resistant.

None of the above alterations to the original SL901 strain altered the SR LPS phenotype. The *rfc* strain SL901 was confirmed to have the phage sensitivity profile characteristic of SR strains (Wilkinson *et al.*, 1972), being $9NA^R$, $P22^R$, $P22I^S$ and $FO^S$. Strains LV203 and LV242 were $9NA^R$, $P22^R$, $P221^S$ and $FO^R$ on NA with glucose, but were $9NA^R$, $P22^R$, $P221^S$ and $FO^S$ on NA with galactose.

### 3.2.2 Insertional inactivation of the *rfc* locus of C5

To facilitate complementation tests with a cloned *rfc* gene, a transposon-generated SR mutant of *S. typhimurium* C5 was isolated. An F' factor, which incorporated transposon Tn*10*, and which was temperature-sensitive for replication (F'$114_{ts}$ *lac*$^+$ *zzf*-21::Tn*10* ) was conjugated from strain TT628 into *S. typhimurium* C5, with selection for $Tc^R$ on minimal medium at 30°C. Transconjugants with transposon-mediated chromosomal mutations were selected by growth at 42°C on L agar containing Tc. Individual $Tc^R$ colonies were screened for resistance to phage 9NA and for sensitivity to phage FO at 37°C. In this manner, strain J208 was identified as having an SR phage phenotype ($9NA^R$, $P22^R$, $FO^S$, $P22I^S$) but was found to be $Lac^+$ on MacConkey lactose medium. This indicated that although an *rfc* locus had been inactivated, the F' factor had not been lost at the non-permissive temperature. Derivatives of J208 which were $Tc^S$ were isolated by plating on fusaric acid-containing medium (Bochner *et*

*al.*, 1980). On this medium, $Tc^R$ cells were killed and $Tc^S$ cells which had eliminated Tn*10* were selected. Two individual $Tc^S$ isolates of J208, J208-B1 and J208-B2, were examined and were found to be $Lac^-$, indicating that the F' factor had been lost concomitant with the loss of the Tc resistance of the Tn*10* element. The *rfc* phenotype (SR phage type) was retained in both J208-B1 and J208-B2. As IS*10* -Right (IS*10* - R) is a fully functional transposon (Roberts *et al.*, 1985), the above results suggested that IS*10* -R transposition from the $F'114_{ts}$ $lac^+$ *zzf*::Tn*10* into the *rfc* locus might have occurred.

A 2.8 kb *Acc* I fragment of transposon Tn*10*, which contained part of the IS*10* -R element (Way *et al.*, 1984) was used as a probe to examine the possibility of an IS*10* insertion in J208 and its derivatives. The 2.8 kb *Acc* I fragment was purified from plasmid pPR11 (ColE1 *tolC*::Tn*10* ) (Morona and Reeves, 1981) and labelled. Whole genomic DNA of C5, J208, J208-B1, J208-B2, and plasmid DNA of pPR11 were probed with the labelled fragment derived from IS*10* -R (Fig. 3.1). No IS*10* -R-like sequences were detected in C5 (Fig. 3.1; lanes 1 and 6). The control plasmid pPR11 had homologous *Hin*dIII and *Acc* I fragments consistent with the presence of the entire Tn*10* on this plasmid. The two upper *Hin*dIII and *Acc*I fragments of J208 which showed strong hybridization corresponded to fragments of the episomal Tn*10* element on the F' factor (Fig.3.1; lanes 2 and 7). The smallest hybridizing fragments in each case (the 3.2 kb *Hin*dIII and the 1.1 kb *Acc*I bands) were thought to represent an insertion of part or all of an IS*10* element in the chromosome. The presence of only these hybridizing fragments in J208-B1 and J208B-2 (Fig. 3.1; lanes 3, 4, 8, 9) was consistent with a chromosomal IS*10* insertion and the absence of the F' factor and the Tn*10* element. The size of the IS*10* -homologous *Hin*dIII band (3.2 kb; Fig. 3.1; lanes 3 and 4) was very close to the size expected for an IS*10* (1.4 kb) insertion in the *rfc* - encoding (1.75 kb) *Hin*dIII fragment (see Section 3.2.4.1). Since there is an *Acc*I site internal to IS*10* -R, a chromosomally integrated IS*10* -R should have two *Acc*I fragments with homology to this probe. As seen (Fig. 3.1; lanes 8,9), only a single fragment was detected. A smaller IS*10* -hybridizing *Acc*I fragment (i.e. <0.6 kb) would not have been retained on the gel of Fig. 3.1. Later in this chapter (Section 3.2.5) evidence is presented which shows that the IS*10* insertion is in the *rfc* region.

**Figure 3.1: Southern hybridization analysis of *rfc* mutants J208, J208-B1, and J208-B2**

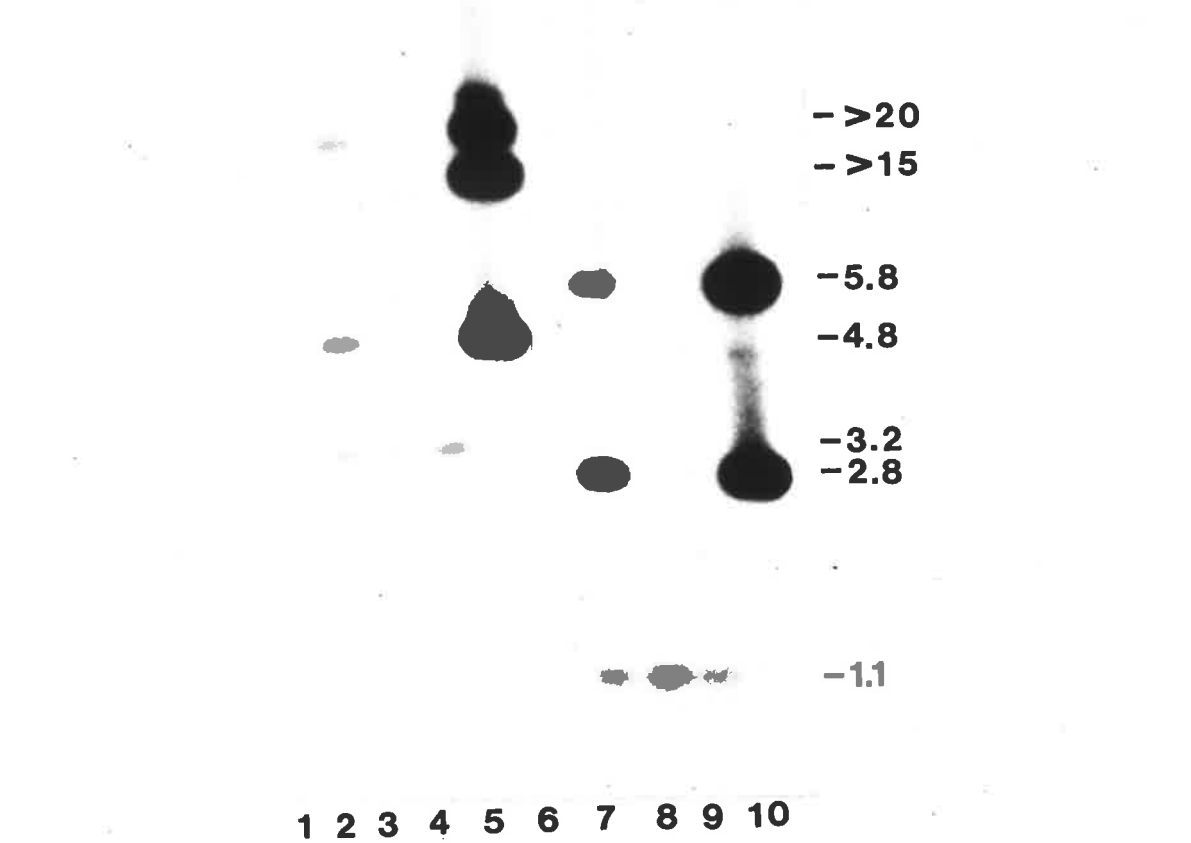
Total DNA of C5, J208, J208-B1, J208-B2, and plasmid DNA of pPR11, were digested with either *Hin*dIII or *Acc* I and electrophoresed on a 0.8% (w/v) agarose gel. After transfer to nitrocellulose the filter was probed with the $\alpha$-[$^{32}$P]-dCTP nick-translated 2.8 kb *Acc* I fragment of transposon Tn*10* (bottom of figure), which contains most of the IS*10* -R element. Following washing, the filter was subjected to autoradiography.

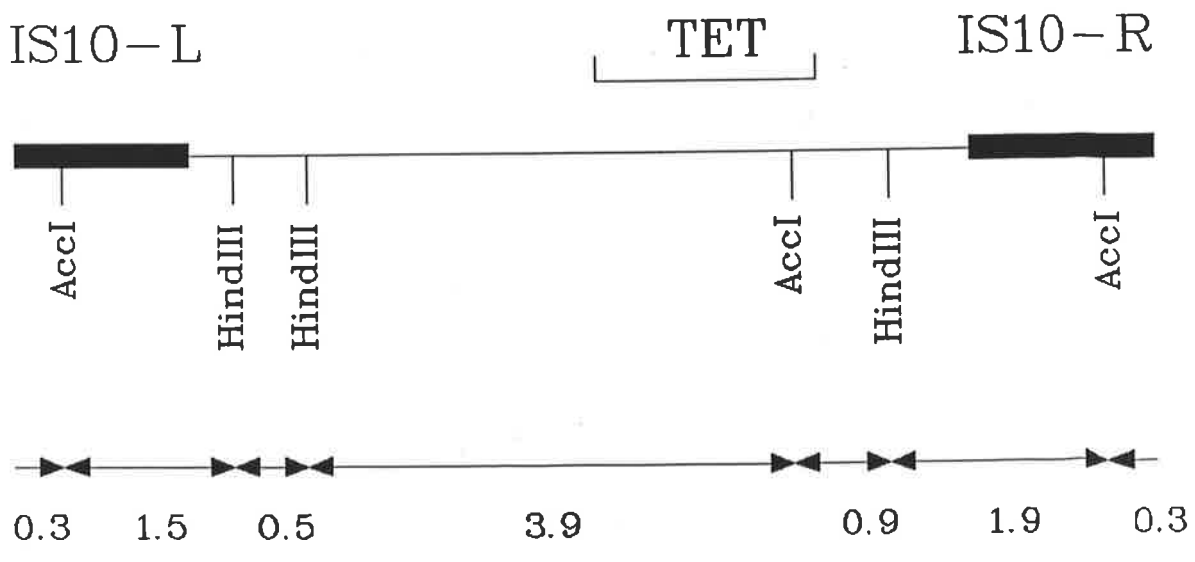
Lane designations (with enzyme used for restriction cut in brackets): 1: C5 (*Hin*dIII); 2: J208 (*Hin*dIII); 3: J208-B1 (*Hin*dIII); 4: J208-B2 (*Hin*dIII); 5: pPR11 (*Hin*dIII); 6: C5 (*Acc* I); 7: J208 (*Acc* I); 8: J208-B1 (*Acc* I); 9: J208-B2 (*Acc* I); 10: pPR11 (*Acc* I).

The probe hybridizes strongly with restriction fragments of the Tn*10* element in pPR11 (lanes 5 and 10): the uppermost *Hin*dIII band (>20 kb) in lane 5 contains IS*10*- L and the second band (ca. 15 kb) contains IS*10* -R. The third *Hin*dIII band (4.8 kb), in lanes 2 and 5, consists of the internal *Hin*dIII fragment of Tn*10*. The 5.8 kb and 2.8 kb *Acc* I -hybridizing fragments (lanes 7 and 10), represent two *Acc* I fragments in Tn*10*. The 5.8 kb *Acc* I band contains part of IS*10* - L and the 2.8 kb *Acc* I band contains part of IS*10* - R, identical to the *Acc* I fragment of Tn*10* used as the probe.

The 3.2 kb *Hin*dIII (lanes 2, 3 and 4) and 1.1 kb *Acc* I bands (lanes 7, 8 and 9) represent IS*10* insertions in the C5-derived strains.

The numbers on the right of the top portion of the Figure are the sizes, in kb, of the indicated fragments. The numbers under the bottom portion of the Figure are the sizes, in kb, of some restriction fragments of Tn*10*. TET: Tc-resistance gene of Tn*10*.


->20
->15
-5.8
-4.8
-3.2
-2.8
-1.1
1 2 3 4 5 6 7 8 9 10


IS10-L
TET
IS10-R
AccI
HindIII
HindIII
AccI
HindIII
AccI
0.3
1.5
0.5
3.9
0.9
1.9
0.3

### 3.2.3 Cosmid cloning of the *S. typhimurium rfc* locus

Strain LV242 was used as the recipient in transfection of an *S. typhimurium* cosmid bank, prepared with vector pHC79. Individual cosmid clones from the bank were screened on NA with galactose for sensitivity to bacteriophage 9NA. Of 300 clones screened, one, containing pADE201 (Fig. 3.2) was $9NA^S$, $P22^S$, $FO^S$, and $P22I^R$. This phage phenotype was consistent with the production of smooth LPS (Wilkinson *et al.*, 1972) and therefore pADE201 was considered to complement the genetic defect in the SR mutant.

### 3.2.4 Physical mapping of the $rfc^+$ cosmid and cutdowns

#### 3.2.4.1 *Hin*dIII Cutdowns of pADE201

Partial digestion with *Hin*dIII, followed by re-ligation, yielded cutdowns of pADE201 (Fig. 3.2). Of plasmids pADE202, pADE203, pADE204 and pADE205 (Fig. 3.2), only one, pADE202, retained the ability to complement the *rfc* defect in LV242. This suggested that the 1.75 kb *Hin*dIII fragment might contain the *rfc* gene. This fragment was subcloned into the *Hin*dIII site of pUC18 (to produce pADE206) and into the *Hin*dIII site of pSUP401, inactivating the $Km^R$ determinant, to produce pADE207. Both plasmids complemented the *rfc* defects in strains LV242 and J208-B1 (*rfc*::IS*10*), as judged by phage efficiency of plaquing tests (Table 3.1). A restriction map of the 1.75 kb *Hin*dIII fragment is shown (Fig 3.3).

#### 3.2.4.2 Transposon mutagenesis with Tn*5* and Tn*1725*

Plasmid pADE206 was subjected to transposon insertion mutagenesis using either Tn*1725* or Tn*5* (Materials and Methods) and the *rfc* status of the plasmids obtained assessed by phage sensitivity observations after transformation into SL901 and J208-B1. The transposons were contained on temperature-sensitive R factors in strains RU2901 (Tn*1725*) (Ubben and Schmitt, 1986) or WR6016 (Tn*5*). The locations of individual transposon insertion points

**Figure 3.2: Plasmid pADE201, and *Hin*dIII-generated deletions thereof**

A restriction map of pADE201 is shown. Open boxes indicate the fragments of pADE201 retained in the listed sub-clones after partial *Hin*dIII digestion and religation. The *rfc* genotype of each clone is shown on the right, and the 1.75 kb *Hin*dIII fragment postulated to encompass the *rfc* gene is indicated by a hatched box at the bottom. ▬ : pHC79.

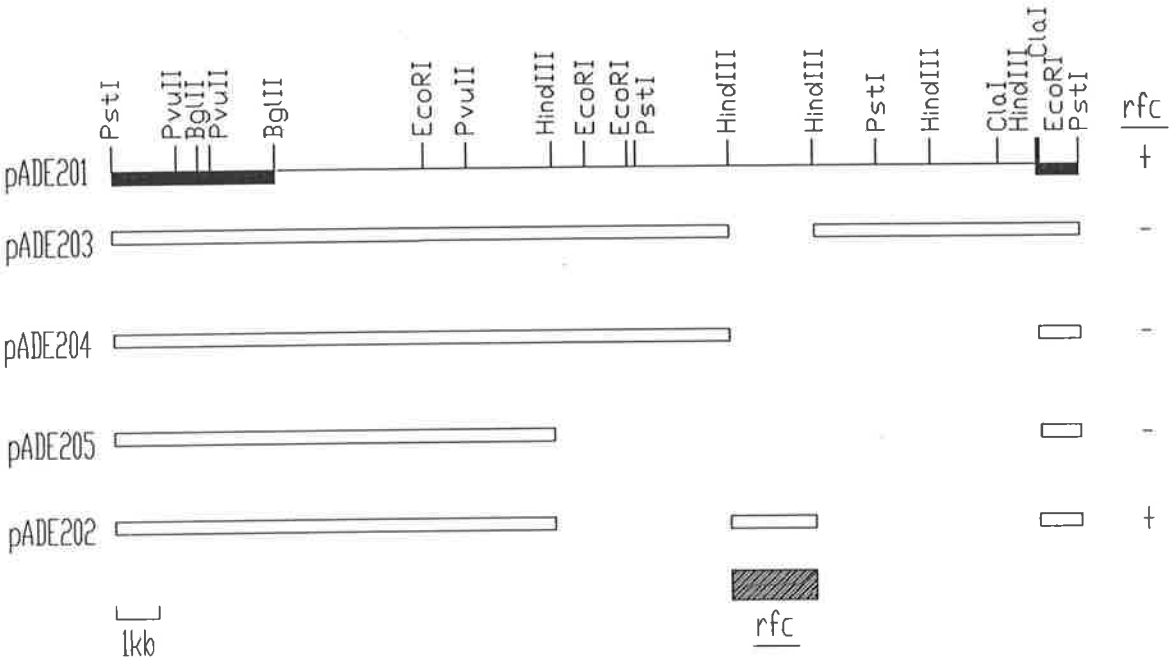

PstI
PvuII
BglII
PvuII
BglII
EcoRI
PvuII
HindIII
EcoRI
EcoRI
PstI
HindIII
HindIII
PstI
HindIII
ClaI
HindIII
EcoRI
ClaI
PstI
rfc
pADE201
+
pADE203
-
pADE204
-
pADE205
-
pADE202
+
1kb
rfc

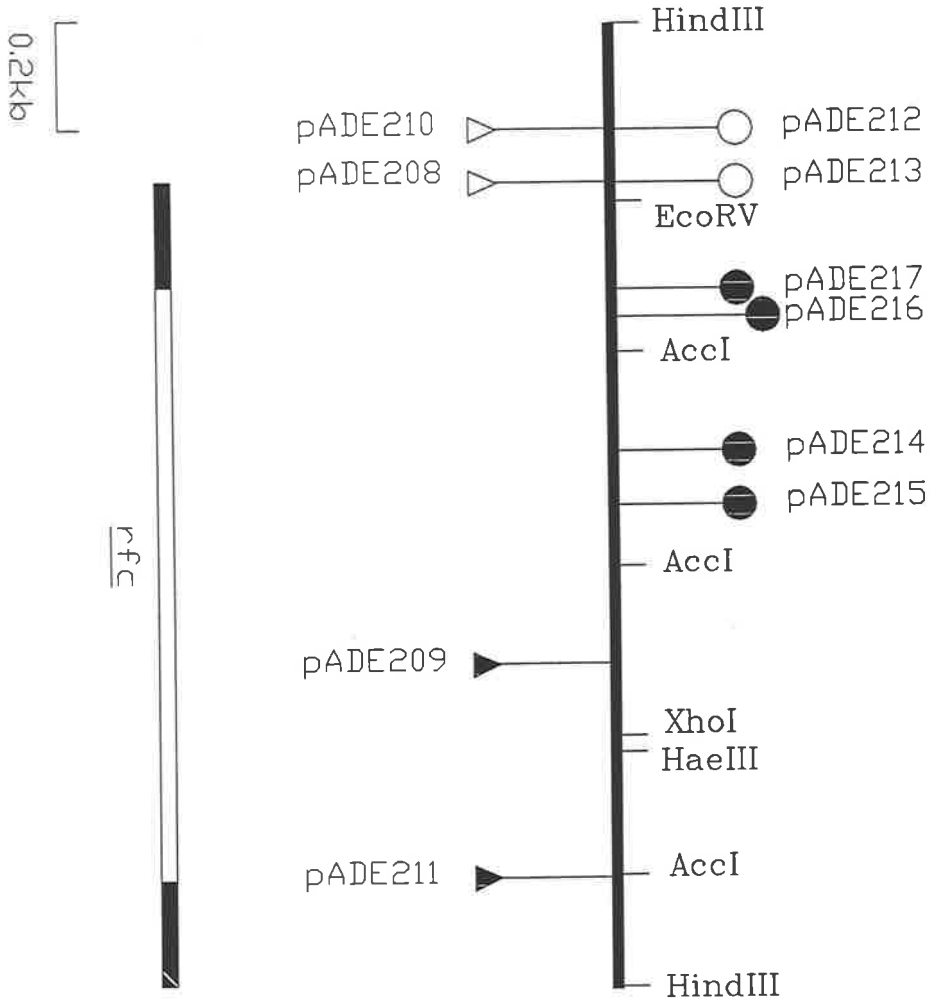
**Table 3.1: The efficiency of plaquing of various bacteriophage on various strains**

| Strain | Phage | | | |
|---|---|---|---|---|
| | P22 | 9NA | FO | P221 |
| C5 | 1 | 1 | 1 | $<0.25X10^{-6}$ |
| LV242 | $<0.25X10^{-8}$ | $<0.25X10^{-8}$ | 5 | 1 |
| J208-B1 | $<0.25X10^{-8}$ | $<0.25X10^{-8}$ | 4 | 1 |
| LV242/ pADE206 | 1 | 1 | 1 | $<0.25X10^{-6}$ |
| J208-B1/ pADE206 | 1 | 1 | 1 | $<0.25X10^{-6}$ |

Bacteria were mixed with phage at various dilutions, and plaques counted after overnight incubation. The efficiencies of plaquing of P22, 9NA, and FO on C5 were taken as unity (underlined above). Similarly, the titre of P221 on LV242 was the reference efficiency of plaquing for this phage (underlined above).

**Figure 3.3: Restriction enzyme, and transposon insertion, map of the 1.75 kb *Hin*dIII fragment of pADE206**

Restriction sites in the 1.75 kb *Hin*dIII fragment of pADE206 are indicated. Plasmids carrying transposon insertions located in this fragment are numbered, and the transposon insertion sites indicated as follows: open circles: Tn*1725* insertions not affecting *rfc*; hatched circles: Tn*1725* insertions inactivating *rfc*; open triangles: Tn*5* insertions not affecting *rfc*; hatched triangles: Tn*5* insertions inactivating *rfc*. The maximum and minimum limits of the *rfc* gene are shown.


0.2kb
orf1
HindIII
pADE210
pADE208
pADE212
pADE213
EcoRV
pADE217
pADE216
AccI
pADE214
pADE215
AccI
pADE209
XhoI
HaeIII
pADE211
AccI
HindIII

were mapped (Fig. 3.3). Plasmids pADE212 and pADE213 both contained Tn*1725* insertions which did not affect *rfc* activity, while non-inactivating Tn*5* insertions were mapped in plasmids pADE208 and pADE210. Transposon insertions which inactivated the *rfc* gene were located in plasmids pADE214, pADE215, pADE216, pADE217 (all Tn*1725* inserts), and pADE209 and pADE211 (Tn*5* inserts) (Fig. 3.3). In summary, transposon insertions to the left of the single *Eco*RV site had no effect on the activity of the *rfc* gene, whereas transposon insertions to the right of the *Eco*RV site all resulted in inactivation of the *rfc* gene (Fig. 3.3). The transposon insertion mutagenesis therefore indicated that the size of the *rfc* gene was between 1.1 kb and 1.5 kb.

### 3.2.5 Confirmation of the location of the IS*10* element in J208-B1 and J208-B2

The IS*10* insertions in J208-B1 and J208-B2 resulted in a phage phenotype characteristic of SR mutants (Section 3.2.2). Introduction of the $rfc^+$ plasmid pADE206 into J208-B1 restored wild-type sensitivities to phage (Section 3.2.4.1). The cloned 1.75 kb *Hin*dIII fragment of pADE206 was used to probe *Hin*dIII-digested total DNA of C5, J208, J208-B1, and J208-B2 (Fig 3.4). As expected, a 1.75 kb *Hin*dIII fragment of C5 hybridized strongly to the probe (Fig 3.4; lane 1). A *Hin*dIII fragment of 3.2 kb with homology to the probe was observed for each of the mutants (Fig 3.4; lanes 2,3,4), in line with the suggestion that an IS*10* element was inserted in the chromosomal *rfc* region in each case.

### 3.2.6 LPS phenotype analysis by SDS-PAGE

Silver-stained SDS-PAGE gels of the LPS of control strains, the *rfc* strains, and their derivatives, are shown (Fig. 3.5). The smooth strain C5 (Fig. 3.5; lane 2) showed the typical ladder-like pattern, presumably representing stepwise elongation of core moieties by addition of O-antigen units. The *rfb* mutant TV119 (Fig. 3.5; lane 1) showed low molecular weight LPS species corresponding to core LPS alone, while in the *rfc* mutant SL901 (Fig. 3.5, lane 3), the TV119 doublet did not appear, but a doublet of lower mobility was seen (Goldman and

**Figure 3.4: IS*10* insertions in the C5 *rfc* gene**

Whole DNA of C5 (lane 1), J208 (lane 2), J208-B1 (lane 3), and J208-B2 (lane 4) were digested with *Hin*dIII and electrophoresed on a 0.8%(w/v) agarose gel. Following transfer to nitrocellulose, the filter was probed for homology with the $\alpha$-[$^{32}$P]-dCTP-labelled 1.75 kb *Hin*dIII fragment of pADE206, washed, and subjected to autoradiography.

The 1.75 kb *Hin*dIII fragment of C5 DNA (lane 1) hybridizes strongly with the probe, as do apparently identical 3.2 kb *Hin*dIII fragments in J208 (lane 2), J208-B1 (lane 3), and J208-B2 (lane 4).

The numbers on the right are the sizes, in kb, of the indicated fragments.

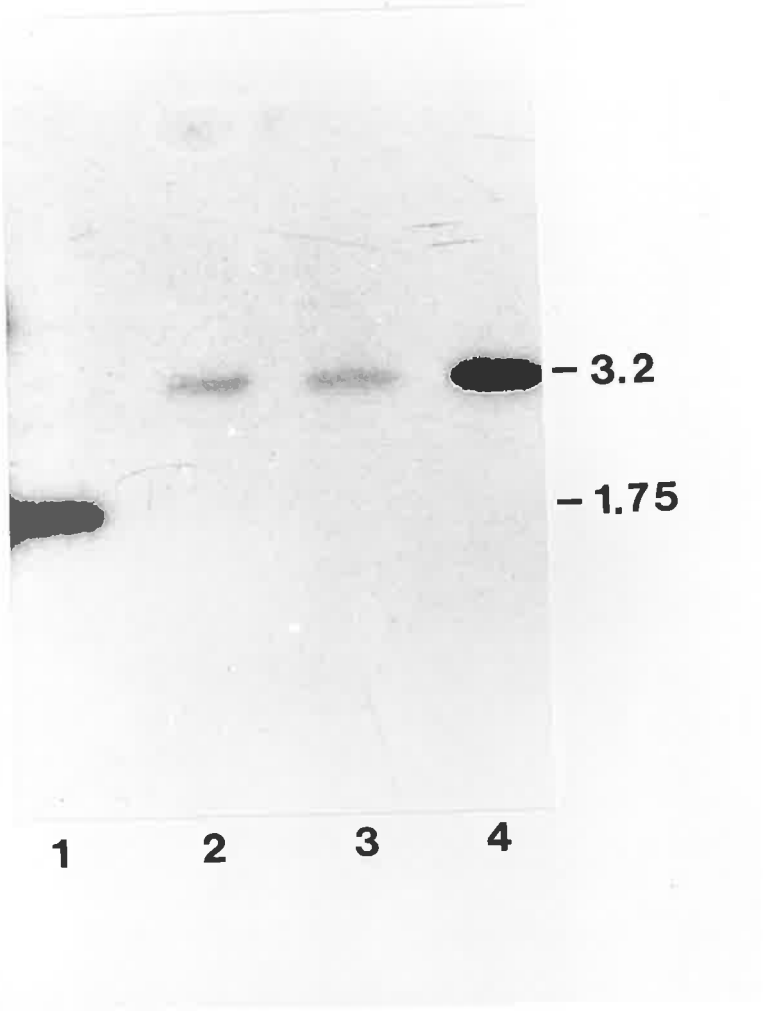
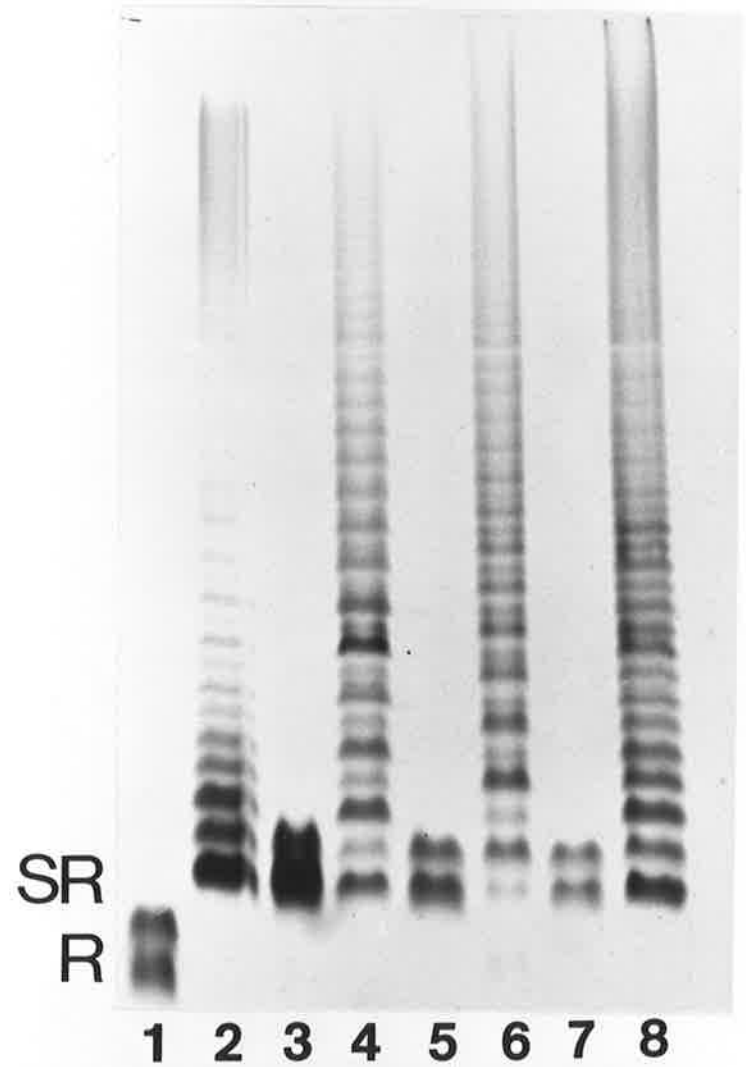

3.2
1.75
1
2
3
4

**Figure 3.5: LPS profiles of various strains in silver-stained SDS-PAGE**

Bacteria were lysed with SDS, treated with Proteinase K, and residual material analysed by SDS-PAGE with LPS-specific silver staining. Lane designations (with relevant genotype in brackets): 1: TV119 (*rfb* ); 2: C5 (wild-type); 3: SL901 (*rfc* ); 4: SL901/pADE206 (*rfc*$^+$); 5: J208-B1 (*rfc*::IS*10* ); 6: J208-B1/pADE206 (*rfc*$^+$); 7: J208-B1/pADE215 (*rfc* ); 8: J208-B1/pADE212 (*rfc*$^+$). "R" denotes the location of rough (core) LPS; "SR" denotes the location of semi-rough LPS.


SR
R
1 2 3 4 5 6 7 8

Leive, 1980; Palva and Mäkelä, 1980). Strain J208-B1 (Fig. 3.5; lane 5) displayed the same SR-type LPS as SL901. Strains SL901/pADE206 (Fig. 3.5; lane 4) and J208-B1/pADE206 (Fig. 3.5; lane 6) made LPS similar to that of wild-type *S. typhimurium* C5. The LPS of J208-B1/pADE215 (*rfc*) (Fig. 3.5; lane 7) was identical to that of J208-B1, while J208-B1/pADE212 ($rfc^+$) (Fig. 3.5; lane 8) made smooth LPS.

In summary, the *rfc* status of strains, as shown by SDS-PAGE LPS profiles, was in agreement with the indications given by the phage sensitivity profiles.

### 3.2.7 Analysis of plasmid-encoded polypeptides

Detection of plasmid-encoded polypeptides may be effected with a number of different techniques. Minicells are *E. coli* mutants (*minA*) defective in cell division. In these mutants, cell division sometimes occurs asymmetrically, resulting in small cells without a chromosome, in which only plasmid-encoded proteins are expressed (Frazer and Curtiss, 1975; Dougan and Kehoe, 1984). Maxicells are *E. coli recA* mutants which are unable to repair DNA damage due to ultraviolet light irradiation. Exposure of plasmid-containing derivatives of such mutants to ultraviolet light results in preferential damage to chromosomal DNA (Sancar *et al.*, 1979). *In vitro* expression of plasmid genes may be effected by a bacterial cell-free coupled transcription-translation system (Zubay, 1973; Collins, 1979). Inducible T7 promoter vectors may be used to analyze the proteins produced by cloned DNA inserts which lack promoter sequences or which are constitutively poorly transcribed. The system uses an inducible T7 RNA polymerase to produce transcripts from genes cloned in front of a T7 promoter. Selective inhibition of host *E. coli* RNA polymerase by treatment with rifampicin permits exclusive labelling and analysis of cloned gene products (Tabor and Richardson, 1985)

### 3.2.7.1 Minicell and maxicell analysis of proteins expressed by *rfc*$^+$ plasmids

The plasmids pSUP401, pADE207 (pSUP401 *rfc*$^+$), pUC18 and pADE206 (pUC18 *rfc*$^+$) were transformed into the minicell-producing strain DS410 (Dougan and Sherratt, 1977). Minicells were purified from whole cells by centrifugation through sucrose gradients. Plasmid-encoded polypeptides were labelled with [$^{35}$S]-methionine (Kennedy *et al.*, 1977; Achtman *et al.*, 1979). Samples were solubilised, and, following electrophoresis, were subjected to autoradiography (Fig. 3.6A). Minicells containing clones of the *rfc* gene in two different vectors were analyzed to minimize the possibility of masking of *rfc* -specified proteins by vector-encoded proteins. Plasmid pSUP401 encoded a major 25kDa polypeptide, corresponding to chloramphenicol acetyltransferase (CAT) and a 29 kDa band corresponding to aminoglycoside phosphotransferase (APH) (Fig. 3.6A; lane 1). Plasmid pUC18 encoded polypeptides of molecular sizes 32, 30, and 26 kDa, (Fig. 3.6A; lane 3) corresponding to products of the β-lactamase gene (Dougan and Kehoe, 1984). The test plasmids, pADE206 and pADE207, produced no polypeptides other than those which were vector-encoded (Fig. 3.6A; lanes 2 and 4). The APH protein was not produced in DS410 / pADE207 (Fig. 3.6A; lane 2) due to the insertion of the cloned *rfc* gene into the APH-encoding region of pSUP401.

Maxicell analysis of protein products of pSUP401 and pADE207 (pSUP401 *rfc*$^+$), was effected by transformation of the plasmids into *E. coli* CSR603, followed by ultraviolet light irradiation (Sancar *et al.*, 1979). Maxicell proteins were labelled with [$^{35}$S]-methionine, separated by electrophoresis, and visualised by autoradiography (Fig.3.6B). The CAT- and APH- protein bands were visible as products of pSUP401 (Fig.3.6B; lane 1), and pADE207 retained the ability to synthesize the CAT protein in maxicells but did not encode the APH protein (Fig.3.6B; lane 2) due to insertional inactivation of the Km$^R$ determinant. No insert-encoded proteins could be distinguished for pADE207.

**Figure 3.6: Minicell and maxicell analysis of polypeptides encoded by various plasmids**

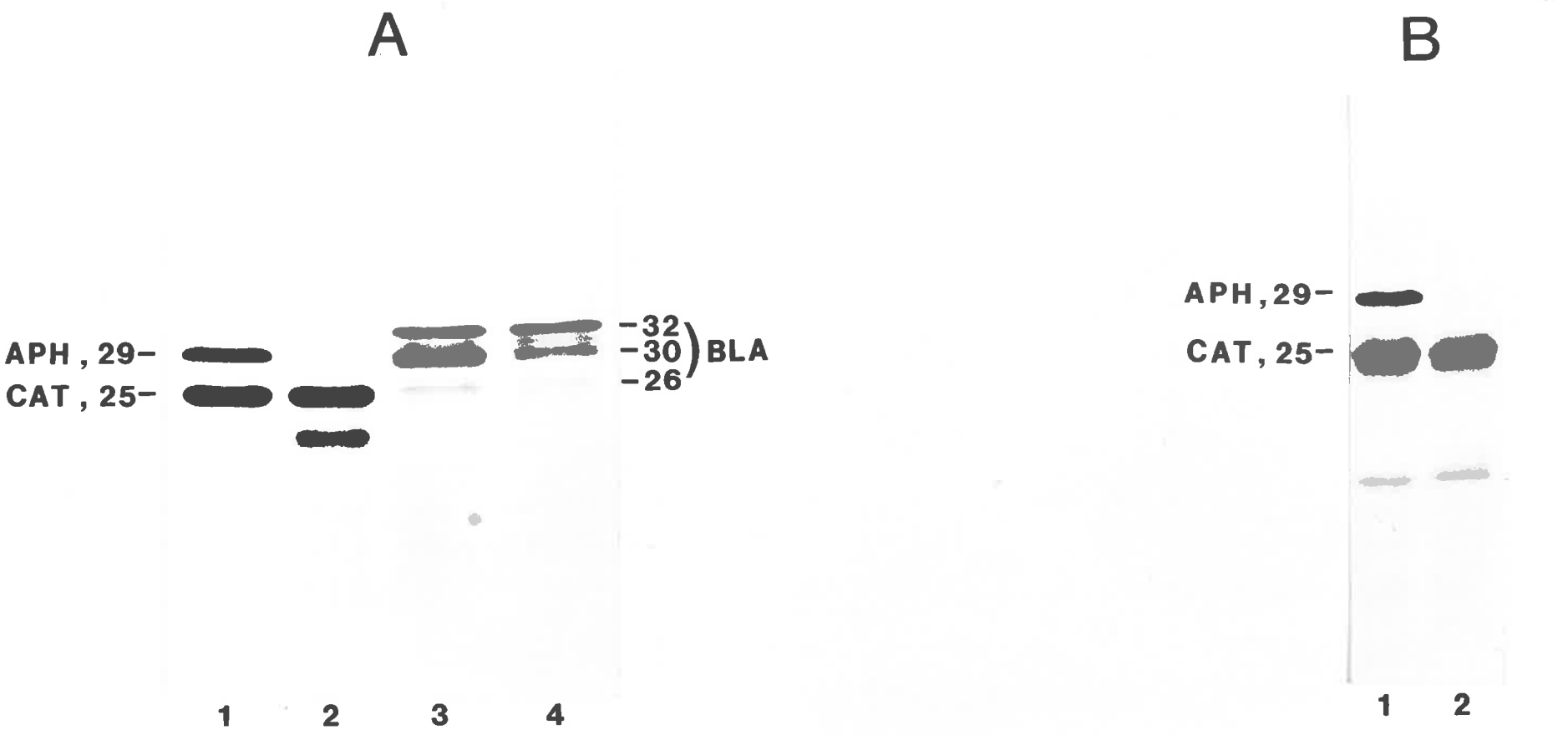
**A:** Plasmid-encoded proteins were analysed using the minicell-producing strain DS410. Minicells harbouring various plasmids were purified on sucrose gradients, labelled with [$^{35}$S]-methionine, and solubilised. Labelled proteins were visualised by autoradiography after electrophoresis on a 0.2% (w/v) SDS, 15% (w/v) polyacrylamide gel. Lane A1: DS410 / pSUP401; lane A2: DS410 / pADE207; lane A3: DS410 / pUC18; lane A4: DS410 / pADE206.

The CAT (chloramphenicol acetyltransferase) and APH (aminoglycoside phosphotransferase) proteins encoded by pSUP401, and the BLA (β - lactamase) proteins encoded by pUC18, are indicated. The protein band in lane 2 at ca. 23 kDa may represent a product of CAT protein breakdown.

**B**: Plasmid encoded proteins were analysed in the maxicell strain CSR603. Following irradiation with ultraviolet light, cells were labelled with [$^{35}$S]-methionine and solubilised. Electrophoresis on a 0.2%(w/v) SDS, 15%(w/v) polyacrylamide gel followed, and separated proteins were visualised by autoradiography. Lane B1: CSR603 / pSUP401; lane B2: CSR603 / pADE207.

The APH and CAT proteins are indicated. The lower band in lanes 1 and 2 represents a vector-encoded protein not expressed in minicells.

The numbers on the sides are the sizes, in kDa, of the indicated proteins.


A
APH , 29–
CAT , 25–
–32
–30
–26
BLA
1
2
3
4
B
APH , 29–
CAT , 25–
1
2

### 3.2.7.2 *In vitro* transcription-translation analysis of proteins encoded by *rfc*$^+$ plasmids

The protein products of the plasmids pSUP401, pADE207 (pSUP401 *rfc*$^+$), pUC18 and pADE206 (pUC18 *rfc*$^+$) were analyzed using a prokaryotic, DNA-directed, coupled transcription-translation kit. The translation products of the CsCl-purified plasmids were labelled with [$^{35}$S]-methionine, separated by electrophoresis, and visualised by autoradiography (Fig. 3.7). Vector-encoded BLA and CAT proteins were evident, while APH protein production was weak (Fig. 3.7; lanes 1 and 3). Some minor protein bands appeared in both the vector and test samples (Fig. 3.7; lanes 1 to 4), probably corresponding to either breakdown products of the antibiotic-resistance proteins or to translated products of contaminating mRNA species. A protein product attributable to translation of the *rfc* gene was not apparent in either pADE207 (Fig. 3.7; lane 2) or pADE206 (Fig. 3.7; lane 4) .

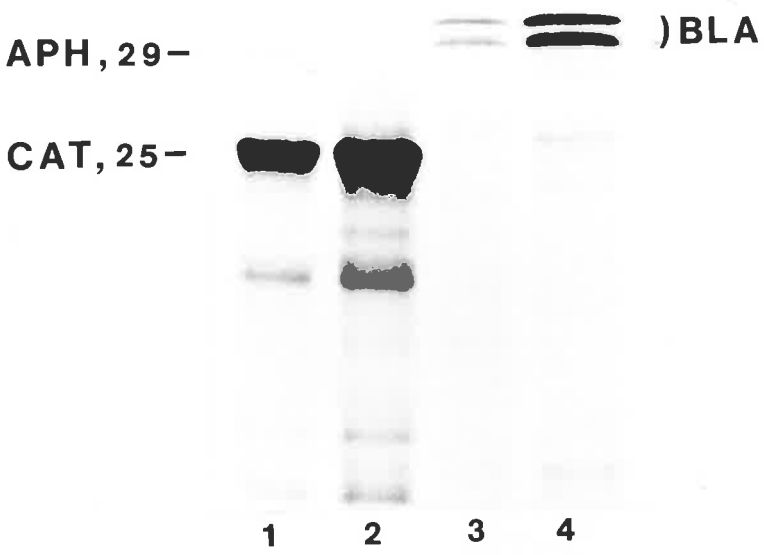
### 3.2.7.3 T7 polymerase / promoter system analysis of plasmid-encoded proteins

The T7 promoter-mediated transcription system was utilised to ascertain if the apparent lack of expression of a protein from the *rfc* gene was due to poor transcriptional efficiency. The 1.75 kb *Hin*dIII fragment of pADE206 was cloned in both orientations before the T7 promoter in the pBLUESCRIPT KS+ vector, to give pADE218 and pADE219. The orientation of the inserted fragment, with respect to the initiation site of T7 transcription, was *Xho*I site proximal, *Eco*RV site distal in pADE218, and *Eco*RV site proximal, *Xho*I site distal in pADE219 (see Fig. 3.3). These two plasmids were transformed into *E. coli* K38 / pGP1-2. The plasmid pGP1-2 carries the T7 RNA polymerase gene under the control of a lambda $P_L$ promoter which is itself under the negative control of the temperature-sensitive lambda cI*857* gene product, all of which are encoded on the plasmid (Tabor and Richardson, 1985). Upon induction of the lambda $P_L$ promoter at 42°C, the T7 polymerase should transcribe the *rfc* gene from the T7 promoter on the pBLUESCRIPT KS+ plasmid. Addition of rifampicin (200 µg / ml) is designed to specifically inhibit the host RNA polymerase. Uninduced cells which were

**Figure 3.7: *In vitro* transcription-translation analysis of plasmids carrying the *rfc* gene**

Plasmid-encoded proteins of pSUP401 (lane 1), pADE207 (lane 2), pUC18 (lane 3) and pADE206 (lane 4) were labelled with [$^{35}$S]-methionine using a prokaryotic DNA-directed transcription and translation kit. Labelled proteins were solubilised and visualised by autoradiography after electrophoresis on a 0.2%(w/v) SDS, 15% (w/v) polyacrylamide gel. The vector-encoded APH, CAT and BLA proteins are indicated. No insert-encoded proteins are obvious although small amounts of some other proteins, probably due to low level cellular mRNA contamination of plasmid DNA, are present.

The numbers on the left are the sizes, in kDa, of the indicated proteins.


APH, 29–
CAT, 25–
)BLA
1
2
3
4

not subjected to T7 polymerase induction and which were treated with rifampicin were used as controls. The protein products were labelled with [$^{35}$S]-methionine, separated by electrophoresis, and visualised by autoradiography (Fig. 3.8).

Several proteins are visible in both the induced and uninduced samples, which may indicate the stability of certain *E. coli* mRNA species after rifampicin treatment (Fig. 3.8; lanes 1 - 6). No major differences in labelled protein profiles were discerned between the induced cells of K38/ pGP1-2 / pBLUESCRIPT KS+, K38 / pGP1-2 / pADE218 and K38 / pGP1-2 / pADE219 (Fig. 3.8; lanes 2, 4, 6). These results suggest that the apparent non-expression of an *rfc* - encoded protein in the minicell, maxicell and *in vitro* transcription / translation systems may not be attributable to inefficient transcription of the *rfc* gene in *E. coli*. Possible explanations for the lack of success in identifying the product of the *rfc* gene in these experiments, are discussed further below.

### 3.2.8 Distribution of the *rfc* locus among *Salmonella* strains

A 0.4 kb *Acc*I fragment internal to the *rfc* region (see Fig. 3.3) was purified from pADE206 (pUC18 *rfc*$^{+}$), labelled with α-[$^{32}$P]-dCTP by the random oligonucleotide primer method, and used to probe *Acc*I- and *Eco*RV-digested chromosomal DNA of various strains (Fig. 3.9). Homologous *Acc*I fragments identical in size to the 0.4 kb *Acc*I probe were detected in *S. paratyphi* A (lane 3), *S. typhi* (lane 4), *Salmonella derby* (lane 5), *S. dublin* (lane 6) and *Salmonella chester* (lane 7), in addition to the parental *S. typhimurium* (lane 2), but no homologous DNA was seen in *Salmonella strasbourg* (lane 8), *Salmonella newington* (lane 9) or in *E. coli* K-12 DH1 (lane 1) .

Similarly, homology with the probe was observed for the *Eco*RV -cut total DNA of *S. typhimurium* (lane 11), *S. paratyphi* A (lane 12), *S. typhi* (lane 13), *S. derby* (lane 14), *S. dublin* (lane 15) and *S. chester* (lane 16) although the hybridizing fragments differed in size between strains. The *Eco*RV-generated DNA fragments of *S. strasbourg* (lane 17), *S.*

**Figure 3.8: Analysis of proteins transcribed from the T7 promoter when present in various plasmids**

Derivatives of K38 / pGP1-2 carrying either pBLUESCRIPT KS+, pADE218 or pADE219, grown at 30°C overnight, were shifted to 42°C to induce production of T7 RNA polymerase. Uninduced control samples were not subjected to induction of the T7 RNA polymerase. Rifampicin was added to all samples to inhibit *E. coli* RNA polymerase and proteins were labelled with [$^{35}$S]-methionine at 30°C. Labelled proteins were solubilised and visualised by autoradiography after electrophoresis on a 0.2% (w/v) SDS, 15% (w/v) polyacrylamide gel.

Samples in lanes 1 to 6 consisted of the base strain K38/pGP1-2 carrying the following plasmids (and treated as indicated in brackets): lane 1: pBLUESCRIPT KS+ (uninduced); lane 2: pBLUESCRIPT KS+ (induced); lane 3: pADE218 (uninduced); lane 4: pADE218 (induced); lane 5: pADE219 (uninduced); lane 6: pADE219 (induced).

The numbers on the left are the sizes, in kDa, of protein standards.

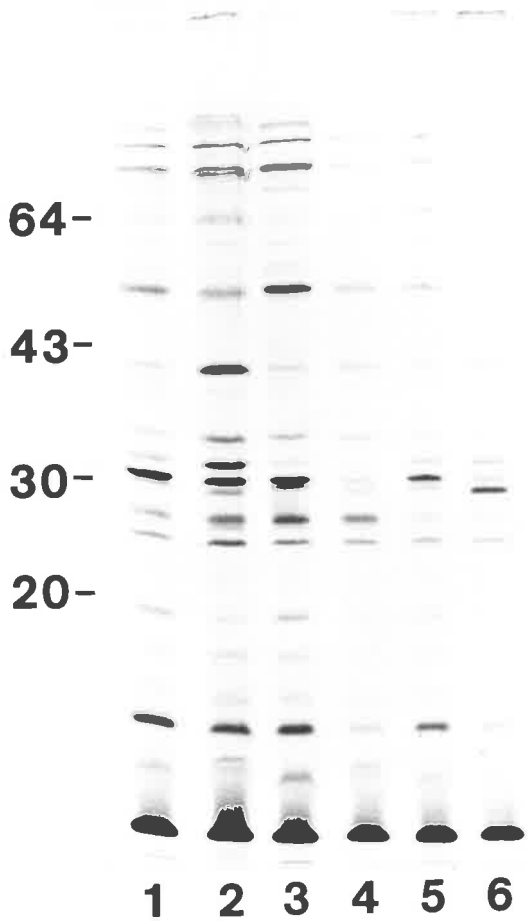
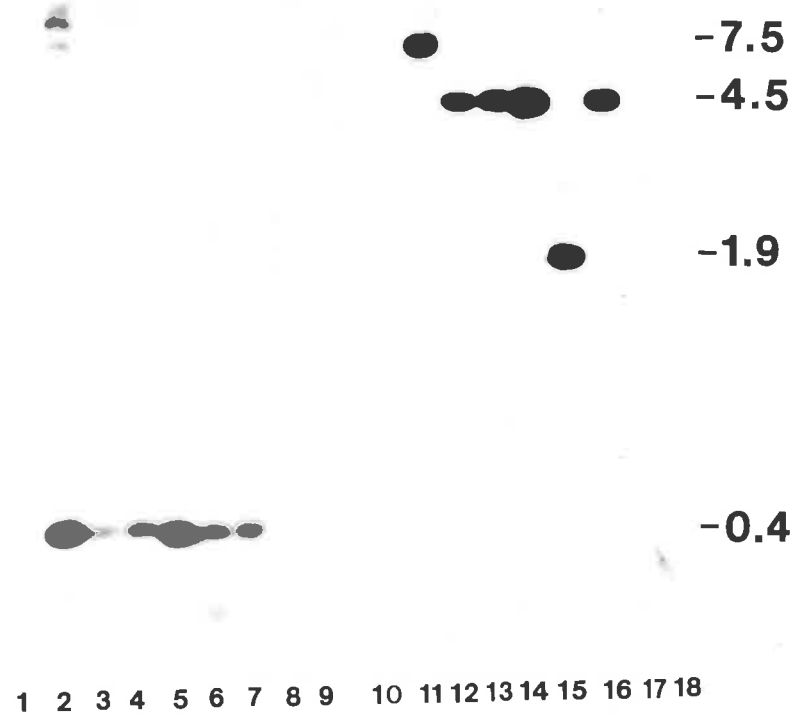

64-
43-
30-
20-
1
2
3
4
5
6

**Figure 3.9: Homology of cloned *S. typhimurium rfc* with DNA from other *Salmonella* strains**

The internal 0.4 kb *Acc* I fragment of pADE206 was labelled with α-[$^{32}$P]-dCTP by oligonucleotide primer labelling. This probe was used in Southern hybridization to test for homology with *Acc* I- and *Eco* RV-generated restriction fragments of total DNA of *E. coli* K-12 and a variety of *Salmonella* strains.

Lane designations (with restriction enzyme used for cut in brackets): 1: *E. coli* K-12 (*Acc* I); 2: *S. typhimurium* (*Acc* I); 3: *S. paratyphi* A (*Acc* I); 4: *S. typhi* (*Acc* I); 5: *S. derby* (*Acc* I); 6: *S. dublin* (*Acc* I); 7: *S. chester* (*Acc* I); 8: *S. strasbourg* (*Acc* I); 9: *S. newington* (*Acc* I ); 10: *E. coli* K-12 (*Eco*RV); 11: *S. typhimurium* (*Eco*RV); 12: *S. paratyphi* A (*Eco*RV); 13: *S. typhi* (*Eco*RV); 14: *S. derby* (*Eco*RV); 15: *S. dublin* (*Eco*RV); 16: *S. chester* (*Eco*RV); 17: *S. strasbourg* (*Eco*RV); 18: *S. newington* (*Eco*RV).

The numbers on the right are the sizes, in kb, of the indicated fragments.


-7.5
-4.5
-1.9
-0.4
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18

*newington* (lane 18) and of *E. coli* K-12 DH1 (lane 10) again showed no homology to the *rfc* probe.

## 3.3 Discussion

The original *rfc* mutant of *S. typhimurium*, SL901 (Naide *et al.*, 1965), made SR LPS, and was thought to be defective in the polymerase function required for assembly of O-antigen repeat units into long-chain LPS (Naide *et al.*, 1965; Yuasa *et al.*, 1970). An *rfc*::IS*10* mutant, J208-B1, isolated here by mutagenesis of C5, was phenotypically similar to SL901. Both strains had SR type phage specificity, and synthesised LPS which, on SDS-PAGE, appeared intermediate between R and smooth (S) forms of LPS.

A cosmid containing *S. typhimurium* DNA was identified which restored the ability to synthesise smooth LPS to both SL901 and J208-B1, and was therefore postulated to carry the *rfc* gene. The *rfc* gene was shown to reside on a 1.75 kb *Hin*dIII fragment subcloned from this cosmid and further localization to a smaller DNA region was possible by transposon mutagenesis. The demonstration of loss of complementing ability in *rfc*::Tn*1725* and *rfc*::Tn*5* plasmids derived from the $rfc^+$ clone strengthened the hypothesis that the cloned gene was the *rfc* gene.

Extensive experimentation employing minicell, maxicell, *in vitro* transcription-translation, and T7 polymerase / promoter systems, failed to demonstrate a protein product of the cloned *rfc* gene. Several reasons for this may be postulated. The utilization of systems which require *E. coli* host strains or sub-cellular components of *E. coli* may not be suitable for studying the products of genes from other bacterial species. This may be especially true in cases where species-specific co-factors or positive regulatory elements are required for gene expression. Poor or inefficient expression of non-lethal genes at the transcriptional level can, in some cases, be overcome by placing a promoter region in front of the gene. Induction of transcription from strong promoters produces mRNA transcripts in far greater quantities than those found in strains wild-type for that gene. The *rfc* gene, cloned in either orientation, in

front of the T7 promoter region, failed to produce an *rfc* -specific protein when transcribed by T7 RNA polymerase. Assuming that no *Salmonella* -encoded trans-acting factors are required for *rfc* gene expression, it seemed likely that the non-appearance of an Rfc protein was due to low translational, rather than low transcriptional, efficiency.

Attenuation of gene expression at the translational level has been documented. Lack of homology to the consensus sequences of strongly expressed *E. coli* proteins at the ribosomal binding and initiation sites, coupled with non-optimal spacing between these two sites, has been implicated in poor translational efficiency (Gold and Stormo, 1987).

When the entire cloned *rfb* region of *S. typhimurium* was introduced into *E. coli* K-12, biosynthesis of the polymerized O-polysaccharide of *S. typhimurium* was not detected since *rfc* function was not provided by the *E. coli* strain (Brahmbhatt *et al.*, 1988). The Southern hybridization data indicated that there were no DNA sequences in *E. coli* K-12 homologous to the *rfc* gene. Since *rfc* performs no known function in *E. coli* it is possible that expression of the *rfc* gene of *S. typhimurium* is sub-optimal in *E. coli*. The hypotheses raised above are investigated further in Chapter 4 in light of the elucidation of the nucleotide and derived amino acid sequence of this gene.

Strains which displayed no homology with the *rfc* gene probe included: (serogroups and O - antigen forms in brackets) *S. strasbourg* (group D2, O 9,46) *S. newington* (group E2, O 3,15) and *E. coli* K-12. The presence of DNA regions homologous to the *rfc* gene of *S. typhimurium* was demonstrated for identical 0.4 kb *Acc*I genomic fragments of : *S. typhimurium* (group B; O 1,4,(5),12), *S. paratyphi* A (group A; O 2,12), *S. typhi* (group D1, O 1,4,12) *S. derby* (group B, O 1,4,(5),12), *S. dublin* (group D1, O 1,9,12), and *S. chester* (group B, O 4,5,12). *Eco*RV fragments of each of these strains also showed strong homology to the *Acc*I probe but inter-strain heterogeneity was observed in the size of *Eco*RV fragments displaying homology. This suggests that among *Salmonella* strains of serogroups A, B and D, the DNA region around the *rfc* gene may not be highly conserved. The *rfc* gene itself appears to be conserved and this is in line with previous work showing that hybrids

between groups B and D involving transfer of *rfb* genes were always smooth (Mäkelä, 1965; Nurminen *et al.*, 1971; Valtonen *et al.*, 1975). In contrast, hybrids in which group B *rfb* genes were transferred to a group E strain were found to be SR, implying differences between the O-antigen subunit polymerases of groups B and E (Nyman *et al.*, 1979). Similarly, hybrids in which the group B *rfb* region was transferred into *Salmonellae* of group C1 or C2, produced SR type LPS (Naide *et al.*, 1965; Mäkelä, 1966). The cloned *rfc* gene showed no homology with the genomic DNA of *S. cholerae-suis* (group C1, O 6,7), *Salmonella bonariensis* (group C2, O 6,8), or *Salmonella virginia* (group C3, O (8)) (data not shown).

# CHAPTER 4

## Nucleotide sequence analysis and genetic organization of the *rfc* gene of *Salmonella typhimurium*

## 4.1 Introduction

The nucleotide sequences of genes may provide insight into the mechanisms of expression and regulation of these genes and their gene products. The nucleotide sequence of very few genes primarily involved in LPS biosynthesis has been determined. Those genes which have been sequenced include the *S. typhimurium rfbJ* (Wyk and Reeves, 1989), and *rfbE* and *rfbS* (Verma and Reeves, 1989). Analysis of the nucleotide and predicted amino acid sequences of these genes provided an understanding of the evolution of antigenic variation within these genes in different strains of *Salmonella.*

Sequencing of the *rfc* gene was undertaken in an attempt to provide an insight into the poor expression of this gene in *E. coli* K-12 and derived systems (Chapter 3) and in order to allow a prediction of the mechanism of synthesis and assembly of a possible Rfc protein. In this chapter, the nucleotide sequence and the genetic organization of the 1.75 kb *Hind* III fragment containing the *rfc* gene is determined. A theoretical Rfc protein is postulated from the predicted amino acid sequence and some characteristics of this protein are discussed.

## 4.2 Results

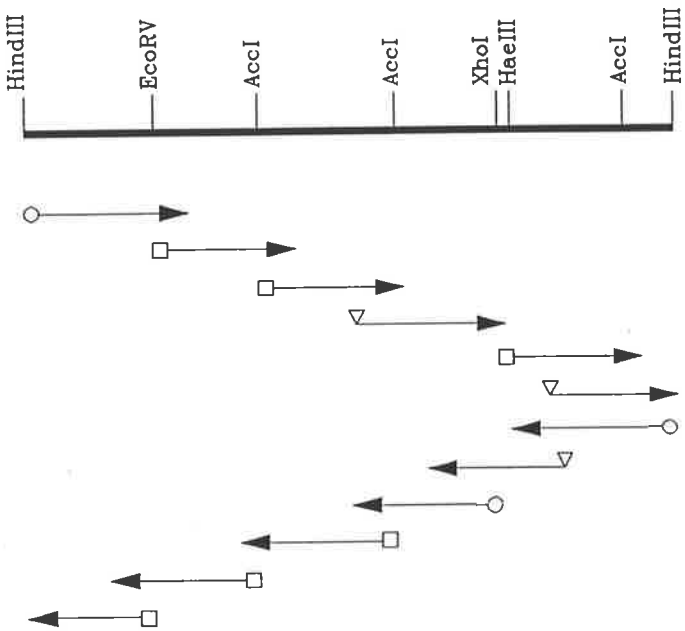
### 4.2.1 *rfc* nucleotide sequence determination and analysis

#### 4.2.1.1 DNA sequencing strategy

The entire 1.75 kb *Hind*III fragment of pADE206 was cloned in both directions into the *Hind*III site of M13mp18 (Fig. 4.1). In addition, DNA fragments of pADE206 were sub-

**Figure 4.1: Strategy used for DNA sequencing of the *rfc* gene**

Indicated restriction fragments of the 1.75 kb *Hin*dIII insert of pADE206 were subcloned in either M13mp18 or M13mp19 for dideoxy sequencing. The sequencing start point of each clone is indicated by a circle for M13mp18 derivatives and by a square for M13mp19 derivatives. The direction and extent of the sequencing carried out on each clone is represented by arrows. The DNA regions preceded by a triangle were sequenced using a specifically synthesised oligodeoxynucleotide primer.




1.75 kb HindIII
1.4 kb EcoRV–HindIII
0.4 kb AccI

0.5 kb XhoI–HindIII

1.75 kb HindIII

1.3 kb XhoI–HindIII
0.4 kb AccI
0.6 kb AccI–HindIII
0.3 kb EcoRV–HindIII

cloned in the multiple cloning site of either M13mp18 or M13mp19 (Vieira and Messing, 1982) (Fig. 4.1). The 17-mer (-20) M13 sequencing primer was employed to sequence the DNA in the M13 clones by the dideoxy method. The 17-mer (-40) M13 sequencing primer was used to determine the sequence of the 1.75 kb *Hin*dIII fragment at the junction with the polylinker in M13mp18.

Synthetic deoxyoligonucleotide sequences were used as primers to sequence DNA regions not overlapped by the M13 subclones of pADE206 (Fig. 4.1).

#### 4.2.1.2 Nucleotide sequence analysis of *rfc*

The complete nucleotide sequence of the 1750 bp *Hin*dIII fragment in pADE206 was determined in both directions (Fig. 4.2). Potential transcriptional and translational signals are described below.

Based on the analysis of conserved sequences among *E. coli* K-12 promoters, two consensus regions have been identified (Rosenberg and Court, 1979; Hawley and McClure, 1983). The "Pribnow Box" (the "-10 region") consensus is $T_{80}$ $A_{95}$ $T_{45}$ $A_{60}$ $A_{50}$ $T_{96}$, where the subscript represents the percentage occurrence of the base most frequently found at that position (Siebenlist *et al.*, 1980; Hawley and McClure, 1983). The second region is located 35 base pairs upstream of the transcriptional initiation site (the "-35 region") and is implicated in the initial recognition by RNA polymerase of the promoter site (Schaller *et al.*, 1975; Pribnow, 1979; Hawley and McClure, 1983; Studnicka, 1987). The -35 region consensus sequence is $T_{82}$ $T_{84}$ $G_{78}$ $A_{65}$ $C_{54}$ $A_{45}$ (Hawley and McClure, 1983).

**Figure 4.2: Nucleotide sequence of the 1,750 bp *Hin*dIII fragment of cloned *S. typhimurium* DNA which carries the *rfc* gene**

The nucleotide sequence from nt 1 to nt 1750 is shown in the same orientation as in Fig. 4.1. The amino acids of the major open reading frame are numbered starting at the presumed *rfc* initiation codon (ATG-Met). In addition to the ATG-Met (nt 461) start, and possible nearby S-D sequence (CTAAAGGC), two alternative potential translation initiation sites at GTG-Val (nt 641) and at ATG-Met (nt 1574), and their associated putative S-D sequences, are italicised and underlined. A nonomer is indicated as the potential S-D sequence for ATG-Met (nt 1574), as either of the contained octamers has 4/8 bp in common with the consensus S-D sequence.

The *E. coli* consensus promoter sequences for the -35 (TTGACA) and -10 (TATAAT) regions are shown for comparison below the potential *rfc* promoter sequences. A *rho* - independent transcriptional terminator region is indicated by head-to-head arrows. Two direct repeats of 10 bases each are indicated by single lines above the sequence.

A synthetic deoxyoligonucleotide homologous to the end of transposon Tn*1725* was used as a primer to sequence the DNA of transposon-bearing plasmids. The Tn*1725* insertion points are indicated by the underlining of the single bases after which the Tn*1725* insertions occurred; the plasmid numbers of the insertion-bearing plasmids are shown (see Fig. 3.3).

```
   1 AAGCTTCTATAACTAGCAACGTCTCACTTATGATGCCGTACTTGTAATTCGTCTGTGTTGCTTGA
        pADE212
  66 AGTATGTCGCGGCACGATGTGAGATGAATTACTCGTCGAGTGATAGCTCTTTCTCTGTATAGTC

 130 TTTTACAGGACTCGGGAACCTCATGATGAATCTTCGAATGCCACTGTATATAAACACAGTAACA
                                                    pADE213
 194 ATCATGTGTAATTATCAAAAGCATTTACATTGTTTGATGTTTGTGCTGCAAATGCTTGATAGCT

 258 TCATGCTATCGGAGGCGCAGTAGATAGGAGTGTTAATGAGTGTTAATATGTCAATAACACGGTT
                           TTGACA                  TATAAT
 322 TTGATATCGGGAAGATAGTGATGAGTAATGATTATAGACTACCAGATTCAGAATATCTTGCCAG
                                                              pADE217
 386 AAGATTTTCCGTACCACACCTTATTTGCCTGATGGTAATATTTTTAATACTAAGCATTTTTTCT

 450 AAAGGCTCTAT ATG CTT ATA ATT TCA TAC ATT GCA TTA TGC TTA TTA TTT
   1             Met Leu Ile Ile Ser Tyr Ile Ala Leu Cys Leu Leu Phe

 500 ATA GTT TAT CTC TAT ACT CTT TCC GTA AGA ATT GAG GGG AAA ATA ATA
  14 Ile Val Tyr Leu Tyr Thr Leu Ser Val Arg Ile Glu Gly Lys Ile Ile
       pADE216
 548 AATGTA ATG GTC CCA TAC CTG ATA ATA ACA GTC CCT ACA CTG TAC GTG
  30 Asn Val Met Val Pro Tyr Leu Ile Ile Thr Val Pro Thr Leu Tyr Val

 596 TTT GAA GGT ATA TTT GTA TAC CTC TCA GAG GTG CAG AAT TAT ACA GTG
  46 Phe Glu Gly Ile Phe Val Tyr Leu Ser Glu Val Gln Asn Tyr Thr Val

 644 GAA TAT TTG TTT TTC TAT ACT TGC TAT ATA ACA TAC ATA GCA TCA TTT
  62 Glu Tyr Leu Phe Phe Tyr Thr Cys Tyr Ile Thr Tyr Ile Ala Ser Phe

 692 GTT ATT TCT TAT CTT TAC ACA CAA AGA AAA CCC ATA TAC AAC AAA TCA
  78 Val Ile Ser Tyr Leu Tyr Thr Gln Arg Lys Pro Ile Tyr Asn Lys Ser

 740 AAC ACG AAA AAT AAA CCA AGG TAT GTG TTT ACT TCA TTG TTA TTC ACC
  94 Asn Thr Lys Asn Lys Pro Arg Tyr Val Phe Thr Ser Leu Leu Phe Thr
               pADE214                    pADE215
 788 TTC CTT GCT TTT ATC ATT TAC CTT CCA GTG TTG ATG GAG TTC AGA GAG
 110 Phe Leu Ala Phe Ile Ile Tyr Leu Pro Val Leu Met Glu Phe Arg Glu

 836 TAT ATA CTT AGC CCA AGA AGA ATA TAC GAA TTA ACC AGA ACA GGG TAT
 126 Tyr Ile Leu Ser Pro Arg Arg Ile Tyr Glu Leu Thr Arg Thr Gly Tyr

 884 GGT ATA TAC TTC TAT CCT TCA TTA ATG TTT TCT CTT GTC GCT TCT ATT
 142 Gly Ile Tyr Phe Tyr Pro Ser Leu Met Phe Ser Leu Val Ala Ser Ile

 932 TGC GCG TTC TTT ACA TAC AAA AAA TCA AAG TTA TTT TGT ATT TCC ATA
 158 Cys Ala Phe Phe Thr Tyr Lys Lys Ser Lys Leu Phe Cys Ile Ser Ile

 980 GTT TTA TTT AAC TGT ATA CTT ATT TTC TTG CAT GGT AAC AAA GGA CCA
 174 Val Leu Phe Asn Cys Ile Leu Ile Phe Leu His Gly Asn Lys Gly Pro

1028 ATA TTT AGT ATA TTT ATA GCA TTC ATC CTT TAC CTT TCA TAT ATT GAA
 190 Ile Phe Ser Ile Phe Ile Ala Phe Ile Leu Tyr Leu Ser Tyr Ile Glu

1076 AAT AAA AAA ATT AAA TTT ATG TTC CTG GTA AAA TCG TTT GCT GTT ATA
 206 Asn Lys Lys Ile Lys Phe Met Phe Leu Val Lys Ser Phe Ala Val Ile
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1124  GCA GTC ATT GTA ACG GCA TTC TTT GCA TAT ACG TTT ACT GAT GGG AAT
 222  Ala Val Ile Val Thr Ala Phe Phe Ala Tyr Thr Phe Thr Asp Gly Asn

1172  CCG ATA GAA AAT ATG GCG AAT TAC TCG GAT TAT ACC CGT AAT GCT GTT
 238  Pro Ile Glu Asn Met Ala Asn Tyr Ser Asp Tyr Thr Arg Asn Ala Val

1220  CTT GTT GCT TCC TCA AAC TTT GAC TTT ATG TAC GGA AAA TTA CTA ATG
 254  Leu Val Ala Ser Ser Asn Phe Asp Phe Met Tyr Gly Lys Leu Leu Met

1268  GAA AGC GAG GTT TAC TCG AGG ATT CCG AGG GCT ATT TGG CCT GAT AAG
 270  Glu Ser Glu Val Tyr Ser Arg Ile Pro Arg Ala Ile Trp Pro Asp Lys

1316  CCT GAA GAT TTT GGC GCA TTG TAT CTG GCA AAA GTA TTT TTC CCT GAT
 286  Pro Glu Asp Phe Gly Ala Leu Tyr Leu Ala Lys Val Phe Phe Pro Asp

1364  GCA TTC TAC AGA AAT CAG GGC GCT CCT GCT TTC GGG TAT GGT GAA CTA
 302  Ala Phe Tyr Arg Asn Gln Gly Ala Pro Ala Phe Gly Tyr Gly Glu Leu

1412  TAC GCA GAT TTC GGG CTT TTT ACA CCA GTT TGG TTA GTT ATA TCT GGA
 318  Tyr Ala Asp Phe Gly Leu Phe Thr Pro Val Trp Leu Val Ile Ser Gly

1460  GTA TTT AAA GGC GTC CTA GCT AAG TAT TTC TCC AAT AAA ACT CAG GAA
 334  Val Phe Lys Gly Val Leu Ala Lys Tyr Phe Ser Asn Lys Thr Gln Glu

1508  ACA AAG TCA GCG CAT TAT TTC ATA ATG TTC CTA TTT TGC ATT GGA ATA
 350  Thr Lys Ser Ala His Tyr Phe Ile Met Phe Leu Phe Cys Ile Gly Ile

1556  AGT GTG ATT CCT GTT AGC ATG GGA TGG TTG TTC CCT GAG CAT TTG ATG
 366  Ser Val Ile Pro Val Ser Met  Gly Trp Leu Phe Pro Glu His Leu Met

1604  ATT GCT TTT ATG GTA TAC ATT GCA TCT TCC TTT GTT TTT TCA GAG CAT
 382  Ile Ala Phe Met Val Tyr Ile Ala Ser Ser Phe Val Phe Ser Glu His

1652  ATA AGA TTC GTT TTA CTA AGA AAC AAT AAA TAAGTTATACGGCGGCAATGCCGC
 398  Ile Arg Phe Val Leu Leu Arg Asn Asn Lys ***     --------> <-----

1706  CGTTTTATTTGGCTCTGAAGCGTAAAAATTGTTCCTGTAAAGCTT 1750
      ---            ***
```

Base-specific mutations in these hexamers have marked effects on promoter function (Rosenberg and Court, 1979; Hawley and McClure, 1983). In general, base changes which increase homology to the -35 consensus result in promoter-up mutations, while base changes which decrease homology to the -35 consensus correspond to promoter-down mutations (Hawley and McClure, 1983).

The spacing between the -10 and -35 regions has been implicated as a factor in the determination of promoter strength . The optimal spacing was deduced to be 17 +/- 1 nt, within spacing limits of 15 to 21 nt (Rosenberg and Court, 1979; Hawley and McClure, 1983). Sequences outside these regions may also have significant impact on promoter activity, therefore care must be exercised in estimation of promoter strength based on homology to the consensus sequences and spacing between -10 and -35 regions (Deuschle *et al.*, 1986). The highly efficient promoters of coliphages T2 and T7, for example, also show homology around position -43 and between +1 and +20 (Gentz and Bujard, 1985; Kammerer *et al.*, 1986).

Analysis of the present nucleotide sequence revealed a potential promoter structure between nt 278 and nt 306 (Fig. 4.2). Within this segment, a potential -10 region reads TAatAT (4 of 6 bases identical to the consensus TATAAT) and a potential -35 region reads TaGAtA (4 of 6 bases identical to the consensus TTGACA). The spacing between these putative -10 and -35 sequences is 17 bp, which is optimum for promoter activity (Fig. 4.2). The spacer sequence between the -10 and -35 regions displays two direct repeats of the 10 bp series GAGTGTTAAT, the second of which overlaps the putative -10 region (Fig. 4.2).

A ribosome binding site (RBS) for translation initiation consists of a region, known also as the Shine-Dalgarno (S-D) sequence, upstream of the start codon (usually AUG), which

displays homology with the free 3' end of the 16S RNA (Shine and Dalgarno, 1974). Deviations from the S-D consensus sequence AAGGAGGU reduce the level of translation initiation (Gold *et al.*, 1981; Kozak, 1983). In addition , the spacing between the S-D sequence and the start codon is optimal for initiation at 7 nt, with spacings of less than 5 nt or greater than 9 nt being rare.

A potential open reading frame (ORF), ORF1, with the translational start codon ATG at nt 461 is preceded by the potential RBS (ctaaAGGc) at a spacing of 5 nt. A second possible ORF (ORF2) starts with GTG at nt 641 and is preceded by a putative RBS (gtGcAGaa) at a spacing of 7 nt (Fig. 4.2). ORF1 and ORF2 read in the same frame and are predicted to encode proteins of $M_r$ 47,472 and $M_r$ 40,511 respectively. Since ATG start codons are, in general, more efficient at initiating translation than GTG start codons (Gold and Stormo, 1987), the $M_r$ 47,472 protein would in theory be produced preferentially.

#### 4.2.1.3 Location of the *rfc*::Tn*1725* insertions, and Tn*1725* insertions not inactivating *rfc*

A synthetic 20-mer deoxyoligonucleotide (GCTGTCACGAGAACACCGTT) with homology to the end of transposon Tn*1725* was used as a primer to sequence the site of Tn*1725* insertion in plasmids pADE212, pADE213, pADE214, pADE215, pADE216 and pADE217. The precise location of each insertion is indicated in Fig. 4.2.

#### 4.2.1.4 Transcriptional termination

Transcriptional termination occurs at inverted repeat sequences with the ability to form stable base-paired stem-and-loop structures, and may be either *rho* -independent or

-dependent (Rosenberg and Court, 1979). Two other features which are characteristic of efficient terminators are: (a) a stretch of U residues at the terminus of the RNA transcript, and (b) G/C rich sequences preceding the transcriptional stop site. In the *rfc* gene sequence from nt 1690 to nt 1708 a region of hyphenated dyad symmetry (inverted repeat sequence) exists with the potential to form a stem and loop structure with a free energy of -17.9 kCal/mol (Tinoco et al., 1973), which is energetically favourable for formation (Fig. 4.3). This region is rich in G/C sequences which may stabilise the stem-and-loop structure and which may aid in transcription termination by impeding the RNA polymerase (Gilbert, 1976). RNA transcripts usually end in a run of 4-8 U residues, which aid in release of the transcript, ending 20 (+/- 4) nucleotides beyond the centre of dyad symmetry (Rosenberg and Court, 1979). The proposed *rfc* transcriptional terminator has runs of 4 and 3 U residues after the stem-and-loop (Fig 4.3).These results suggest that transcription of the *rfc* gene undergoes *rho* -independent termination within this region.

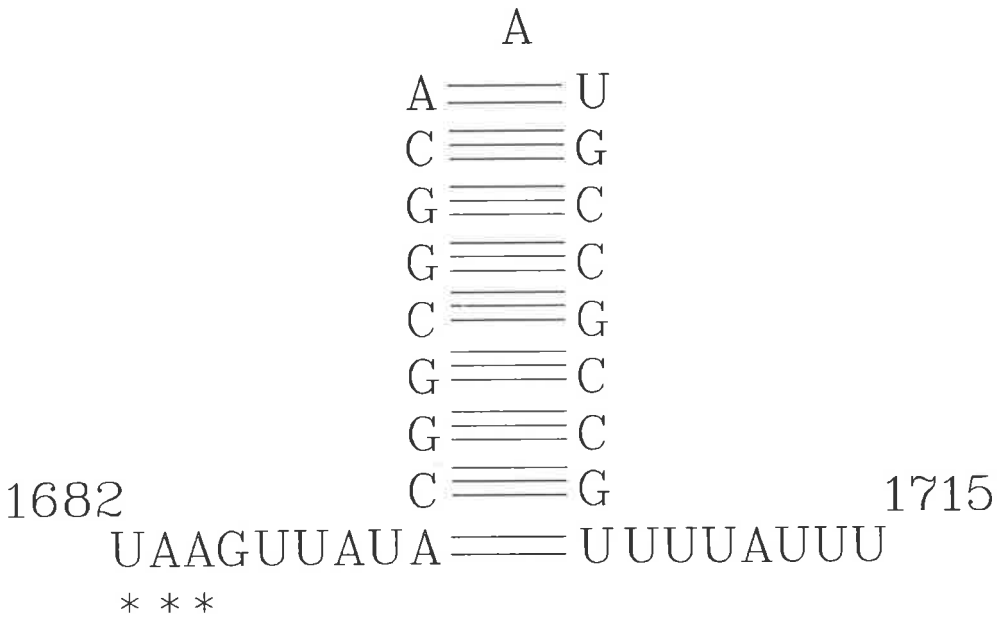
#### 4.2.1.5 Codon and base usage

In what follows, the *rfc* gene is assumed to run from nt 461 to nt 1681 (Fig. 4.2).

For most amino acids, although there are several different codon choices, the codon usage is non-random. In the case of highly-expressed genes, codon usage is biased towards those codons which correspond to the most abundant tRNA species (Ikemura, 1981). Ikemura and Ozeki (1982) have proposed a set of rules which attribute the non-random choices of synonymous codons to differences in populations of iso-accepting tRNA species, and to varying levels of modified nucleotides at the anticodon wobble position. The synonymous codon choices have significant impact on the expressivity of individual genes. Codons preferred by individual amino acids are termed "optimal codons" and should be the more efficiently utilised by the translational machinery. The use of non-

**Figure 4.3: Putative transcriptional terminator of the *rfc* gene**

A typical *rho*-independent terminator exists at the end of the *rfc* gene, from nt 1682-1715. This stem-and-loop structure has a potential free energy of -17.9 kCal/mol (Tinoco *et al.*, 1973). The UAA translational termination codon is indicated by asterisks.


A
A U
C G
G C
G C
C G
G C
G C
C G
1682
1715
UAAGUUAUA UUUUAUUU
* * *

optimal codons requires the presence of minor species of isoaccepting tRNAs which increases the energy requirements of translation.

It is possible to quantitatively examine the relationship between the frequency of optimal codon usage ($F_{op}$) and to relate this value to gene expressivity (Ikemura and Ozeki ,1982). The $F_{op}$ values for several *E. coli* genes have been determined and found to correlate well with the production level of the encoded protein (Ikemura and Ozeki, 1982).

Grosjean and Fiers (1982) proposed that efficient translation was facilitated by the selection of specific synonymous codon choices which promoted codon-anticodon interactions of intermediate strength over those with very strong or very weak interaction energy. A group of "modulating" codons corresponding to minor or weakly interacting tRNAs was evident at lower frequencies in strongly expressed genes than in weakly expressed genes, suggesting that regulation of translation occurred in weakly expressed genes by stalling of the translational machinery at these codons (Grosjean and Fiers, 1982). The codons AUA (for isoleucine), and CGG/AGA/AGG (for arginine) were considered modulating codons since their cognate tRNAs were very minor species in *E. coli*. In addition, CUA (for leucine), CGA (for arginine) and GGA/GGG (for glycine) were also considered as modulating codons since they were found in abundance in weakly expressed genes and not found in appreciable numbers in strongly expressed genes (Grosjean and Fiers, 1982).

The codon usage table for the *rfc* gene (Table 4.1) shows that modulating codons (indicated by asterisks in Table 4.1) account for 12.5% of the total codon content, with 51 of the 407 aa residues encoded in this manner. The $F_{op}$ value for the *rfc* gene was found

## Table 4.1: Codon usage within the *rfc* gene

| CODON | CT | %AGE | CODON | CT | %AGE | CODON | CT | %AGE | CODON | CT | %AGE |
|---|---|---|---|---|---|---|---|---|---|---|---|
| TTT-Phe | 27 | 6.6% | TCT-Ser | 5 | 1.2% | TAT-Tyr | 18 | 4.4% | TGT-Cys | 2 | 0.5% |
| TTC-Phe | 19 | 4.7% | TCC-Ser | 5 | 1.2% | TAC-Tyr | 18 | 4.4% | TGC-Cys | 4 | 1.0% |
| TTA-Leu | 11 | 2.7% | TCA-Ser | 11 | 2.7% | TAA-*** | 0 | 0.0% | TGA-*** | 0 | 0.0% |
| TTG-Leu | 7 | 1.7% | TCG-Ser | 3 | 0.7% | TAG-*** | 0 | 0.0% | TGG-Trp | 3 | 0.7% |
| CTT-Leu | 12 | 2.9% | CCT-Pro | 8 | 2.0% | CAT-His | 4 | 1.0% | CGT-Arg | 1 | 0.2% |
| CTC-Leu | 2 | 0.5% | CCC-Pro | 1 | 0.2% | CAC-His | 0 | 0.0% | CGC-Arg | 0 | 0.0% |
| CTA-Leu* | 5 | 1.2% | CCA-Pro | 6 | 1.5% | CAA-Gln | 1 | 0.2% | CGA-Arg* | 0 | 0.0% |
| CTG-Leu | 4 | 1.0% | CCG-Pro | 2 | 0.5% | CAG-Gln | 3 | 0.7% | CGG-Arg* | 0 | 0.0% |
| ATT-Ile | 17 | 4.2% | ACT-Thr | 5 | 1.2% | AAT-Asn | 11 | 2.7% | AGT-Ser | 2 | 0.5% |
| ATC-Ile | 2 | 0.5% | ACC-Thr | 3 | 0.7% | AAC-Asn | 6 | 1.5% | AGC-Ser | 3 | 0.7% |
| ATA-Ile* | 24 | 5.9% | ACA-Thr | 9 | 2.2% | AAA-Lys | 17 | 4.2% | AGA-Arg* | 9 | 2.2% |
| ATG-Met | 12 | 2.9% | ACG-Thr | 3 | 0.7% | AAG-Lys | 4 | 1.0% | AGG-Arg* | 3 | 0.7% |
| GTT-Val | 12 | 2.9% | GCT-Ala | 10 | 2.5% | GAT-Asp | 6 | 1.5% | GGT-Gly | 4 | 1.0% |
| GTC-Val | 5 | 1.2% | GCC-Ala | 0 | 0.0% | GAC-Asp | 1 | 0.2% | GGC-Gly | 3 | 0.7% |
| GTA-Val | 8 | 2.0% | GCA-Ala | 11 | 2.7% | GAA-Glu | 9 | 2.2% | GGA-Gly* | 5 | 1.2% |
| GTG-Val | 6 | 1.5% | GCG-Ala | 3 | 0.7% | GAG-Glu | 7 | 1.7% | GGG-Gly* | 5 | 1.2% |

CT: number of times a given codon used; %age: percentage of total codons.
Modulating codons (see the text) are marked with a single asterisk.

to be 0.42 (Table 4.2). This value places the *rfc* gene in the range of poorly expressed genes (Ikemura and Ozeki, 1982).

The G+C content of the 1750 bp *Hin*dIII fragment was 34.9 %, and of the putative Rfc-encoding region was 33.5%. Compared with the average G+C content of *Salmonella* strains, 52%, the *rfc* gene is extremely A+T rich. The %A+T in the *rfc* gene promoter (nt 278 to 460) region is 67.2%. A zone of exceptionally high A+T content (78%) exists between nt 420 and the start codon at nt 461. It is possible that this region represents a site for the the binding of regulatory proteins.

### 4.2.2 The putative Rfc protein

In the following sections the $M_r$ 47,472 protein is regarded as the Rfc protein, for the reasons given earlier.

#### 4.2.2.1 Hydropathic profile of the Rfc protein

The hydropathic plot of Rfc (Fig. 4.4), according to the methods of Kyte and Doolittle (1982), indicated that the overall protein was hydrophobic in character with a mean index of + 0.7. Many alternating hydrophobic-hydrophilic domains are indicative of spatial phase transitions for various regions of the protein.

#### 4.2.2.2 Predicted secondary structure of the Rfc protein

The secondary structure of the 407 aa Rfc (Fig. 4.5) was predicted using the methods of Chou and Fasman (1974, 1974a, 1978). The structure embodies a predominance of β-sheet structure and β-turn-β segments with very few α -helical sections.

**Table 4.2: $F_{op}$ calculation for *rfc***

| AMINO ACID | OPTIMAL CODON | NO. | NON-OPTIMAL CODON | NO. |
|---|---|---|---|---|
| PHE | UUC | 19 | UUU | 27 |
| TYR | UAC | 18 | UAU | 18 |
| LEU | CUG | 4 | CUU,CUC,CUA,UUA,UUG | 37 |
| PRO | CCG | 2 | CCU,CCC,CCA | 15 |
| ARG | CGU,CGC | 1 | CGA,CGG,AGA,AGG | 12 |
| GLN | CAG | 3 | CAA | 1 |
| ILE | AUC | 2 | AUU,AUA | 41 |
| ASN | AAC | 6 | AAU | 11 |
| LYS | AAA | 17 | AAG | 4 |
| VAL | GUU,GUA,GUG | 26 | GUC | 5 |
| ALA | GCU,GCA,GCG | 24 | GCC | 0 |
| GLY | GGU,GGC | 7 | GGA,GGG | 10 |
| GLU | GAA | 9 | GAG | 7 |
| THR | ACU,ACC | 8 | ACA,ACG | 12 |
| | TOTAL | 146 | | 200 |

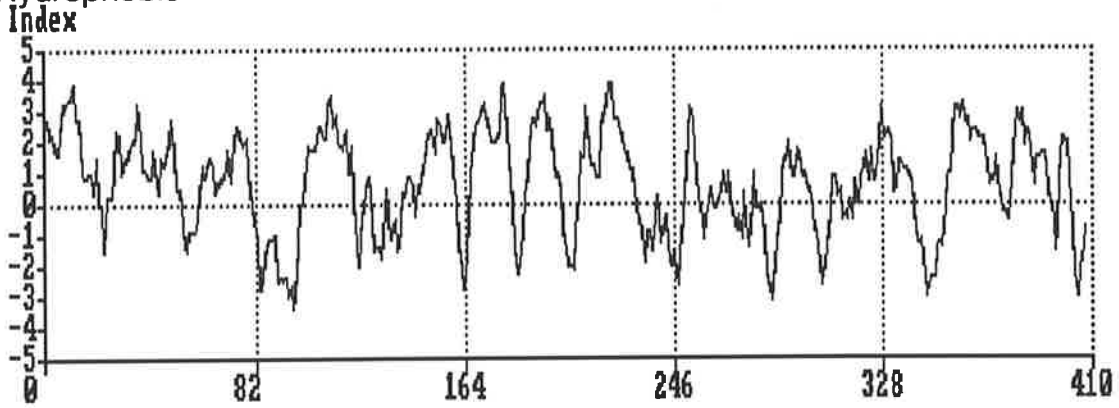
$$F_{op}\, rfc = 0.42$$

**Figure 4.4 : Hydropathic plot of the Rfc protein**

The 407 aa sequence of the predicted Rfc protein was analyzed according to Kyte and Doolittle (1982) using a window of 9 aa. The average hydropathic index for this protein is 0.7.

**HYDROPATHIC PLOT**
***Salmonella* RFC PROTEIN**

Hydrophobic




Hydrophilic

Amino Acid Number

**Figure 4.5: Predicted secondary structure of the Rfc protein**

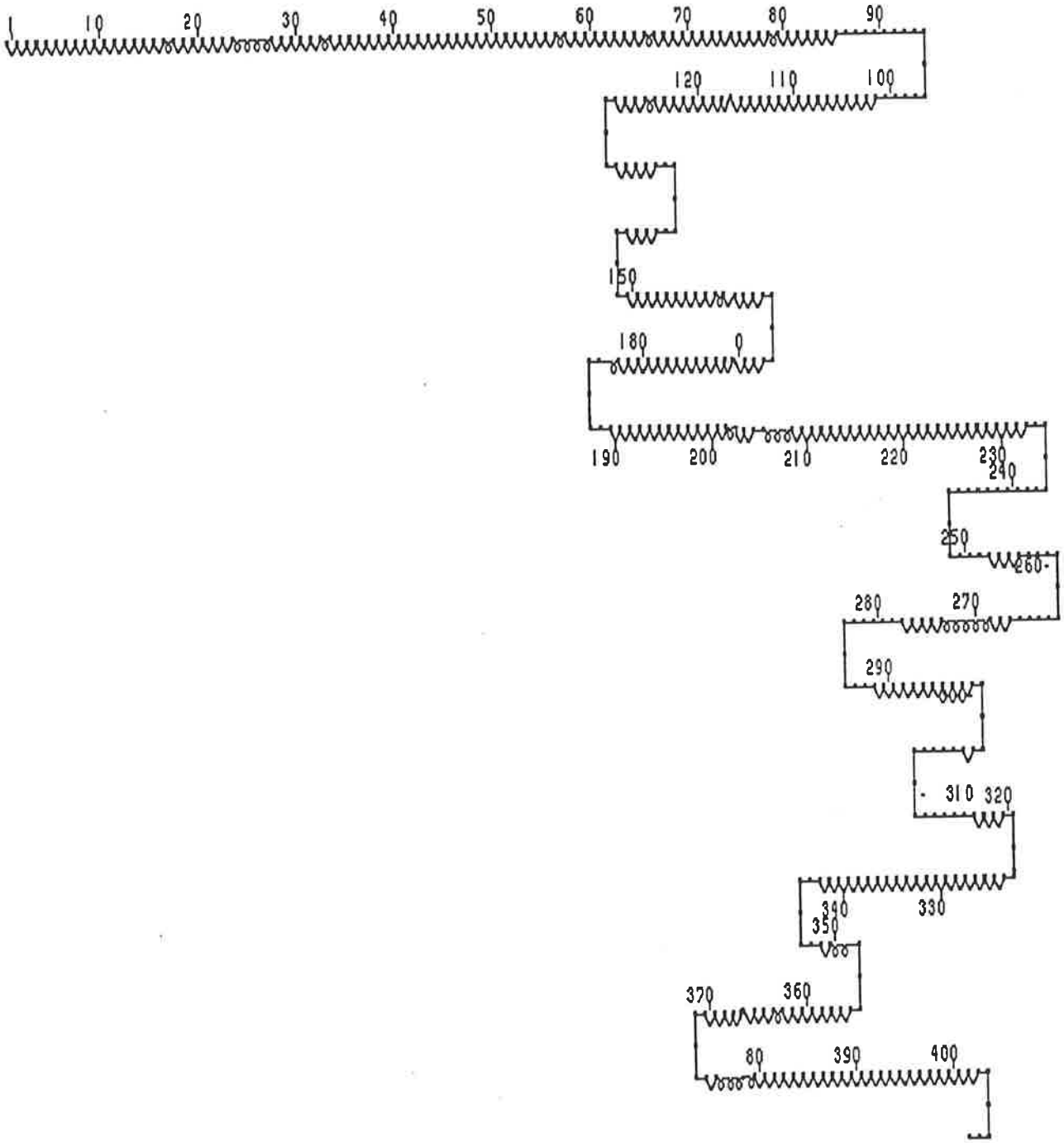
The 407 aa sequence of the Rfc protein was analyzed using the algorithm of Chou and Fasman (1978). The various domains are illustrated as follows:

α - Helix : 

β- Sheet : 

β - Turn : 

Random Coil : 


1
10
20
30
40
50
60
70
80
90
120
110
100
150
180
0
190
200
210
220
230
240
250
260
280
270
290
310
320
340
330
350
370
360
380
390
400

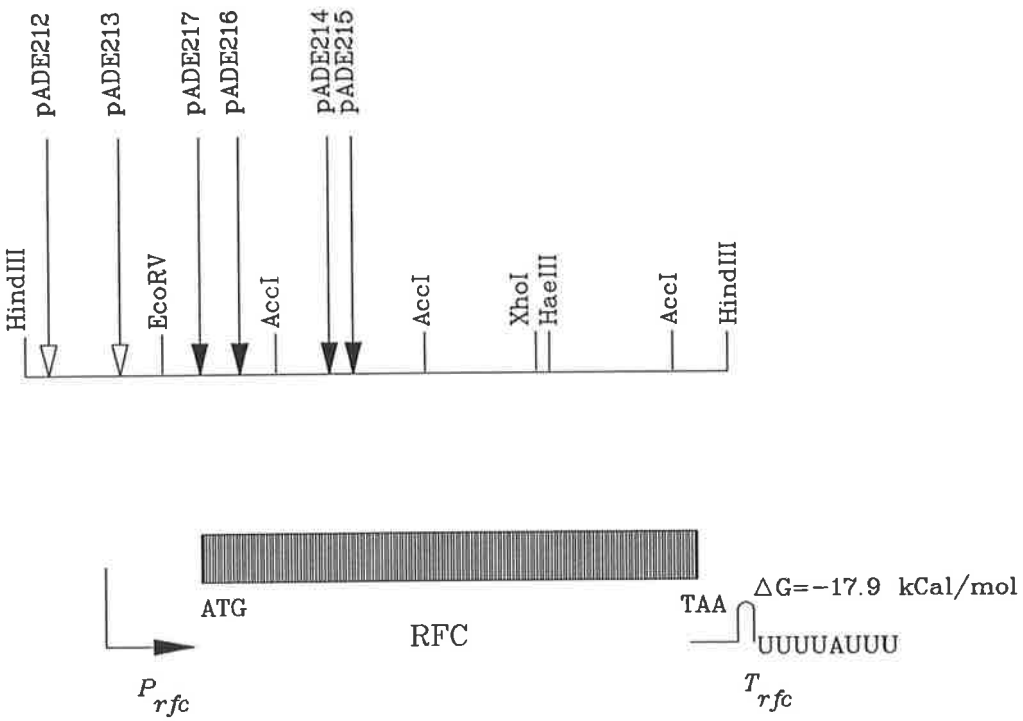
## 4.3 Discussion

A summary of the genetic organization of the *rfc* gene is given in Fig. 4.6. The salient features of the the sequenced 1.75 kb *Hin*dIII fragment include: (A): a promoter region with good homology to *E. coli* K-12 consensus sequences; (B): a potentially strong stem-and-loop structure which probably acts as a transcriptional terminator; (C): definition of the genetic unit boundaries by location of Tn*1725* insertions, and (D): a potential ORF of 407 aa which is proposed to represent the Rfc protein (Fig 4.6). Computer homology searches of both the nt and aa sequences, using the Genbank and EMBL Gene / Protein Sequence Database, failed to uncover any prokaryotic genes with significant homology to *rfc*.

Analysis of the codon usage in the *rfc* gene revealed a large proportion of modulating codons. The frequency of modulating codon usage for the strongly expressed ribosomal proteins (combined sequences of *rpoA*, *rpoB*, *rpoD* and part of *rpoC* ) was 0.65% (15/2274), which contrasted with a frequency for the weakly expressed repressor proteins (combined sequences of *lacI*, *trpR*, *araC* and the Tn*3* -coded repressor) of 6.45% (59/915) (derived from the data of Grosjean and Fiers, 1982). The corresponding frequency for the *rfc* gene was 12.5%, suggesting that translational attenuation of gene expression is likely. The prediction of poor expression may explain the inability to visualise an Rfc protein in the experiments described in Chapter 3. Constitutive low levels of polymerase production may be a strategy employed by enteric bacteria to regulate the synthesis of high molecular weight LPS. Production of long-chain LPS has been shown to be temperature- and growth rate-dependent; regulation of polymerase activity is therefore plausible.

**Figure 4.6: Genetic organization of the 1,750 bp *Hin*dIII *rfc*+ fragment of cloned *S. typhimurium* DNA**

A partial restriction map of the 1,750 bp *Hin*dIII fragment of pADE206, which encodes the proposed 407 aa Rfc protein (hatched area), is shown. The rightward arrow indicates the proposed site and direction of the *rfc* gene promoter ($P_{rfc}$), which precedes the ATG start codon. The stem-and-loop structure incorporating the TAA stop codon, and the succeeding stretch of uridine residues, constitutes the *rfc* gene terminator ($T_{rfc}$). Downward arrows indicate the sites at which Tn*1725* inserts were located by sequencing in derivatives of pADE206. Filled arrowheads: insertions inactivating *rfc*; empty arrowheads: insertions not inactivating *rfc*. The designations of the transposon-bearing plasmids are shown.


pADE212
pADE213
pADE217
pADE216
pADE214
pADE215
HindIII
EcoRV
AccI
AccI
XhoI
HaeIII
AccI
HindIII
ATG
RFC
TAA
ΔG=−17.9 kCal/mol
UUUUAUUU
$P_{rfc}$
$T_{rfc}$

The G+C content of the *Enterobacteriaceae* ranges from 38% to 60%. Variations in G+C content in different bacterial species are thought to be due to differences in the mutation rates of (A.T) to (G.C), compared with the mutation rates of (G.C) to (A.T) (where (A.T) refers to an AT or TA base pair and (G.C) refers to an GC or CG base pair)(Sueoka, 1988). Localized high A+T content has been observed in several genes. The highly expressed *E. coli* K-12 *lpp* gene for lipoprotein has a DNA stretch of 70% A+T content for 261 bp preceding the transcriptional start site, in contrast to an A+T content of 53% for the *lpp* mRNA region and an average A+T content of 49% for *E. coli* K-12 (Nakamura and Inouye, 1979). The *E. coli* K-12 *ompF* gene has a similar 69% A+T region in an area before the transcriptional start, which decreases to 57% A+T in the mRNA coding region (Inokuchi *et al.*, 1982). A+T rich regions, such as those described above in the vicinity of gene promoters, are considered to play a role in helix structure destabilization, as a prelude to the RNA polymerase-mediated unwinding necessary for transcriptional initiation. The A+T richness of the *rfc* gene, however, prevails throughout the promoter and protein-encoding region. Similar A+T richness has been observed for the *S. typhimurium rfbJ, rfbE,* and *rfbS* genes; it has been proposed that these genes evolved outide of the species *Salmonella* (Verma and Reeves, 1989).

The presence of several hydrophobic domains, interposed with hydrophilic stretches, in the predicted secondary structure of the Rfc protein (Fig. 4.4) is indicative of transmembrane domains. These regions are composed almost exclusively of β-sheet structure with several β-turn-β domains. The most common configuration of transmembrane protein is that of hydrophobic α-helical bundles. However, bacterial porins, which form aqueous channels in the outer membrane, are made up of an assembly of β-sheets (Popot and Engelman, 1990). A macromolecular structure consisting of parallel interacting transmembrane β-sheets is conceivable for the Rfc protein but is highly speculative until the the existence and cellular localization of the protein is confirmed.

Identification of the O-antigen polymerase may be possible by using antibodies raised against a synthetic peptide, the primary sequence of which would be taken from the predicted amino acid sequence of the *rfc* gene. The cellular location of the Rfc protein could also be elucidated, using these antibodies.

Polymerization of O-antigen units is thought to occur *via* membrane-bound enzymes at the inner membrane (Osborn *et al.*, 1972a, 1972b). An understanding of the Rfc protein topology is fundamental to the understanding of the possible roles this protein may play in both polymerization and translocation. A model for LPS synthesis and assembly has been proposed where synthesis of core LPS and O-antigen units takes place initially at the cytoplasmic face of the inner membrane, with both components then transposed to the periplasmic face of the inner membrane, where polymerization and attachment of O-units to core may occur (Mulford and Osborn, 1983). Translocation of completed LPS to the outer membrane would then occur at zones of adhesion between the inner and outer membranes. In support of this model was the finding that in an LPS core-deficient mutant, undecaprenol-linked O-antigenic polysaccharide was detected on the periplasmic face of the inner membrane (Mulford and Osborn, 1983). The Rfc protein may therefore be multi-functional, being involved in O-unit polymerization and translocation.

# CHAPTER 5

## Molecular cloning and characterization of the phosphomannose isomerase gene of *Salmonella typhimurium*

## 5.1 Introduction

The enzyme phosphomannose isomerase converts fructose-6-phosphate to mannose -6-phosphate in a step intermediate in the production of capsular polysaccharide (in *E. coli* K-12), alginate (*Pseudomonas aeruginosa* ) and LPS O-antigen (*S. typhimurium* ). Mannose-negative mutants, which were unable to synthesize the enzyme, and made no capsular polysaccharide when grown on glucose-containing media, had mutations which mapped between the *his* and *trp* markers on the *E. coli* K-12 chromosome (Markovitz *et al.*, 1967). Pmi$^-$ mutants of *E. coli* K-12 were characterized by an inability to grow on mannose as a sole source of carbon and by the absence of colanic acid production. The *manA* gene for the Pmi protein mapped at position 35.5 on the *E. coli* K-12 linkage map (Bachmann, 1983).

Darzins *et al.* (1985) cloned the *E. coli* K-12 *manA* gene on a 1.5 kb *Hin*dIII - *Hpa* I fragment encoding a protein of $M_r$ 45,000, which restored alginate production in an Alg$^-$ mutant of *P. aeruginosa*. The *manA* gene has been sequenced and the size of the Pmi monomer more accurately deduced as being of $M_r$ 42,716 (Miles and Guest, 1984a).

Production of GDP-mannose is essential to the biosynthesis of the O-antigen repeat units of *S. typhimurium* ( Mäkelä and Stocker, 1984). Mutants of *S. typhimurium* defective in Pmi protein synthesis displayed an altered LPS phenotype, with a phage sensitivity pattern characteristic of SR mutants, when grown in mannose-free medium (Wilkinson *et al.*,1972). When exogenous mannose was supplied, the mutants became phenotypically smooth, indicating that the abilities to transport and utilise mannose had not been impaired (Rosen *et al.*, 1965).

This Chapter describes the molecular cloning, and the genetic nature and organization, of the *S. typhimurium* Pmi protein-encoding region. In addition, the size of the *Salmonella*

Pmi protein is estimated, and the cloned material is used to probe total DNA from a variety of *Salmonella* strains. The gene cloned here will be referred to as $pmi_{stm}$, although initial experiments involved complementation of an *E. coli* K-12*manA* mutant with the *S. typhimurium* material.

## 5.2 Results

### 5.2.1 Cosmid cloning of $pmi_{stm}$

One cosmid, from a genomic library of *S. typhimurium* strain C5 in the vector pHC79, appeared to contain the $pmi_{stm}$ gene based on its ability to restore the $Man^+$ phenotype to the *E. coli* K-12 *manA* strain CD1. To facilitate gene manipulation and generation of a restriction fragment map, the cosmid was reduced in size by limited, *Sau* 3A1-generated, deletion. One of these deletions, pADE249 (11.5 kb), retained $Man^+$ complementing ability in CD1 and consisted of four *Hin*dIII fragments of 5.6, 2.9, 1.9 and 1.1 kb (Fig. 5.1). Partial *Hin*dIII deletion of pADE249 produced plasmids pADE250, pADE251 and pADE252, all of which were $Man^+$ (Fig 5.1). The 5.6 kb *Hin*dIII fragment of pADE250 represented the smallest of the cutdowns, consisting of some of the original insert DNA and part of the pHC79 cosmid vector. When pADE250 was transformed into CD5 (a *manA*, *cap* mutant of *E. coli* ), the $Man^+$ phenotype and production of capsular polysaccharide (mucoid colony appearance on mannose-free medium) were observed. Transformation of pADE250 into the *S. typhimurium pmi rfc* double mutant SL1101 (Wilkinson *et al.*, 1972) restored the ability of the strain to ferment mannose and allowed the strain to grow on mannose as a sole source of carbon ($Pmi^+$ phenotype). In addition, the SL1101/pADE250 recombinant, in the absence of exogenous mannose, had a phage efficiency of plaquing profile characteristic of SR strains (i.e. $9NA^R$, $P22^R$, $FO^S$, and $P22I^S$)(Table 5.1). In silver-stained SDS-PAGE, the LPS of SL1101 grown without added mannose (Fig. 5.2; lane 1) was shorter than that of the same strain grown with mannose (Fig. 5.2; lane 2). A small amount of R-type LPS was also apparent in lane 2.

**Figure 5.1: Plasmid pADE249, and *Hin*dIII-generated deletions thereof**

A restriction map of pADE249 is shown. Heavy lines below the restriction map indicate the amount of DNA of pADE249 retained in the indicated plasmids after partial *Hin*dIII digestion and religation. All of the plasmids shown expressed the $pmi_{stm}$ gene.

▬▬ in pADE249 : pHC79 DNA.

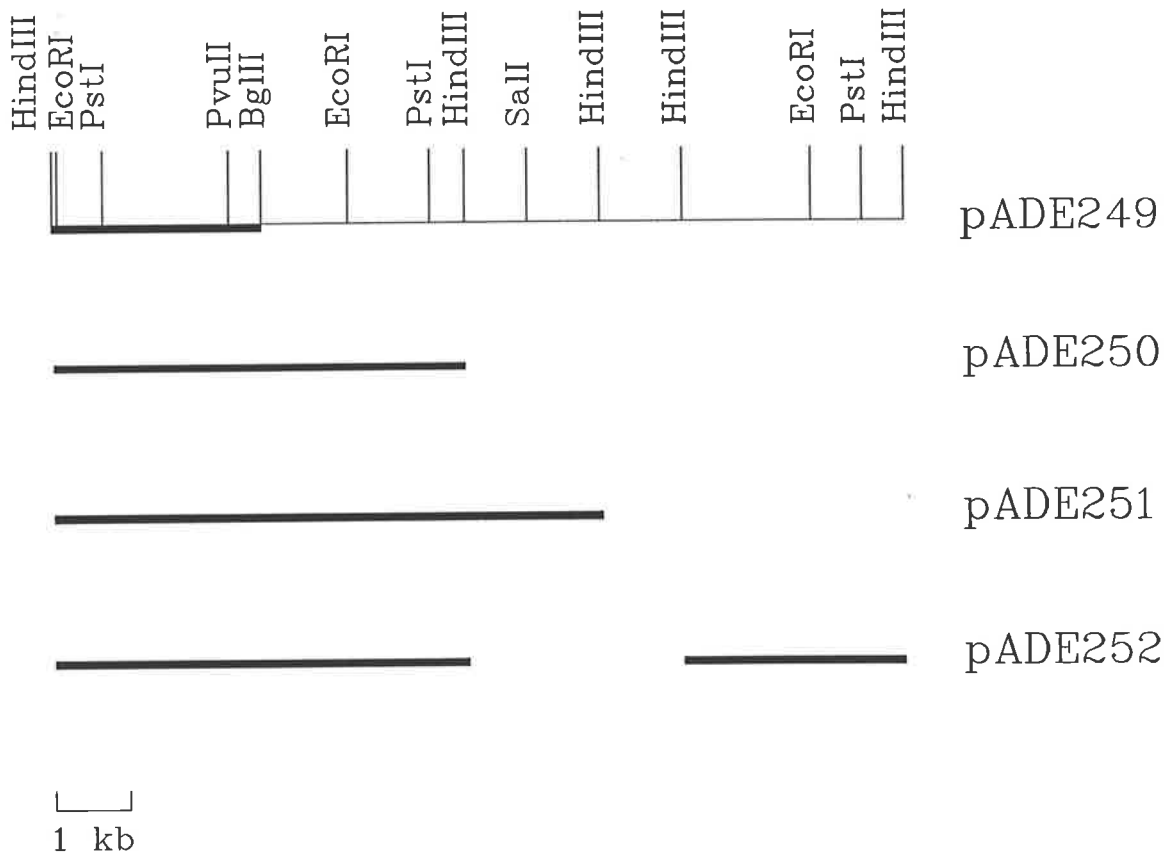

HindIII
EcoRI
PstI
PvuII
BglII
EcoRI
PstI
HindIII
SalI
HindIII
HindIII
EcoRI
PstI
HindIII
pADE249
pADE250
pADE251
pADE252
1 kb

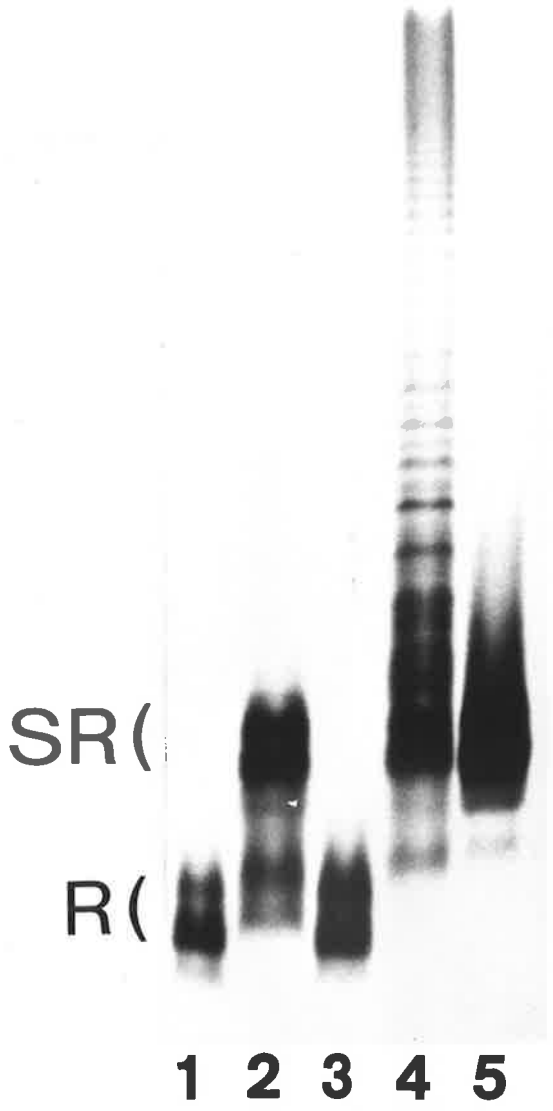
**Table 5.1: The efficiency of plaquing of various bacteriophage on various strains**

| Strain | Phage | | | |
|---|---|---|---|---|
| | P22 | 9NA | FO | P221 |
| C5 | 1 | 1 | 1 | $<0.25X10^{-6}$ |
| SL1101, glu | $<0.25X10^{-8}$ | $<0.25X10^{-8}$ | $<0.25X10^{-6}$ | 1 |
| SL1101, glu, man | $<0.25X10^{-8}$ | $<0.25X10^{-8}$ | 3 | 1 |
| SL1101/ pHC79, glu | $<0.25X10^{-8}$ | $<0.25X10^{-8}$ | $<0.25X10^{-6}$ | 1 |
| SL1101/ pHC79, glu, man | $<0.25X10^{-8}$ | $<0.25X10^{-8}$ | 3 | 1 |
| SL1101/ pADE250, glu | $<0.25X10^{-8}$ | $<0.25X10^{-8}$ | 3 | 1 |
| SL1101/ pADE250, glu, man | $<0.25X10^{-8}$ | $<0.25X10^{-8}$ | 3 | 1 |

Bacteria, grown either in NB (C5), or in NB with either glucose alone (glu), or glucose and mannose (glu, man) were mixed with phage at various dilutions, and plaques counted after overnight incubation. The efficiencies of plaquing of P22, 9NA, and FO on C5 were taken as unity (underlined above). Similarly, the titre of P221 on glucose-grown SL1101 was the reference efficiency of plaquing for this phage (underlined above).

**Figure 5.2: LPS profiles of various strains in silver-stained SDS-PAGE**

Bacteria were lysed with SDS, treated with Proteinase K, and residual material analysed by SDS-PAGE, followed by LPS-specific silver staining. Lane designations (with relevant genotypes in square brackets, and growth conditions in round brackets): 1: SL1101 [*pmi, rfc* ] (grown in 0.5% glucose); 2: SL1101 [*pmi, rfc* ] (grown in 0.5% glucose plus 0.5% mannose); 3: SL1101/pHC79 [*pmi, rfc* ] (grown in 0.5% glucose); 4: C5 [wild-type](grown in 0.5% glucose); 5: SL1101/pADE250 [*rfc* ] (grown in 0.5% glucose). "R" denotes the location of rough (core) LPS, "SR" denotes the location of semi-rough LPS.


SR(
R(
1 2 3 4 5

The LPS of SL1101/pADE250 (grown without mannose) (Fig. 5.2; lane 5) is longer than that of SL1101/pHC79 (grown without mannose) (Fig. 5.2; lane 3) and is indicative of SR LPS, being intermediate between the R LPS of SL1101 (Fig. 5.2; lane 1) and the S LPS of C5 (Fig. 5.2; lane 4). The transition from R LPS synthesis (SL1101) to SR-type LPS production (SL1101/pADE250) is more complete than that seen by growth of SL1101 in mannose, suggesting that addition of mannose to SL1101 does not fully neutralise the Pmi phenotypic defect of the strain, as it affects LPS production.

### 5.2.2 Transposon mutagenesis of pADE250

Transposon Tn*1725* (Ubben and Schmitt, 1986) insertions in plasmid pADE250 were isolated (Materials and Methods). Individual derivatives of pADE250 carrying Tn*1725* insertions were transformed into CD1 and screened for the $Man^+$ phenotype on mannose-tetrazolium plates. The Tn*1725* -containing plasmids pADE258, pADE259 and pADE260 all gave a $Man^-$ phenotype whereas the plasmids pADE256 and pADE257 both gave a $Man^+$ phenotype. The site of insertion of each transposon was identified by restriction enzyme analysis (Fig 5.3).

These results indicated that one end of the $pmi_{stm}$ gene lay between the transposon insertion sites in pADE257 and pADE258 and that the *pmi* gene extended rightward to or beyond the insertion point in pADE260 (Fig. 5.3).

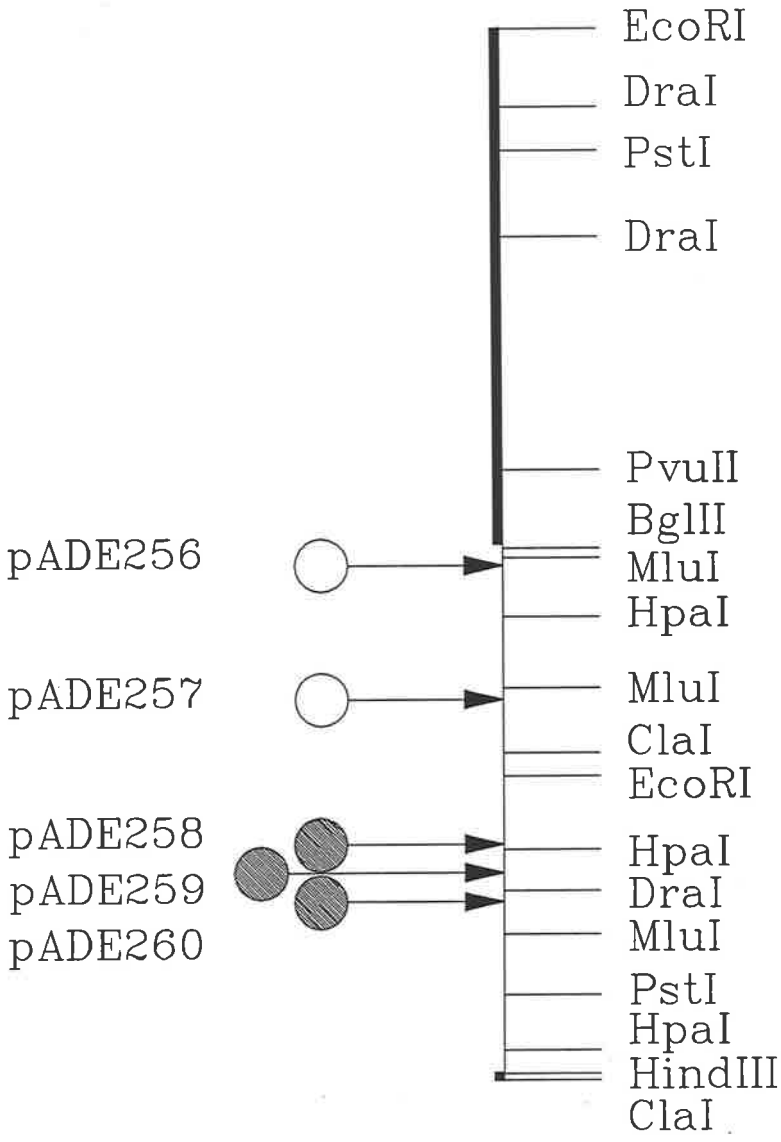
### 5.2.3 Sub-cloning the $pmi_{stm}$ gene from pADE250

The 1.6 kb *Cla* I fragment of pADE250 (Fig. 5.3) was ligated into the *Cla* I site of pBR322, producing pADE253, which complemented the CD1 ($Man^-$) and SL1101 ($Pmi^-$) mutants. A restriction fragment map of the *Cla* I insert in pADE253 was obtained (Fig. 5.4). A deletion mutation in pADE253 was effected by *Mlu* I digestion and end-filling, followed by partial *Hpa* I digestion and religation. After transformation into CD1, plasmid

**Figure 5.3: Restriction enzyme, and transposon insertion, map of plasmid pADE250**

Restriction sites in the 5.6 kb pADE250 plasmid are indicated. Plasmids, with Tn*1725* insertions, derived from pADE250, are numbered below the restriction map, and the transposon insertion sites are defined with open circles (for Tn*1725* insertions not affecting *pmi* $_{stm}$) or with hatched circles (for Tn*1725* insertions inactivating *pmi* $_{stm}$ ·

▬▬▬ : pHC79 DNA.


EcoRI
DraI
PstI
DraI
PvuII
BglII
MluI
HpaI
MluI
ClaI
EcoRI
HpaI
DraI
MluI
PstI
HpaI
HindIII
ClaI
pADE256
pADE257
pADE258
pADE259
pADE260

pADE254, which contained a deletion of the 0.4 kb *Mlu* I-*Hpa* I fragment of pADE253 (Fig. 5.4), was identified. Plasmid pADE254 did not alter the Man$^-$ phenotype of CD1. A part or all of this deleted DNA fragment was therefore essential for $pmi_{stm}$ activity.

### 5.2.4 Analysis of the $pmi_{stm}$ -encoded polypeptide

#### 5.2.4.1 Minicell analysis

Plasmids pBR322, pADE253 ($pmi_{stm}^+$), pADE254 ($pmi_{stm}^-$) and pADE260($pmi_{stm}^-$) were transformed into the minicell-producing strain DS410 (Dougan and Sherratt, 1977), and plasmid-encoded proteins viewed by SDS-PAGE (Fig. 5.5). Strain DS410/pBR322 (lane 1) displayed an obvious protein of 30 kDa corresponding to the plasmid β-lactamase (Dougan and Kehoe, 1987). The Pmi$^+$ clone, DS410/pADE253 (lane 2), had an additional protein at 42 kDa. Strains DS410/pADE254 (lane 3) and DS410/pADE260 (lane 4) (Fig. 5.5) lacked this protein. The labelled protein band at 28 kDa (lane 3) suggested that DS410/pADE254 produced a truncated version of the 42 kDa protein due to the deletion described in Section 5.2.3 above. The Tn*1725* insertion in pADE260 (Fig 5.3) inactivated $pmi_{stm}$, and DS410/pADE260 (lane 4) did not have the 42 kDa protein but a band at ca. 25 kDa was visible, corresponding to the chloramphenicol acetyltransferase of Tn*1725*. The absence of an additional protein of any significant size in lane D suggested that the Tn*1725* transposon insertion interrupted the Pmi protein-encoding region close to either the transcriptional or translational start signals. The precise location of this transposon insertion in the *pmi* gene is determined in Chapter 6.

#### 5.2.4.2 Determination of the extent of processing of the Pmi polypeptide

To determine if the 42 kDa Pmi protein was subjected to proteolytic cleavage *in vivo*, the plasmids pHC79 and pADE250 were transformed into DS410 and minicells prepared as before (Section 5.2.4.1). Labelling with [$^{35}$S]-methionine subsequently proceeded with

**Figure 5.4: Restriction enzyme map of the 1.6kb *Cla*1 fragment of pADE253**

The restriction sites in the 1.6 kb *Cla* I insert in plasmid pADE253 are indicated with the scale (in kb) below. The triangle represents the *Hpa* I - *Mlu* I fragment deleted from pADE253 to generate pADE254.

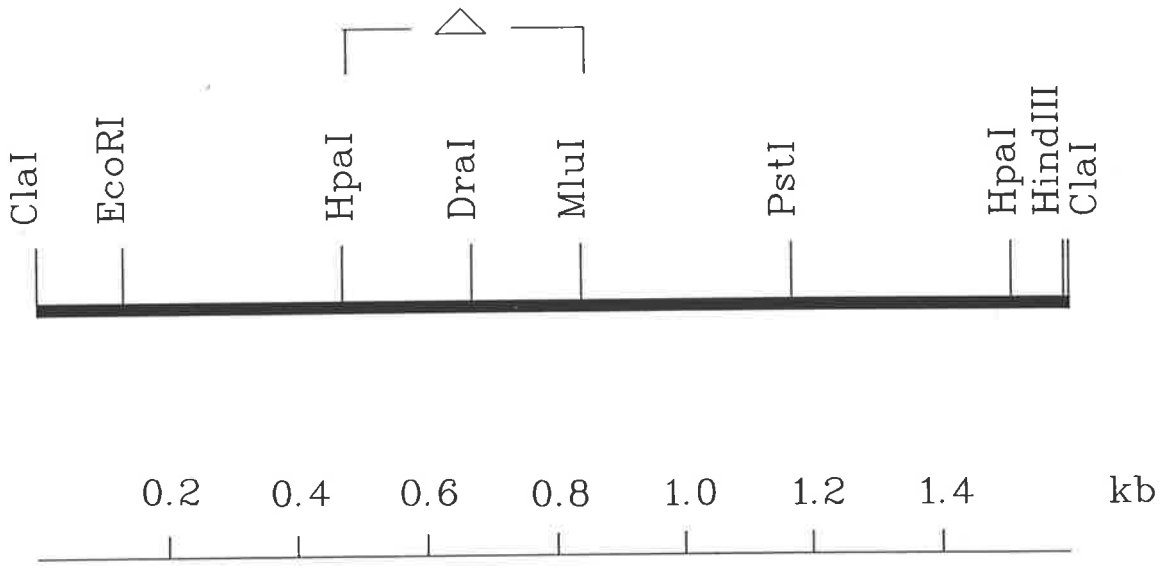
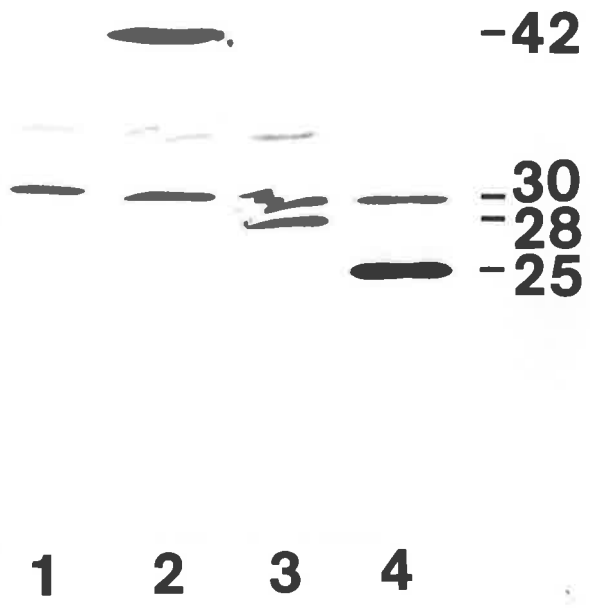

ClaI
EcoRI
HpaI
DraI
MluI
PstI
HpaI
HindIII
ClaI
0.2
0.4
0.6
0.8
1.0
1.2
1.4
kb

**Figure 5.5: Minicell analysis of the Pmi polypeptide**

Plasmid-encoded proteins were analyzed using the minicell-producing strain DS410. Minicells harbouring various plasmids were purified on sucrose gradients, labelled with [$^{35}$S]-methionine, and solubilised. Labelled proteins were visualised by autoradiography after electrophoresis on a 0.2%(w/v) SDS, 15% (w/v)polyacrylamide gel.

Lane designations: lane 1: DS410/pBR322; lane 2: DS410/pADE253; lane 3: DS410/pADE254; lane 4: DS410/pADE260. The 42 kDa Pmi, the 30 kDa β-lactamase, the truncated 28 kDa version of Pmi, and the 25 kDa chloramphenicol acetyltransferase proteins are indicated.


42
30
28
25
1
2
3
4

the following modifications: (a) 0.9%(w/v) ethanol was added prior to labelling, or (b) minicells were treated with Cm (200 ug/ ml final concentration) prior to labelling, or (c) minicells were pulse-labelled for 2 min and chased with cold methionine, with the addition of Cm to 200 ug/ ml, with aliquots withdrawn at intervals of 30, 60 and 120 min. Total cell lysates (Materials and Methods) were prepared from each sample and analysed by SDS-PAGE followed by autoradiography (Fig. 5.6).

Inhibition of proteolysis by addition of 0.9%(w/v) ethanol during labelling of DS410/pHC79 led to the accumulation of the precursor form of β-lactamase (Pre-Bla; Fig. 5.6; lane 2). In the presence of Cm, *de novo* protein synthesis was inhibited and thus no labelled proteins appeared (Fig. 5.6; lane 3). When minicells were pulse-labelled followed by Cm treatment, the Pre-Bla protein (Fig. 5.6; lane 4) underwent progressive proteolytic cleavage to remove the signal peptide (Fig. 5.6; lanes 5-7) resulting in a predominance of the mature (Mat-Bla) form of the enzyme (Fig. 5.6; lane 7).

The 42 kDa Pmi protein produced in minicells of DS410/pADE250 (Fig. 5.6; lane 7) was present in the ethanol-treated sample (Fig. 5.6; lane 8) and this protein band remained unchanged in the pulse-labelled, chased, Cm-treated samples (Fig. 5.6; lanes 11-14). This suggested that the 42 kDa Pmi protein was not synthesised in a precursor form.

### 5.2.5 Cellular localization of the Pmi protein

Minicells of DS410/pADE250 were prepared and labelled with [$^{35}$S]-methionine as described previously (Section 5.2.4.1). The minicells were separated into periplasmic, cytoplasmic and inner and outer membrane fractions (Materials and Methods). The protein components of each fraction were analyzed by SDS-PAGE, followed by autoradiography (Fig. 5.7). The Pmi protein was found in greatest abundance in the cytoplasmic fraction (Fig. 5.7, lane 5) and was essentially absent in the periplasmic and membrane fractions

**Figure 5.6 : A search for possible processing of the Pmi polypeptide**

Aliquots of purified minicells of DS410/pHC79 and DS410/pADE250 were treated by one of the following methods: routine labelling with [$^{35}$S]-methionine (lanes 1, 8); treatment with 0.9% ethanol prior to labelling with [$^{35}$S]-methionine (lanes 2, 9); treatment with Cm (200 μg/ ml) prior to labelling with [$^{35}$S]-methionine (lanes 3, 10); pulse labelling with [$^{35}$S]-methionine followed by cold methionine chase, and Cm (200 μg/ ml) addition, with aliquots withdrawn at 0, 30, 60 and 120 minutes post labelling (lanes 4-7 and 11-14).

The 42 kDa Pmi protein, and the precursor (PRE-BLA), and mature (MAT-BLA) forms, of the β - lactamase protein, are indicated.

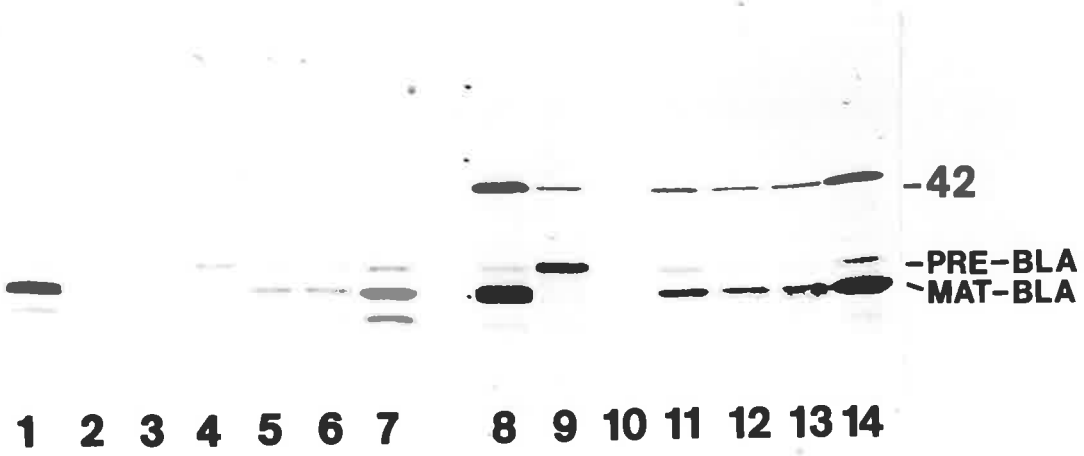
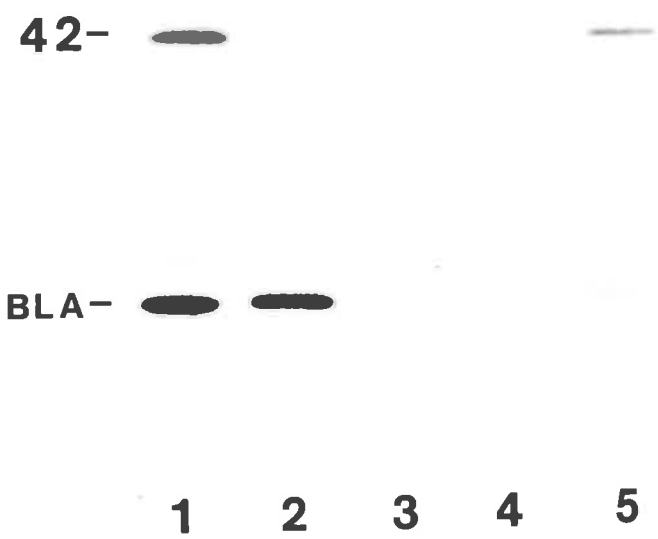

-42
-PRE-BLA
-MAT-BLA
1 2 3 4 5 6 7 8 9 10 11 12 13 14

**Figure 5.7 Localization of the Pmi polypeptide**

*E. coli* minicells harbouring pADE250 were purified on sucrose gradients and labelled with [$^{35}$S]-methionine (lane 1). The minicells were sphaeroplasted to release the periplasmic fraction (lane 2). Sonication followed by ultracentrifugation was used to separate the membranes from the cytoplasmic fraction (lane 5). Membrane fractions were then extracted with sarkosyl. The sarkosyl-insoluble fraction consisted of outer membrane (lane 3) while the inner membrane (lane 4) was sarkosyl-soluble. All fractions were solubilised and subjected to SDS-PAGE, followed by autoradiography. Each gel track received material from equal amounts of minicells.

The 42 kDa Pmi and the 30 kDa β-lactamase (BLA) proteins are indicated.


42-
BLA-
1
2
3
4
5

(Fig. 5.7; lanes 2, 3 and 4). The Bla protein was localized predominantly in the periplasmic fraction (Fig. 5.7; lane 2).

### 5.2.6 Assays for Pmi enzyme activity

The levels of Pmi enzyme activity in cell extracts of wild-type, mutant, and complemented stains of *E. coli* K-12,were measured as described (Materials and Methods)(Table 5.2). No appreciable Pmi enzyme activity was observed for either CD1 or CD1/pBR322. Extracts of the $Pmi^+$ recombinant CD1/pADE253 yielded a specific activity of 206, compared to a value of 117 for the wild-type, parental strain of CD1, AC80 (Table 5.2). This variation in the levels of Pmi enzyme specific activity may be due to a gene dosage effect, as multiple, plasmid-encoded copies of $pmi_{stm}$ were present in the $Pmi^+$ recombinant.

### 5.2.7 Homology of $pmi_{stm}$ with *E. coli*, *Shigella*, and *Salmonella* spp.

In order to ascertain whether DNA homology existed between the cloned $pmi_{stm}$ gene, and total DNA of strains of *Salmonella* other than *S. typhimurium*, the 1.6 kb fragment of pADE253 (pBR322 $pmi^+$) was purified and labelled with $\alpha$-[$^{32}$P]-dCTP for use as a $pmi_{stm}$ probe. Southern hyridization with this probe was performed against chromosomal preparations of *E. coli* K-12 ,*Shigella flexneri*, and 5 *Salmonella* strains (Fig. 5.8).

The $pmi_{stm}$ probe hybridized with a single *Acc* I fragment of 3.3 kb (Fig. 5.8; lanes 1-5) and with two *Pst* I fragments of 3.6 kb and 0.7 kb (Fig. 5.8, lanes 6-10) of the *Salmonella* strains: *S. typhimurium* (group B), *S. paratyphi* A (group A), *S. typhi* (group D1), *S. derby* (group B), and *S. dublin* (group D1). *Pst* I fragments of this size were not detected in the *S. typhimurium* $Pmi^+$ cosmid cutdown, pADE249, due probably to displacement of normally contiguous chromosomal sequences during ligation of *Sau* 3A1-generated DNA fragments. The $pmi_{stm}$ gene probe also hybridized with a 12 kb *Pst* I

**Table 5.2: Specific activity of the Pmi enzyme in various strains**

| STRAIN | Pmi enzyme activity* |
|---|---|
| AC80 | 117 |
| CD1 | < 5 |
| CD1/pBR322 | < 5 |
| CD1/pADE253 | 206 |

* One unit of activity is that converting one nanomole of mannose-6-phosphate to fructose-6-phosphate per min, per mg protein.

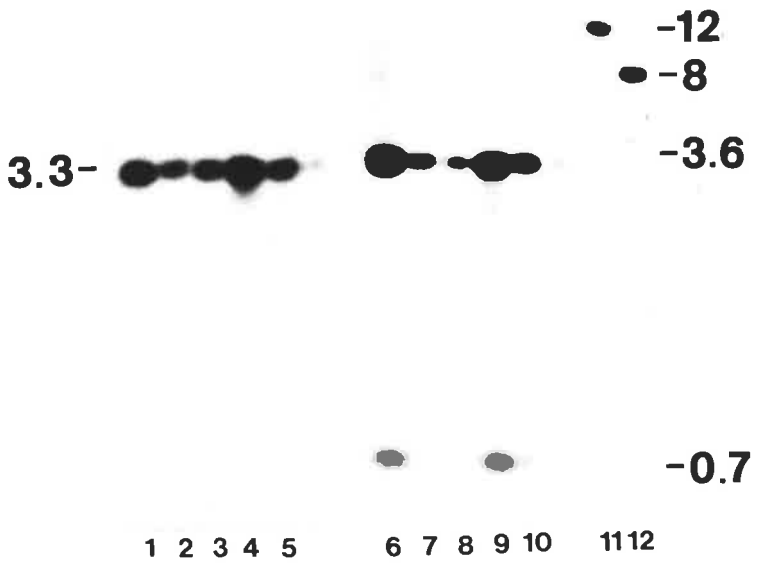
**Figure 5.8: Homology of cloned *pmi*$_{stm}$ with DNA from other bacterial strains**

Whole DNA of each strain was digested with either *Acc*I or *Pst*I and electrophoresed on a 1% (w/v) agarose gel. Following transfer to nitrocellulose, the filter was probed for homology with the α-[$^{32}$P]-dCTP- labelled 1.6 kb *Cla*I fragment of pADE253, washed, and subjected to autoradiography.

The probe hybridized strongly with all five of the *Salmonella* strains examined. The 0.7 kb *Pst*I-hybridising fragment was present in each of the *Salmonella Pst*I digests but showed up weakly in some tracks due to variation in the amounts of DNA loaded. Single *Pst*I fragments of each of the *E. coli* and *S. flexneri* strains also showed homology to the probe.

Lane designations (with restriction enzyme used for cut in brackets): 1: *S. typhimurium* (*Acc*I); 2: *S. paratyphi* A (*Acc*I); 3: *S. typhi* (*Acc*I); 4: *S. derby* (*Acc*I); 5: *S. dublin* (*Acc*I); 6: *S. typhimurium* (*Pst*I); 7. *S. paratyphi* A (*Pst*I); 8: *S. typhi* (*Pst*I); 9: *S. derby* (*Pst*I); 10: *S. dublin* (*Pst*I); 11: *E. coli* K-12 (*Pst*I); 12: *Shigella flexneri* (*Pst*I)

The numbers at the sides are the sizes (kb) of indicated fragments.


-12
-8
3.3-
-3.6
-0.7
1 2 3 4 5 6 7 8 9 10 11 12

fragment of *E. coli* K-12 DH1 genomic DNA (Fig. 5.8; lane 11) and with an 8 kb genomic DNA fragment of *S. flexneri* PE523 (Fig. 5.8; lane 12).

## 5.3 Discussion

The $pmi_{stm}$ gene was isolated from a genomic bank of *S. typhimurium* C5 by complementation of a *manA* mutant of *E. coli* K-12. The $Pmi^+$ clone enabled the *manA* strain to utilise mannose as a source of carbon, and restored to a *manA cap* mutant the ability to synthesize capsular polysaccharide without the provision of exogenous mannose. This suggested that the products of the $pmi_{stm}$ and *manA* genes were similar.

Rosen *et al.* (1965) described *pmi* mutants as being unable to synthesize GDP-mannose, a prerequisite for the synthesis of the O-repeating unit of group B *Salmonella* strains, unless supplied with exogenous mannose. The introduction of the $Pmi^+$ clone into a *pmi* mutant of *S. typhimurium* restored the ability to grow on mannose as a sole source of carbon, and also resulted in production of SR LPS when a *pmi, rfc* strain harbouring the $Pmi^+$ plasmid was grown without mannose. The O-antigen unit assembly was here possible due to the acquisition of the ability to synthesize GDP-mannose.

Transposon mutagenesis with Tn*1725* established the approximate location and extent of the $pmi_{stm}$ gene on pADE250. The $pmi_{stm}{}^+$ activity was preserved on the 1.6 kb sub-cloned *Cla* I fragment of pADE253. By deletion construction it was demonstrated that the internal 0.4 kb *Hpa* I -*Mlu* I fragment of pADE253 contained DNA sequences essential for $pmi_{stm}$ activity.

The polypeptide encoded in minicells by the $pmi_{stm}$ gene was shown to be 42 kDa in size. The 42 kDa polypeptide could be eliminated either by deletion (as in pADE254) or by transposon insertion in the $pmi_{stm}$ gene (as in pADE258-60). The *S. typhimurium* Pmi protein was similar in size to the 42.716 kDa *E. coli* K-12 Pmi protein (Miles and Guest,

1984), but significantly smaller than the 52.86 kDa Pmi protein of *P. aeruginosa* (Darzins *et al.*, 1986).

Pulse-chase experiments using [$^{35}$S]-methionine labelled minicells, or treatment of the minicells with ethanol during labelling, were used to ascertain whether the 42 kDa protein represented the mature form or a precursor form of the Pmi protein. There was no evidence of processing of the 42 kDa protein, or of the derivation of the 42 kDa protein from a larger precursor, although processing of the β - lactamase protein from precursor to mature form took place in these minicells.

In the minicell fractionation experiments the cytoplasm was shown to be the major location of the Pmi protein in *E. coli* K-12, with no appreciable amounts of this protein being found in the periplasmic or membrane fractions. The assignation of the Pmi protein to the cytoplasmic fraction is consistent with the fundamental metabolic roles of this enzyme, in addition to its roles in LPS and capsular polysaccharide production.

Conservation of the *pmi*$_{stm}$ gene was strong among selected *Salmonella* strains (name in brackets) representative of O-serotype groups : A (*S. paratyphi* A), B (*S. derby* ), and D1 (*S. typhi* and *S. dublin* ), as judged from strong hybridization between the chromosomal DNA of these strains and a DNA probe containing the entire *pmi*$_{stm}$ gene. The *pmi*$_{stm}$ gene also shared homology with DNA sequences in both *E. coli* K-12 and *S. flexneri*. The *pmi*$_{stm}$ probe hybridized with a 12 kb *Pst* I fragment of *E. coli* K-12, indicating that the analogous gene in *E. coli* K-12 may be present on a single *Pst* I fragment. In support of this is the mapping of a *man*::Mu insertion in *E. coli* K-12 to a *Pst*I fragment of between 11.6 kb and 12.35 kb in size (Bouché *et al.*, 1982) and the absence of a *Pst* I restriction site in the *manA* nucleotide sequence (Miles and Guest, 1984a).

Since the *pmi*$_{stm}$ gene can replace the *E. coli* K-12 *manA* gene to restore Pmi enzyme activity, the Pmi enzyme may be very similar in both species. A similar, albeit less

complete, complementation of Pmi function was observed when the *P. aeruginosa pmi* gene was transferred into an *E. coli* K-12 *manA* mutant. The *P. aeruginosa* gene restored colanic acid synthesis from glucose, but not the ability to grow on mannose as a sole source of carbon, to a Pmi-deficient mutant of *E. coli* K-12 derepressed for capsular polysaccharide synthesis (Darzins *et al.*, 1985). This suggested a unidirectional function (i.e. conversion of fructose-6-phosphate to mannose-6-phosphate with negligible activity in the reverse direction) for the *Pseudomonas* Pmi protein (Darzins *et al.*, 1985). Furthermore, the cloned *manA* gene of *E. coli* K-12 could restore alginate production to an $Alg^-$ mutant of *P. aeruginosa*, although the Pmi enzyme levels in these recombinants was only ca. 5% of the Pmi enzyme activity seen in *E. coli* K-12 *manA* mutants containing the *manA* clone (Darzins *et al.*, 1985). Interestingly, no DNA homology was detected between the Pmi-encoding sequence of *P. aeruginosa* and chromosomal sequences of *E. coli* K-12 or *vice versa* (Darzins *et al.*, 1985) and no significant similarities in the nucleotide sequences of the *E. coli* K-12 *manA* and *P. aeruginosa pmi* genes could be noted(Darzins *et al.*, 1986). The hybridization experiment with the $pmi_{stm}$ probe indicated that strong DNA homology existed between this gene and sequences on the *E. coli* K-12 chromosome. The degree of homology between $pmi_{stm}$ and the analogous *E. coli* K-12 *manA* gene is investigated further in Chapter 6.

# CHAPTER 6

## Nucleotide sequence analysis and genetic organization of the *pmi* stm gene of *Salmonella typhimurium*

## 6.1 Introduction

The nucleotide sequences for the genes encoding the Pmi protein in both *E. coli* K-12 and *P. aeruginosa* have been reported (Miles and Guest, 1984a; Darzins *et al.*, 1986). The cloned *E. coli manA* gene restored the ability to synthesise alginate to an $Alg^-$ mutant of *P. aeruginosa* ,and, conversely, the *pmi* gene of *P. aeruginosa* ($pmi_{ps}$) restored the facility for capsular polysaccharide production to a $Man^-$ mutant of *E. coli* K-12(Darzins *et al.*, 1985). A probe of *E. coli* K-12 *manA* DNA hybridized with chromosomal sequences of *Klebsiella pneumoniae* and *Enterobacter aerogenes*, but not with the DNA of *Pseudomonas* spp.. A $pmi_{ps}$ DNA probe had homology with DNA of other *Pseudomonas* spp. but not with chromosomal sequences of *E. coli* K-12, *K. pneumoniae* and *E. aerogenes* (Darzins *et al.*, 1986). In support of these results, comparisons of the DNA sequences of the $pmi_{ps}$ and *manA* genes revealed the absence of areas of significant homology. It was postulated that two types of *pmi* gene existed, albeit with similar enzymatic properties. The possibility of a third *pmi* gene class present in either or both of *Arthrobacter viscosus* or *Agrobacterium tumefaciens* was also proposed, since neither of these species had DNA which hybridized with the $pmi_{ps}$ or *manA* probes, although both were known to utilise mannose as a carbon source (Darzins *et al.*, 1986).

The $pmi_{stm}$ gene restored the $Man^+$ phenotype to an *E. coli* K-12 *manA* mutant, and the $Pmi^+$ phenotype to an *S. typhimurium pmi* mutant (Chapter 5). Therefore, it was of interest to ascertain the degree of conservation of the *pmi* genes and their protein products between these species.

This chapter details the primary nucleotide sequence, and the regulatory signals, of the *S.typhimurium* $pmi_{stm}$ gene, and the characteristics of the encoded Pmi protein. In addition, a comparison of the $pmi_{stm}$ gene sequence with the previously reported sequence for the *manA* gene of *E. coli* K-12 is effected.

## 6.2 Results

### 6.2.1 *pmi* $_{stm}$ nucleotide sequence determination and analysis

#### 6.2.1.1 DNA sequencing strategy

The *pmi*$_{stm}$ gene of *S. typhimurium* was localised on a 1.6 kb *Cla* I fragment on plasmid pADE253 (Section 5.2.3). The 1.6 kb *Cla* I fragment was cloned in both orientations into the *Acc*I site of M13mp18 (Vieira and Messing, 1982). In addition, various restriction fragments of the pADE253 insert were subcloned into the multiple cloning sites of M13mp18 or M13mp19 (Fig. 6.1). The M13 -20 universal primer (17-mer) was used to sequence the M13 clones by the dideoxy sequencing method. The M13 -40 primer (17-mer) was used with the M13 clones containing the entire 1.6 kb *Cla* I fragment in order to precisely determine the DNA sequence at the junctions with the polylinker. Synthetic oligodeoxynucleotide primers were used in the sequencing of those areas where the cloned fragments did not overlap (Fig. 6.1).

#### 6.2.1.2 Nucleotide sequence analysis of *pmi*$_{stm}$

The complete nucleotide sequence of 1650 bp was determined in both directions; a single ORF was found (Fig 6.2). The transcriptional and translational regulatory signals of this ORF are described below. In addition, the degree of homology between this sequence and that of other *pmi* genes is examined.

Analysis of the nucleotide sequence (Fig. 6.2) revealed the presence of an ORF beginning at nt 432 and terminating at nt 1604. This frame encoded 391 amino acids producing a protein of predicted $M_r$ 42,600.

Analysis of the nucleotide sequence for potential transcriptional signals identified regions similar to the -10 ('Pribnow box') and -35 consensus sequences (Rosenberg and Court, 1979; Hawley

**Figure 6.1: Strategy used for DNA sequencing of the *pmi*$_{stm}$ gene**

Clones containing the indicated DNA sub-fragments of the 1.6 kb *Cla*I insert of pADE253 were constructed in either M13mp18 or M13mp19 . The sequencing start point of each clone is indicated by a circle for M13mp18 derivatives and by a square for M13mp19 derivatives. The direction and extent of the sequencing carried out on each clone is represented by arrows. The DNA regions preceded by a triangle were sequenced using a specifically synthesized deoxyoligonucleotide primer.

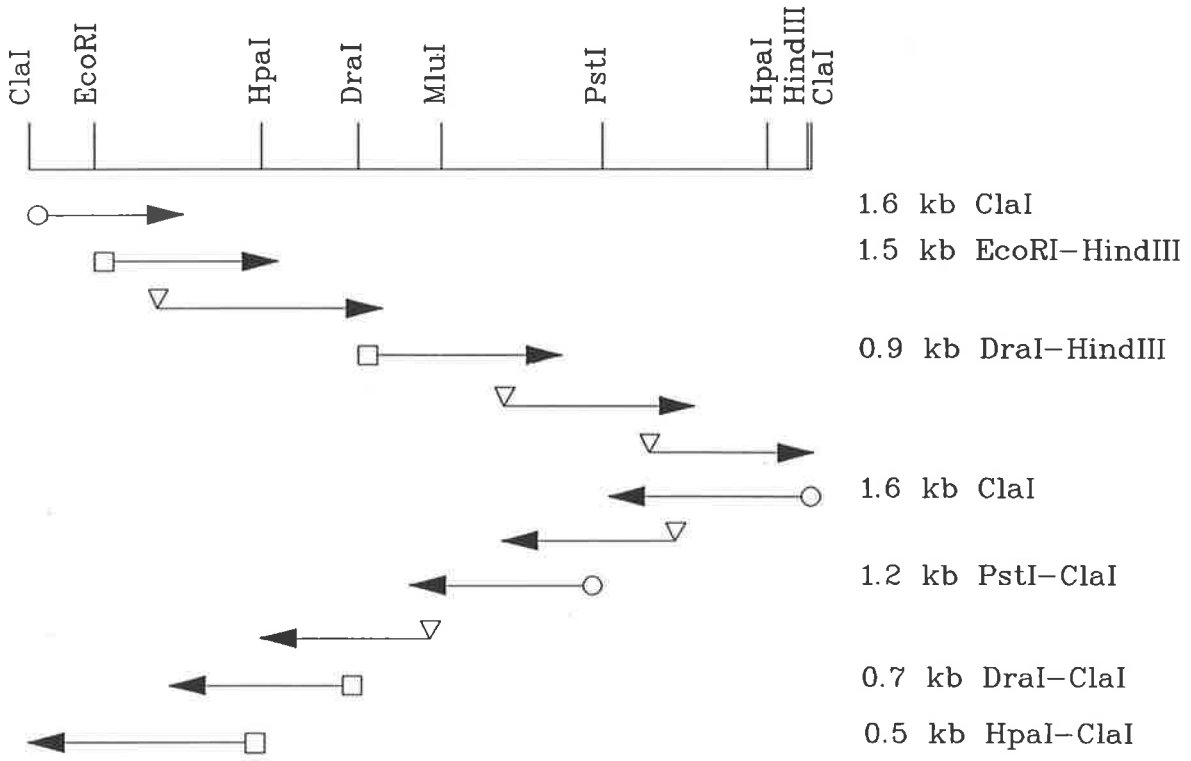

ClaI
EcoRI
HpaI
DraI
MluI
PstI
HpaI
HindIII
ClaI
1.6 kb ClaI
1.5 kb EcoRI–HindIII
0.9 kb DraI–HindIII
1.6 kb ClaI
1.2 kb PstI–ClaI
0.7 kb DraI–ClaI
0.5 kb HpaI–ClaI

**Figure 6.2: Nucleotide sequence of the 1,650 bp *Cla*I fragment of cloned *S. typhimurium* DNA which carries the $pmi_{stm}$ gene**

The nucleotide sequence is numbered from nt 1 to nt 1650 and shown in the same orientation as in Fig. 6.1. Sequences with homology to the *E. coli* -35 (TTGACA) and -10 (TATAAT) consensus sequences (shown beneath their homologues) are underlined. A potential CRP binding site (from nt 332 - 353) is shown thus: +-------+. The amino acids of the 391 aa open reading frame are indicated below the nucleotide sequence and are numbered starting at the initiation codon (ATG-Met). A potential S-D sequence is italicised and underlined, 7 bp upstream of the start codon, also italicised and underlined.

A synthetic deoxyoligonucleotide homologous to the end of transposon Tn*1725* was used as a primer to sequence the DNA of a transposon-bearing plasmid derivative of pADE253, in which the transposon inactivated the $pmi_{stm}$ gene. The Tn*1725* insertion point in pADE260 is indicated by the underlining of the single base after which the Tn*1725* insertion occurred.

```
1    ATCGATAACTTTCCACGCGATGTCGCAGAGCTGGTGGACTGGTTCGACGCTCGCGACCCTAAC   63

64   CGCATGTGCGCCCGGTGCCCGCTACGCGAGCAGATCCCGGTCTGGCTGTTGGGATCTCTTGTCC  127

128  TTCGAATTCGGCGACGGAAACATGTTCGCTGGTCAACAAGTAGTACTCGGTATCGTCCTTTTTG  191

192  AGGGGAAAAGGGTCTTGATAAAAGAAGGGTTTGTTTGACATTGTGCTCTCACTTACCGCTCGGT  255

256  ATGGTTATTCTCTGGGCAGGTGTTCCATTGCCCGACTCAAAGCGAGTAACACTATCCTACACAA  319
                +--------------------+
320  TTTTTTAACAAAAACTGAGACAAGTACGACTTTTTACGCCCGGAGGTTACTTCATGCGGGTTTC  383
                                                    TTGACA
384  TTGGTTTAATACCTCCCATTGATCTCCACATTGAAACAGGGCTTGATA ATG CAA AAA CTC  443
1        TATAAT                                          Met Gln Lys Leu    4

444  ATT AAC TCA GTG CAA AAC TAT GCC TGG GGA AGT AAA ACT GCG TTA ACG    491
5    Ile Asn Ser Val Gln Asn Tyr Ala Trp Gly Ser Lys Thr Ala Leu Thr    20

492  GAA CTT TAT GGC ATC GCC AAT CCG CAG CAG CAG CCA ATG GCT GAA CTC    539
21   Glu Leu Tyr Gly Ile Ala Asn Pro Gln Gln Gln Pro Met Ala Glu Leu    36

540  TGG ATG GGC GCG CAT CCC AAA AGC AGC TCG CGA ATC ACC ACC GCC AAC    587
37   Trp Met Gly Ala His Pro Lys Ser Ser Ser Arg Ile Thr Thr Ala Asn    52

588  GGC GAA ACC GTC TCC CTG CGT GAC GCC ATC GAA AAG AAT AAA ACC GCC    635
53   Gly Glu Thr Val Ser Leu Arg Asp Ala Ile Glu Lys Asn Lys Thr Ala    68

636  ATG CTG GGC GAA GCG GTA GCC AAC CGT TTC GGC GAA CTG CCG TTT CTG    683
69   Met Leu Gly Glu Ala Val Ala Asn Arg Phe Gly Glu Leu Pro Phe Leu    84
                                                pADE260
684  TTT AAA GTA CTG TGC GCC GCC AAA CCG CTC TCT ATT CAG GTG CAC CCG    731
85   Phe Lys Val Leu Cys Ala Ala Lys Pro Leu Ser Ile Gln Val His Pro   100

732  AAT AAA CGC AAC TCC GAA ATC GGT TTC GCG AAA GAA AAT GCG GCG GGT    779
101  Asn Lys Arg Asn Ser Glu Ile Gly Phe Ala Lys Glu Asn Ala Ala Gly   116

780  ATC CCC ATG GAT GCC GCA GAG CGG AAC TAT AAA GAT CCT AAC CAT AAA    827
117  Ile Pro Met Asp Ala Ala Glu Arg Asn Tyr Lys Asp Pro Asn His Lys   132

828  CCA GAG CTG GTT TTT GCC CTG ACG CCT TTC CTG GCG ATG AAC GCG TTC    875
133  Pro Glu Leu Val Phe Ala Leu Thr Pro Phe Leu Ala Met Asn Ala Phe   148

876  CGC GAA TTT TCT GAC ATT GTC TCT TTA CTG CAA CCT GTC GCC GGC GCG    923
149  Arg Glu Phe Ser Asp Ile Val Ser Leu Leu Gln Pro Val Ala Gly Ala   164

924  CAT TCC GCT ATC GCC CAC TTT TTG CAG GTG CCG AAT GCT GAA CGT CTG    971
165  His Ser Ala Ile Ala His Phe Leu Gln Val Pro Asn Ala Glu Arg Leu   180

972  AGC CAG CTT TTC GCC AGC CTG TTG AAT ATG CAA GGC GAA GAA AAA TCC   1019
181  Ser Gln Leu Phe Ala Ser Leu Leu Asn Met Gln Gly Glu Glu Lys Ser   196

1020 CGC GCG TTA GCC GTA CTC AAA GCG GCG CTT AAC AGC CAG CAA GGC GAA   1067
197  Arg Ala Leu Ala Val Leu Lys Ala Ala Leu Asn Ser Gln Gln Gly Glu   212

1068 CCG TGG CAA ACG ATC CGC GTG ATT TCA GAG TAT TAT CCT GAC GAC AGC   1115
213  Pro Trp Gln Thr Ile Arg Val Ile Ser Glu Tyr Tyr Pro Asp Asp Ser   228
```

```
1116  GGG CTT TTC TCT CCT TTG TTG CTG AAT GTG GTC AAA CTG AAT CCC GGC  1163
 229  Gly Leu Phe Ser Pro Leu Leu Leu Asn Val Val Lys Leu Asn Pro Gly   244

1164  GAG GCG ATG TTC CTG TTT GCT GAA ACG CCT CAT GCT TAT CTG CAG GGC  1211
 245  Glu Ala Met Phe Leu Phe Ala Glu Thr Pro His Ala Tyr Leu Gln Gly   260

1212  GTT GCG CTG GAA GTC ATG GCG AAC TCC GAT AAC GTT CTG CGC GCT GGC  1259
 261  Val Ala Leu Glu Val Met Ala Asn Ser Asp Asn Val Leu Arg Ala Gly   276

1260  CTT ACG CCA AAA TAT ATC GAC ATC CCT GAG CTG GTC GCG AAC GTG AAG  1307
 277  Leu Thr Pro Lys Tyr Ile Asp Ile Pro Glu Leu Val Ala Asn Val Lys   292

1308  TTC GAA CCT AAG CCT GCC GGC GAG TTG CTG ACT GCC CCG GTG AAA AGC  1355
 293  Phe Glu Pro Lys Pro Ala Gly Glu Leu Leu Thr Ala Pro Val Lys Ser   308

1356  GGC GCG GAG CTG GAC TTC CCA ATT CCG GTT GAC GAT TTT GCT TTT TCA  1403
 309  Gly Ala Glu Leu Asp Phe Pro Ile Pro Val Asp Asp Phe Ala Phe Ser   324

1404  CTG CAC GAC CTG GCG CTT CAG GAG ACG AGC ATC GGC CAA CAC AGC GCC  1451
 325  Leu His Asp Leu Ala Leu Gln Glu Thr Ser Ile Gly Gln His Ser Ala   340

1452  GCG ATT CTG TTC TGC GTT GAG GGT GAG GCG GTG TTA CGT AAA GAT GAA  1499
 341  Ala Ile Leu Phe Cys Val Glu Gly Glu Ala Val Leu Arg Lys Asp Glu   356

1500  CAG CGT CTG GTA CTG AAG CCG GGT GAA TCT GCC TTT ATC GGC GCG GAT  1547
 357  Gln Arg Leu Val Leu Lys Pro Gly Glu Ser Ala Phe Ile Gly Ala Asp   372

1548  GAG TCT CCG GTT AAC GCC AGC GGC ACG GGC CGT TTA GCG CGT GTT TAT  1595
 373  Glu Ser Pro Val Asn Ala Ser Gly Thr Gly Arg Leu Ala Arg Val Tyr   388

1596  AAC AAG CTG TAG CAACGTACTGAATTTTTTAACAACTCTTGCTAAGCTTATCGAT      1650
 389  Asn Lys Leu ***                                  ***              392
```

and McClure, 1983; Siebenlist *et al.*, 1980; Pribnow, 1979)(see Chapter 4.2.1.2). A putative -10 region having 5 out of 6 nt positions identical to the consensus sequence (TATAAT ;Rosenberg and Court, 1979) was located between nt 388 and nt 393 and reads TtTAAt. A corresponding -35 region between nt 365 and nt 370 reading gTtACt had 3 out of 6 positions in common with the -35 consensus sequence (TTGACA; Hawley and McClure, 1983) (Fig. 6.2). The distance between these postulated -10 and -35 regions was17 bp, the optimal spacing for efficient promoter activity (Hawley and McClure, 1983; Rosenberg and Court, 1979).

It has been postulated that the production of the Pmi protein in *E. coli* K-12 is subject to catabolite repression, mediated by cAMP and the cAMP receptor protein, the cAMP receptor protein (CRP) (Miles and Guest, 1984). A consensus sequence has been proposed for the CRP binding site (AA-TGTGA--TA--TCA[C/A]ATTT; Chapon and Kolb, 1983). A similar sequence, identical in 11 of the 17 designated base positions of the consensus sequence, existed in the $pmi_{stm}$ sequence between nt 332 and nt 353. It read AAcTGaGAcaagtacgACtTTT (Fig. 6.2). This 22 bp region also shared 95% homology (21 of 22 bases identical) with the postulated CRP binding site of the *manA* gene of *E. coli* K-12 (Miles and Guest, 1984a). The CRP binding site of the *lac* operon was positioned at approx. -70 to -50 nt to the transcriptional start site (Simpson, 1980). The proposed CRP binding site of the $pmi_{stm}$ gene was similarly positioned with respect to the putative transcriptional start site (at nt 397) (Fig. 6.2).

A potential Shine-Dalgarno (S-D) sequence AAacAGGg (nt 417 to nt 424), showing good homology to the S-D consensus sequence (AAGGAGGU; Shine and Dalgarno, 1974), and an initiation codon ATG (nt 432 to nt 434) were detected (Fig. 6.2). The 7 nt spacing between the S-D region and the initiation codon is the optimal spacing for efficient translation (Gold *et al.*, 1980; Kozak, 1983).

### 6.2.1.3 Location of a *pmi*::Tn*1725* insertion

A transposon Tn*1725* insertion in pADE260 inactivated *pmi* $_{stm}$ and abolished production of the Pmi protein in minicells (Section 5.2.4.1). A synthetic 20-mer deoxyoligonucleotide (GCTGTCACGAGAACACCGTT), with homology to the end of transposon Tn*1725* ,was used as a primer to sequence the site of Tn*1725* insertion in plasmid pADE260. The transposon was found to be inserted at nt 715 in the nucleotide sequence. A protein of ca. 11 kDa would be predicted for translation of the nt 432-715 open reading frame. A protein of this size, if synthesized in minicells bearing pADE260, would not have been visible in Fig. 5.5.

### 6.2.1.4 Codon and base usage

The codon usage for the *pmi*$_{stm}$ gene is shown (Table 6.1). Modulator codons (indicated in Table 6.1 with asterisks) account for 1.0% of the total codon content, suggesting that they play only a minor role in the expression of this gene. The frequency of optimal codon usage ($F_{op}$) values for *pmi*$_{stm}$ (Table 6.2 A) and *manA* (Table 6.2 B) were calculated using the method of Ikemura and Ozeki (1982). The values obtained were similar, 0.64 for *manA* and 0.62 for *pmi*$_{stm}$, and are indicative of moderately expressed genes (Ikemura and Ozeki, 1982). The G+C content of the *pmi*$_{stm}$ gene was 54.3%, which is slightly above the average for *E. coli* (51%).

## 6.2.2 The Pmi protein

### 6.2.2.1 Hydropathic profile and secondary structure of the Pmi protein

The hydropathic profile of the Pmi protein, according to the method of Kyte and Doolittle (1982), is indicative of overall hydrophobicity with a mean value of -0.1 (Fig. 6.3). This result suggests that Pmi is a globular protein.

## Table 6.1: Codon usage within the *pmi*$_{stm}$ gene

| CODON | CT. | %AGE | CODON | CT. | %AGE | CODON | CT. | %AGE | CODON | CT. | %AGE |
|---|---|---|---|---|---|---|---|---|---|---|---|
| TTT-Phe | 9 | 2.3% | TCT-Ser | 6 | 1.5% | TAT-Tyr | 8 | 2.0% | TGT-Cys | 0 | 0.0% |
| TTC-Phe | 10 | 2.6% | TCC-Ser | 5 | 1.3% | TAC-Tyr | 0 | 0.0% | TGC-Cys | 2 | 0.5% |
| TTA-Leu | 5 | 1.3% | TCA-Ser | 3 | 0.8% | TAA-*** | 0 | 0.0% | TGA-*** | 0 | 0.0% |
| TTG-Leu | 5 | 1.3% | TCG-Ser | 1 | 0.3% | TAG-*** | 0 | 0.0% | TGG-Trp | 3 | 0.8% |
| CTT-Leu | 6 | 1.5% | CCT-Pro | 9 | 2.3% | CAT-His | 4 | 1.0% | CGT-Arg | 7 | 1.8% |
| CTC-Leu | 4 | 1.0% | CCC-Pro | 3 | 0.8% | CAC-His | 4 | 1.0% | CGC-Arg | 5 | 1.3% |
| CTA-Leu* | 0 | 0.0% | CCA-Pro | 4 | 1.0% | CAA-Gln | 7 | 1.8% | CGA-Arg* | 1 | 0.3% |
| CTG-Leu | 26 | 6.6% | CCG-Pro | 10 | 2.6% | CAG-Gln | 10 | 2.6% | CGG-Arg* | 1 | 0.3% |
| ATT-Ile | 6 | 1.5% | ACT-Thr | 2 | 0.5% | AAT-Asn | 8 | 2.0% | AGT-Ser | 1 | 0.3% |
| ATC-Ile | 11 | 2.8% | ACC-Thr | 4 | 1.0% | AAC-Asn | 14 | 3.6% | AGC-Ser | 10 | 2.6% |
| ATA-Ile* | 0 | 0.0% | ACA-Thr | 0 | 0.0% | AAA-Lys | 16 | 4.1% | AGA-Arg* | 0 | 0.0% |
| ATG-Met | 9 | 2.3% | ACG-Thr | 7 | 1.8% | AAG-Lys | 5 | 1.3% | AGG-Arg* | 0 | 0.0% |
| GTT-Val | 7 | 1.8% | GCT-Ala | 7 | 1.8% | GAT-Asp | 6 | 1.5% | GGT-Gly | 4 | 1.0% |
| GTC-Val | 6 | 1.5% | GCC-Ala | 19 | 4.9% | GAC-Asp | 8 | 2.0% | GGC-Gly | 17 | 4.3% |
| GTA-Val | 4 | 1.0% | GCA-Ala | 1 | 0.3% | GAA-Glu | 18 | 4.6% | GGA-Gly* | 1 | 0.3% |
| GTG-Val | 8 | 2.0% | GCG-Ala | 22 | 5.6% | GAG-Glu | 11 | 2.8% | GGG-Gly* | 1 | 0.3% |

CT: number of times a given codon used; %age: percentage of total codons.
Modulating codons (see the text) are marked with a single asterisk.

**Table 6.2: Frequency of optimal codon utilization for *pmi*$_{stm}$ and *manA* (*E. coli* ).**

## (A) $F_{op}$ Calculation for *pmi*$_{stm}$

| AMINO ACID | OPTIMAL CODON | NO. | NON-OPTIMAL CODON | NO. |
|---|---|---|---|---|
| PHE | UUC | 10 | UUU | 9 |
| TYR | UAC | 0 | UAU | 8 |
| LEU | CUG | 26 | CUU,CUC,CUA,UUA,UUG | 20 |
| PRO | CCG | 10 | CCU,CCC,CCA | 16 |
| ARG | CGU,CGC | 12 | CGA,CGG,AGA,AGG | 2 |
| GLN | CAG | 10 | CAA | 7 |
| ILE | AUC | 11 | AUU,AUA | 6 |
| ASN | AAC | 14 | AAU | 8 |
| LYS | AAA | 16 | AAG | 5 |
| VAL | GUU,GUA,GUG | 19 | GUC | 6 |
| ALA | GCU,GCA,GCG | 30 | GCC | 19 |
| GLY | GGU,GGC | 21 | GGA,GGG | 2 |
| GLU | GAA | 18 | GAG | 11 |
| THR | ACU,ACC | 6 | ACA,ACG | 7 |
| | TOTAL | 203 | | 126 |

$$F_{op}\, pmi_{stm} = 0.62$$

## (B) $F_{op}$ Calculation for *manA*

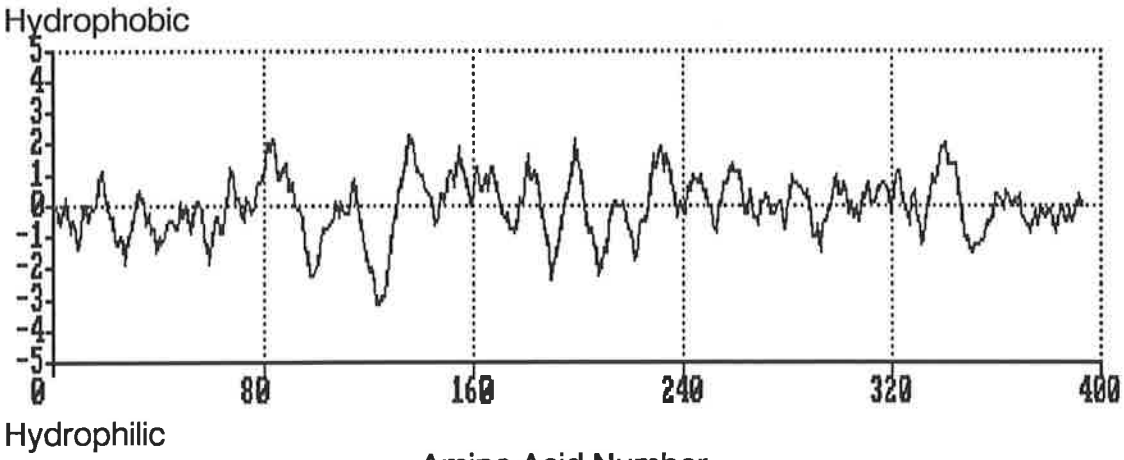
| AMINO ACID | OPTIMAL CODON | NO. | NON-OPTIMAL CODON | NO. |
|---|---|---|---|---|
| PHE | UUC | 11 | UUU | 9 |
| TYR | UAC | 4 | UAU | 3 |
| LEU | CUG | 21 | CUU,CUC,CUA,UUA,UUG | 26 |
| PRO | CCG | 17 | CCU,CCC,CCA | 9 |
| ARG | CGU,CGC | 10 | CGA,CGG,AGA,AGG | 1 |
| GLN | CAG | 16 | CAA | 6 |
| ILE | AUC | 3 | AUU,AUA | 14 |
| ASN | AAC | 12 | AAU | 7 |
| LYS | AAA | 20 | AAG | 2 |
| VAL | GUU,GUA,GUG | 19 | GUC | 4 |
| ALA | GCU,GCA,GCG | 25 | GCC | 20 |
| GLY | GGU,GGC | 19 | GGA,GGG | 2 |
| GLU | GAA | 23 | GAG | 6 |
| THR | ACU,ACC | 6 | ACA,ACG | 6 |
| | TOTAL | 206 | | 115 |

$$F_{op}\, manA = 0.64$$

**Figure 6.3: Hydropathic plot of the Pmi protein**

The 391 aa sequence of the Pmi protein was analyzed according to Kyte and Doolittle (1982) using a window of 9 aa. The average hydropathic index for this protein of -0.1 indicates overall hydrophilicity.

**HYDROPATHIC PLOT**
***Salmonella* PMI PROTEIN**



A predicted secondary structure for Pmi, according to the rules of Chou and Fasman (1974a, 1974b, 1978) is shown is Fig. 6.4.

### 6.2.2.2 Comparison of $pmi_{stm}$ with related sequences

A comparison of the DNA sequence of $pmi_{stm}$ with that of *manA* (Miles and Guest, 1984a) showed, firstly, that each protein contained 391 aa, encoded by 1173 bp. The DNA homology between the 2 genes was 77.5% (Fig. 6.5). The DNA homology was significantly higher in specific DNA regions, for example , at the proposed CRP binding site (95.5%), at the -10 and -35 regions (100%) and at the ribosome binding site (100%). The deduced amino acid sequences of $pmi_{stm}$ and *manA* shared homology of 86.2%. The aa differences between the 2 genes are shown (Table 6.3). No significant areas of homology were identified in a comparison of the $pmi_{stm}$ sequence with that of the *pmi* gene of *P. aeruginosa* ($pmi_{ps}$) (Darzins *et al.*, 1986).

The *fumA* gene for fumarase (EC 4.2.1.2) is positioned adjacent to the *manA* gene in *E. coli* K-12 and is transcribed from a promoter with opposite polarity to that of the *manA* gene (Miles and Guest, 1984a, 1984b). The cloned *S. typhimurium* DNA in pADE253 contained 364 bp of DNA upstream of the proposed -35 region of the $pmi_{stm}$ gene (Fig. 6.5). Within this region were sequences with strong homology to the -35 and -10 regions of *fumA* (Miles and Guest, 1984b). Also highly conserved were the RBS for this gene, and, at nt 232 from the start of the insert in pADE253, the *fumA* initiation codon (Fig. 6.5). The predicted amino acid coded by the region nt 232 to nt 1 was compared with the N-terminal amino acid sequence of the *fumA* gene (Miles and Guest, 1984b) and found to be 92.3% homologous for amino acids 1-39 (3 aa different) and to be non-homologous for amino acids 40-77 (Fig. 6.6). The DNA homology between the regions encoding aa 1-39 of FumA was 79.5% (Fig. 6.5). The dramatic loss of amino acid homology at aa 40 may be explained by the presence of a *Sau*3A1 site at nt 116 (Fig. 6.6) which may indicate the juxtapositioning of non-contiguous *Sau*3A1 fragments during the present cloning process.

**Figure 6.4 : Predicted secondary structure of the Pmi protein**

The 391 aa sequence of the PMI protein was analyzed using the algorithm of Chou and Fasman (1978). The various domains are illustrated as follows:

α - Helix : 

β - Sheet : 

β - Turn : 

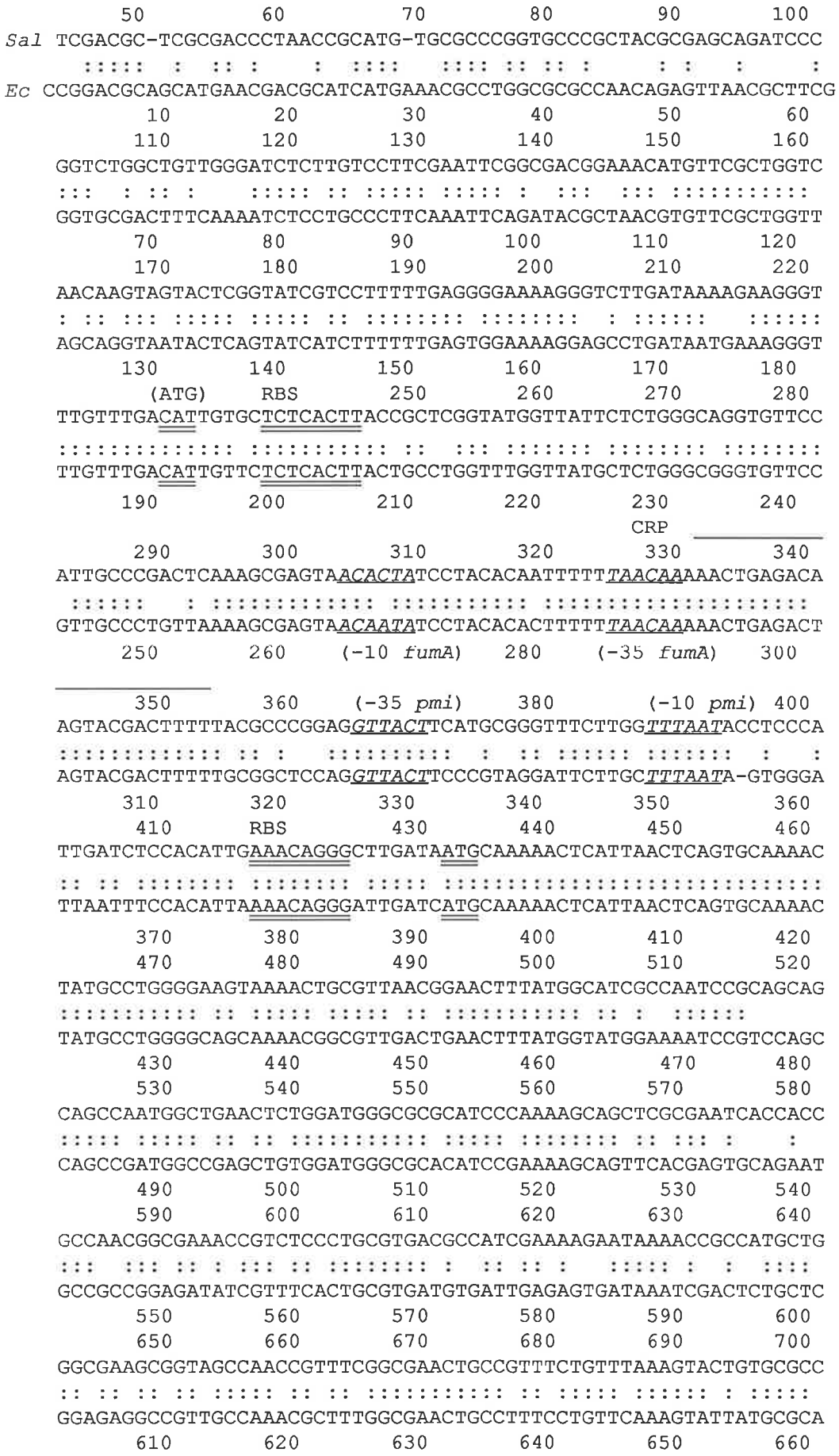
Random Coil : 


1
10
20
30
40
50
60
70
100
90
80
110
120
160
150
140
130
170
200
190
180
210
220
230
240
250
260
270
280
290
300
310
320
330
360
350
340
370
390
380

**Figure 6.5: Nucleotide sequence comparison of *pmi*$_{stm}$ and *manA***

Regions of DNA homology between the *pmi*$_{stm}$ (upper sequence) and *manA* (lower sequence) genes are illustrated. Nucleotide sequence matches are indicated by colons between the matching bases. Nucleotide co-ordinates attributed to each sequence are taken from Fig. 6.2 for *pmi*$_{stm}$ and from Miles and Guest (1984a) for *manA*. Potential -10 and -35 regions are italicised and underlined. A putative CRP binding site is overlined, and putative translational start sites and RBS regions are double underlined.

The region of nt 183-242 (*E. coli* K-12 sequence) contains the RBS and the initiation codon of the *E. coli* K-12 *fumA* gene (Miles and Guest, 1984b). Homology between *E. coli* K-12 DNA and *S. typhimurium* DNA (nt 222-281) may be seen in this region.


50 60 70 80 90 100
Sal TCGACGC-TCGCGACCCTAACCGCATG-TGCGCCCGGTGCCCGCTACGCGAGCAGATCCC
Ec CCGGACGCAGCATGAACGACGCATCATGAAACGCCTGGCGCGCCAACAGAGTTAACGCTTCG
10 20 30 40 50 60
110 120 130 140 150 160
GGTCTGGCTGTTGGGATCTCTTGTCCTTCGAATTCGGCGACGGAAACATGTTCGCTGGTC
GGTGCGACTTTCAAAATCTCCTGCCCTTCAAATTCAGATACGCTAACGTGTTCGCTGGTT
70 80 90 100 110 120
170 180 190 200 210 220
AACAAGTAGTACTCGGTATCGTCCTTTTTGAGGGGAAAAGGGTCTTGATAAAAGAAGGGT
AGCAGGTAATACTCAGTATCATCTTTTTTGAGTGGAAAAGGAGCCTGATAATGAAAGGGT
130 140 150 160 170 180
(ATG) RBS 250 260 270 280
TTGTTTGACATTGTGCTCTCACTTACCGCTCGGTATGGTTATTCTCTGGGCAGGTGTTCC
TTGTTTGACATTGTTCTCTCACTTACTGCCTGGTTTGGTTATGCTCTGGGCGGGTGTTCC
190 200 210 220 230 240
CRP
290 300 310 320 330 340
ATTGCCCGACTCAAAGCGAGTAACACTATCCTACACAATTTTTTAACAAAAACTGAGACA
GTTGCCCTGTTAAAAGCGAGTAACAATATCCTACACACTTTTTTAACAAAAACTGAGACT
250 260 (-10 fumA) 280 (-35 fumA) 300
350 360 (-35 pmi) 380 (-10 pmi) 400
AGTACGACTTTTTACGCCCGGAGGTTACTTCATGCGGGTTTCTTGGTTTAATACCTCCCA
AGTACGACTTTTTGCGGCTCCAGGTTACTTCCCGTAGGATTCTTGCTTTAATA-GTGGGA
310 320 330 340 350 360
410 RBS 430 440 450 460
TTGATCTCCACATTGAAACAGGGCTTGATAATGCAAAAACTCATTAACTCAGTGCAAAAC
TTAATTTCCACATTAAAACAGGGATTGATCATGCAAAAACTCATTAACTCAGTGCAAAAC
370 380 390 400 410 420
470 480 490 500 510 520
TATGCCTGGGGAAGTAAAACTGCGTTAACGGAACTTTATGGCATCGCCAATCCGCAGCAG
TATGCCTGGGGCAGCAAAACGGCGTTGACTGAACTTTATGGTATGGAAAATCCGTCCAGC
430 440 450 460 470 480
530 540 550 560 570 580
CAGCCAATGGCTGAACTCTGGATGGGCGCGCATCCCAAAAGCAGCTCGCGAATCACCACC
CAGCCGATGGCCGAGCTGTGGATGGGCGCACATCCGAAAAGCAGTTCACGAGTGCAGAAT
490 500 510 520 530 540
590 600 610 620 630 640
GCCAACGGCGAAACCGTCTCCCTGCGTGACGCCATCGAAAAGAATAAAACCGCCATGCTG
GCCGCCGGAGATATCGTTTCACTGCGTGATGTGATTGAGAGTGATAAATCGACTCTGCTC
550 560 570 580 590 600
650 660 670 680 690 700
GGCGAAGCGGTAGCCAACCGTTTCGGCGAACTGCCGTTTCTGTTTAAAGTACTGTGCGCC
GGAGAGGCCGTTGCCAAACGCTTTGGCGAACTGCCTTTCCTGTTCAAAGTATTATGCGCA
610 620 630 640 650 660

```
      710        720        730        740        750        760
GCCAAACCGCTCTCTATTCAGGTGCACCCGAATAAACGCAACTCCGAAATCGGTTTCGCG
::   : :: ::::: :::::::: :: :: :: :::: ::: :: :::::::::::: ::
GCACAGCCACTCTCCATTCAGGTTCATCCAAACAAACACAATTCTGAAATCGGTTTTGCC
      670        680        690        700        710        720
      770        780        790        800        810        820
AAAGAAAATGCGGCGGGTATCCCCATGGATGCCGCAGAGCGGAACTATAAAGATCCTAAC
:::::::::: :: :::::::: :::::::::: ::::: ::::::::::::::::::
AAAGAAAATGCCGCAGGTATCCCGATGGATGCCGCCGAGCGTAACTATAAAGATCCTAAC
      730        740        750        760        770        780
      830        840        850        860        870        880
CATAAACCAGAGCTGGTTTTTGCCCTGACGCCTTTCCTGGCGATGAACGCGTTCCGCGAA
:: :: :: :::::::::::::: :::::::::::::: :::::::::::::: :: :::
CACAAGCCGGAGCTGGTTTTTGCGCTGACGCCTTTCCTTGCGATGAACGCGTTTCGTGAA
      790        800        810        820        830        840
      890        900        910        920        930        940
TTTTCTGACATTGTCTCTTTACTGCAACCTGTCGCCGGCGCGCATTCCGCTATCGCCCAC
::::: :: ::::::::  :::: :: :: ::::: :: :: ::: : :: :: :: :::
TTTTCCGAGATTGTCTCCCTACTCCAGCCGGTCGCAGGTGCACATCCGGCGATTGCTCAC
      850        860        870        880        890        900
      950        960        970        980        990       1000
TTTTTGCAGGTGCCGAATGCTGAACGTCTGAGCCAGCTTTTCGCCAGCCTGTTGAATATG
::::: ::   :::  :::: :::::: : ::: : :: ::::::::::::::::::::::
TTTTTACAACAGCCTGATGCCGAACGTTTAAGCGAACTGTTCGCCAGCCTGTTGAATATG
      910        920        930        940        950        960
     1010       1020       1030       1040       1050       1060
CAAGGCGAAGAAAAATCCCGCGCGTTAGCCGTACTCAAAGCGGCGCTTAACAGCCAGCAA
:: :: :::::::::::::::::: : ::  :  : ::: :::: ::  : ::::::::
CAGGGTGAAGAAAAATCCCGCGCGCTGGCGATTTTAAAATCGGCCCTCGATAGCCAGCAG
      970        980        990       1000       1010       1020
     1070       1080       1090       1100       1110       1120
GGCGAACCGTGGCAAACGATCCGCGTGATTTCAGAGTATTATCCTGACGACAGCGGGCTT
:: :::::::::::::::: ::  : ::::: :: : ::: :: :: :::::::: ::
GGTGAACCGTGGCAAACGATTCGTTTAATTTCTGAATTTTACCCGGAAGACAGCGGTCTG
     1030       1040       1050       1060       1070       1080
     1130       1140       1150       1160       1170       1180
TTCTCTCCTTTGTTGCTGAATGTGGTCAAACTGAATCCCGGCGAGGCGATGTTCCTGTTT
::::: ::  : :::::::::::::: ::: :::: :: ::::: ::::::::::::::
TTCTCCCCGCTATTGCTGAATGTGGTGAAATTGAACCCTGGCGAAGCGATGTTCCTGTTC
     1090       1100       1110        1120      1130       1140
     1190       1200       1210       1220       1230       1240
GCTGAAACGCCTCATGCTTATCTGCAGGGCGTTGCGCTGGAAGTCATGGCGAACTCCGAT
:::::::: :: :: ::::: ::::: ::::: :::::::::: ::::: :::::::::
GCTGAAACACCGCACGCTTACCTGCAAGGCGTGGCGCTGGAAGTGATGGCAAACTCCGAT
     1150       1160       1170       1180       1190       1200
     1250       1260       1270       1280       1290       1300
AACGTTCTGCGCGCTGGCCTTACGCCAAAATATATCGACATCCCTGAGCTGGTCGCGAAC
::::: ::::: :: :: :: ::::: ::::: :: :: :: :: :: ::::: :: ::
AACGTGCTGCGTGCGGGTCTGACGCCTAAATACATTGATATTCCGGAACTGGTTGCCAAT
     1210       1220       1230       1240       1250       1260
     1310       1320       1330       1340       1350       1360
GTGAAGTTCGAACCTAAGCCTGCCGGCGAGTTGCTGACTGCCCCGGTGAAAAGCGGCGCG
::::: :::::: : :: :: ::   : ::::: ::::    :::::::::   :: ::
GTGAAATTCGAAGCCAAACCGGCTAACCAGTTGTTGACCCAGCCGGTGAAACAAGGTGCA
     1270       1280       1290       1300       1310       1320
```

```
     1370       1380       1390       1400       1410       1420
GAGCTGGACTTCCCAATTCCGGTTGACGATTTTGCTTTTTCACTGCACGACCTGGCGCTT
:: :::::::::::: ::::: :: :: :::::::: :: :: ::::: :::::      :
GAACTGGACTTCCCGATTCCAGTGGATGATTTTGCCTTCTCGCTGCATGACCTTAGTGAT
     1330       1340       1350       1360       1370       1380
     1430       1440       1450       1460       1470       1480
CAGGAGACGAGCATCGGCCAACACAGCGCCGCGATTCTGTTCTGCGTTGAGGGTGAGGCG
 : :: :: : :::  :::: :: :: ::::: ::: :::::::::: :: :: :: ::
AAAGAAACCACCATTAGCCAGCAGAGTGCCGCCATTTTGTTCTGCGTCGAAGGCGATGCA
     1390       1400       1410       1420       1430       1440
     1490       1500       1510       1520       1530       1540
GTGTTACGTAAAGATGAACAGCGTCTGGTACTGAAGCCGGGTGAATCTGCCTTTATCGGC
  :::  : :::: :   ::::   :    :: :: :::::::::::: :: ::::: : :
ACGTTGTGGAAAGGTTCTCAGCAGTTACAGCTTAAACCGGGTGAATCAGCGTTTATTGCC
     1450       1460       1470       1480       1490       1500
     1550       1560       1570       1580       1590       1600
GCGGATGAGTCTCCGGTTAACGCCAGCGGCACGGGCCGTTTAGCGCGTGTTTATAACAAG
::  : :: :: ::::: :  : ::  :::: ::::::::::::::::::::: ::::::
GCCAACGAATCACCGGTGACTGTCAAAGGCCACGGCCGTTTAGCGCGTGTTTACAACAAG
     1510       1520       1530       1540       1550       1560
     1610       1620       1630       1640
CTGTAGCAACGTACTGAATTTTTTAACAACTCTTGCTAAGCTT
:::::  : : :::::::    :::::: ::::::::::::::
CTGTAAGAGCTTACTGAAAAAATTAACATCTCTTGCTAAGCTT
     1570       1580       1590       1600
```

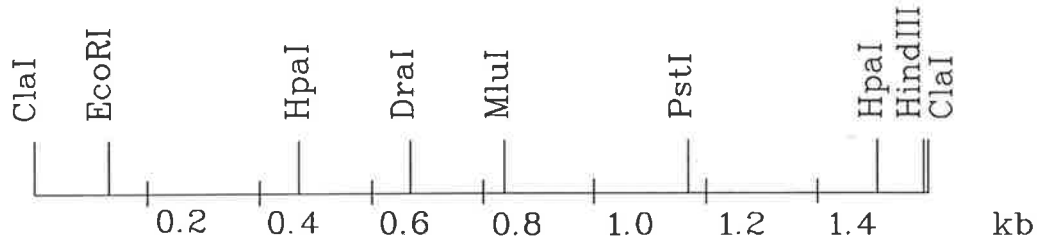
**Table 6.3: Amino acid differences between the Pmi proteins of *S. typhimurium* and *E. coli* K-12**

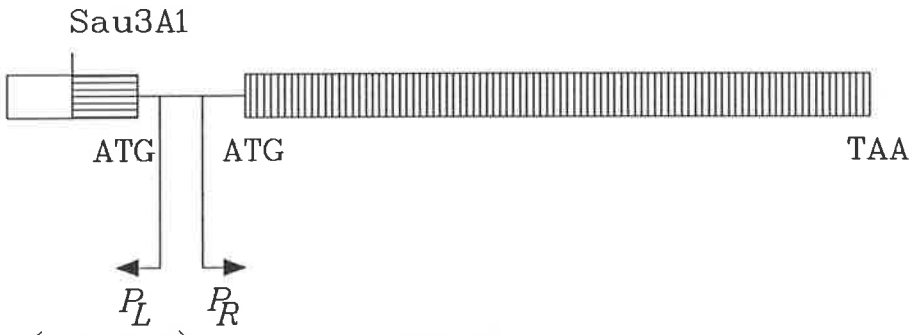
| No. | Amino acid residue | |
|---|---|---|
| | *S. typhimurium* | *E.coli K-12* |
| 25 | Ile | Met |
| 26 | Ala | Glu |
| 29 | Gln | Ser |
| 30 | Gln | Ser |
| 48 | Ile | Val |
| 49 | Thr | Gln |
| 50 | Thr | Asn |
| 52 | Asn | Ala |
| 54 | Glu | Asp |
| 55 | Thr | Ile |
| 61 | Ala | Val |
| 64 | Lys | Ser |
| 65 | Asn | Asp |
| 67 | Thr | Ser |
| 68 | Ala | Thr |
| 69 | Met | Leu |
| 76 | Asn | Lys |
| 92 | Lys | Gln |
| 103 | Arg | His |
| 153 | Asp | Glu |
| 166 | Ser | Pro |
| 174 | Val | Gln |
| 176 | Asn | Asp |
| 182 | Gln | Glu |
| 201 | Val | Ile |
| 204 | Ala | Ser |
| 207 | Asn | Asp |
| 219 | Val | Leu |
| 223 | Tyr | Phe |
| 226 | Asp | Glu |
| 295 | Pro | Ala |
| 299 | Gly | Asn |
| 300 | Glu | Gln |
| 304 | Ala | Gln |
| 308 | Ser | Gln |
| 329 | Ala | Ser |
| 330 | Leu | Asp |
| 331 | Gln | Lys |
| 334 | Ser | Thr |

| | | |
|---|---|---|
| 336 | Gly | Ser |
| 338 | His | Gln |
| 349 | Glu | Asp |
| 351 | Val | Thr |
| 353 | Arg | Trp |
| 355 | Asp | Gly |
| 356 | Glu | Ser |
| 358 | Arg | Gln |
| 360 | Val | Gln |
| 370 | Gly | Ala |
| 372 | Asp | Asn |
| 377 | Asn | Thr |
| 378 | Ala | Val |
| 379 | Ser | Lys |
| 381 | Thr | His |

**Figure 6.6 : Genetic organization of the 1,650 bp *Cla*I $pmi_{stm}$ fragment of cloned *S. typhimurium* DNA**

A partial restriction map of the 1,650 bp *Cla*I fragment of pADE253, which encodes the 391 aa Pmi protein (hatched area) is shown. The rightward promoter ($P_r$) is the promoter of the $pmi_{stm}$ gene. The translational start and stop codons for the $pmi_{stm}$ gene are shown. The leftward promoter ($P_l$) may be the promoter for a *fumA*-like gene (see the text); the translational start codon for this gene is shown. The demarcation point between the *fumA* -like (horizontal lines) and the non *fumA* -like (open box) coding regions of this ORF, leftwards of $pmi_{stm}$, is indicated. The junction corresponds to a *Sau*3A1 site, as mentioned in the text.


ClaI
EcoRI
HpaI
DraI
MluI
PstI
HpaI
HindIII
ClaI
0.2
0.4
0.6
0.8
1.0
1.2
1.4
kb


Sau3A1
ATG
ATG
TAA
$P_L$
$P_R$

(FUM) PMI

## 6.3 Discussion

Determination of the nucleotide sequence of the 1650 bp *Cla* I DNA fragment in pADE253 identified a 391 aa ORF which encoded a protein of $M_r$ 42,600. This protein size was consistent with the protein of 42 kDa seen in minicell protein analysis of DS410/pADE253 (Section 5.2.4.1). The 0.4 kb *Hpa* I - *Mlu* I deletion of $pmi_{stm}$ in pADE254 (Section 5.2.3) represented a deletion from nt 487 to nt 868. From the sequence data it is predicted that this deletion would not involve a frameshift in the ORF of $pmi_{stm}$ and would result in the production of a truncated ($M_r$ 28,705) version of the Pmi protein. This size is in good agreement with that of 28 kDa assigned to the truncated protein detected in minicells of DS410/pADE254 (Section 5.2.4.1). The $M_r$ 42,600 Pmi protein of *S. typhimurium* was very similar in size to the $M_r$ 42,716 Pmi protein of *E. coli* K-12 (Miles and Guest, 1984a), but was significantly different in size from the $M_r$ 52,860 Pmi protein of *P. aeruginosa* (Darzins *et al.*, 1986).

The nucleotide sequence upstream of the ORF start revealed a potential promoter structure with good homology to the -10 and -35 consensus sequences for RNA polymerase recognition and binding domains. The 17 bp distance between the -10 and -35 regions is optimal for promoter activity. Upstream from these regions was a potential CRP binding site suggesting that the gene is subject to catabolite repression.

Downstream of the promoter region a S-D site, with strong homology to the consensus S-D sequence, was evident. The 7 nt spacing between the S-D domain and the start codon suggests that the probability of efficient ribosome binding and translation initiation at this site is high.

The Pmi protein of *S. typhimurium* may be moderately well expressed in *S. typhimurium*. The frequency of optimal codon usage is almost identical for $pmi_{stm}$ ($F_{op}$ 0.61) and *manA* ($F_{op}$ 0.61) and similar to that of $pmi_{ps}$ ($F_{op}$ 0.67; calculated from data of Darzins *et al.*, 1986).

The hydropathic profile of the *S. typhimurium* Pmi protein suggested that the protein was water-soluble, consistent with the localization of this protein in the cytoplasmic compartment (Section 5.2.5).

A probe consisting of the $pmi_{stm}$ gene displayed strong homology with chromosomal sequences of *E. coli* DH1 (Section 5.2.7). Comparison of the sequences of $pmi_{stm}$ and *manA* revealed 77.5% nucleotide and 86.2% amino acid homology (Section 6.2.2.2).

Fumarase is a component of the TCA cycle involved in the interconversion of fumarate and malate. Three genes involved in fumarase biosynthesis have been cloned (*fumA*, *fumB* and *fumC* ) and it has been postulated that *fumC* is the structural gene for fumarase while *fumA* and *fumB* are positive regulatory elements (Guest and Roberts, 1983). In *E. coli* K-12,the *fumA* gene lies adjacent to the *E. coli* K-12 *manA* gene (Miles and Guest, 1984a, 1984b). Sequences with homology to the 5' end of the *E. coli* K-12 *fumA* gene were present upstream of the $pmi_{stm}$ gene in pADE253 (Section 6.2.2.2). Some 36 of the first 39 amino acids of the *fumA* gene were conserved in the *S. typhimurium* clone. The *manA* - *fumA* intergenic region , including the CRP binding sites for both genes (Miles and Guest, 1984a, 1984b), was highly conserved in pADE253. It is therefore suggested that a gene similar to that of *E. coli* K-12 *fumA* exists in *S. typhimurium*.

A summary of the genetic organization of pADE253 and predicted protein-encoding regions, as discussed above, is shown (Fig. 6.6).

# CHAPTER 7

## Construction and characterisation of site-directed mutations in the chromosome of *Salmonella typhimurium*, and analysis of the effects of these mutations on the virulence of *S. typhimurium* for mice.

## 7.1 Introduction

The increasing body of knowledge of the genes encoding virulence factors, and associated with regulatory networks involved in virulence, has necessitated the development of systems for modifying the genomes of pathogenic bacteria in a defined manner. Non-specific mutagenesis as a technique for the definition of virulence genes has serious drawbacks, including the possibility of simultaneous induction of multiple genetic lesions and concomitant difficulties with precise definition of the extent of DNA modification. The *galE* mutant Ty21a was derived by non-specific mutagenesis of *S. typhi* strain Ty2 (Germanier and Furer, 1975). In subsequent tests Ty21a was shown to have several additional genetic lesions not present in Ty2, including a *via* mutation (Germanier and Furer, 1983),an unknown mutation which slows growth (Germanier and Furer, 1975), and an inability to produce $H_2S$ (Germanier and Furer, 1983). The demonstration by Hone *et al.* (1988) that a *galE, via* double mutant of *S. typhi* was pathogenic in humans, indicated that the attenuation of virulence observed with Ty21a was due to some uncharacterized genetic defects.

The isolation of the non-toxinogenic *V.cholerae* strain Texas Star-SR (Honda and Finkelstein, 1979) by nitrosoguanidine mutagenesis, involved a mutation in one of the cholera toxin subunit genes, and an unknown mutation affecting colonizing ability. Evaluation of the effects of cholera toxin elimination on pathogenesis had to await the cloning of the toxin genes (Pearson and Mekalanos, 1982; Gennaro *et al.*, 1982), allowing the introduction of defined toxin subunit gene mutations into the chromosome of a wild-type *V.cholerae* strain (Mekalanos *et al.*, 1983; Kaper *et al.*, 1984).

Random chromosomal lesions have been generated by imprecise excision of transposon Tn*10* (Bochner *et al.*, 1980; Kleckner *et al.*, 1977), or by P2-mediated excision (Sunshine and Kelly, 1971). However the precise extent of mutations generated in this way is often difficult to ascertain.

The construction of site-specific mutations, followed by demonstration of complementation with the cloned wild-type gene, is therefore fundamental to the appraisal of the contribution of individual determinants to virulence. Replacement of specific chromosomal genes with mutated analogues has been acheived by F' homogenotization (Miller, 1972), by the formation and resolution of ColE1 plasmid cointegrates in a *polA* genetic background (Gutterson and Koshland, 1983), by integration and excision of engineered lambda *c* I857 vectors (Joyce and Grindley, 1984), and by transformation with linear DNA of strains carrying *recBC*, *sbcB* (Jason and Schimmel, 1984; Winans *et al.*, 1985) or *recD* (Shevell *et al.*, 1988) mutations. Recently, Hamilton *et al* (1989) have used a temperature-sensitive pSC101 replicon to promote replacement by homologous recombination of chromosomal genes with modified cloned genes.

In this chapter, the construction of site-directed mutations in the cloned *rfc* and *pmi* $_{stm}$ genes is described. Modifications to the temperature-sensitive pSC101-based replicon pHSG422, to facilitate plasmid mobilization and the cloning of specific DNA sequences, are outlined. Ultimately, the application of this vector to the recombination of the site directed *rfc* and $pmi_{stm}$ mutations into the chromosome of *S. typhimurium* is demonstrated, and the mutants are compared with the wild-type strain for virulence and colonisation ability in mice.

## 7.2 Results

### 7.2.1 Modifications of pHSG422

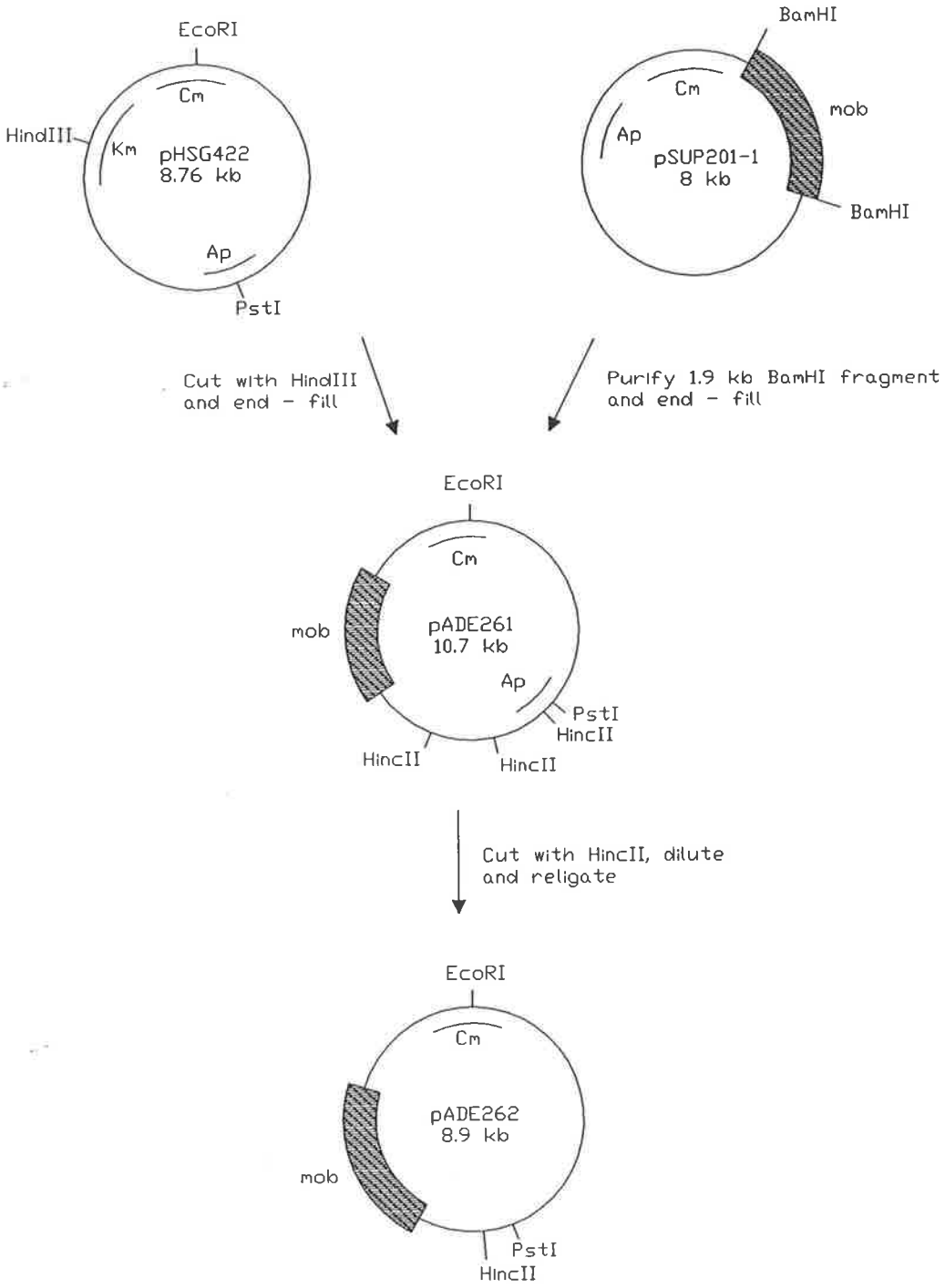
Plasmid pHSG422 is a low copy-number, mobilization-defective, pSC101-derived cloning vector which exhibits temperature-sensitive replication (Hashimoto-Gotoh and Sekiguchi, 1977; Hashimoto-Gotoh *et al.*, 1981 ). In order to expedite the transfer of mutated sequences cloned in pHSG422 into *S. typhimurium*, a mobilizable derivative was constructed. The recognition site for mobilization (*mob* ) of RP4 was isolated on a 1.9 kb *Bam*HI fragment from plasmid pSUP201-1 (Simon *et al.*, 1983). The *Bam*HI ends were made flush with DNA polymerase I, and ligated to the blunt-ended *Hin*dIII site of pHSG422 (Fig. 7.1). The resultant plasmid, pADE261, had lost Km-resistance and could be conjugated from *E. coli* S17-1 into *S. typhimurium* C5 by selection for $Ap^R$ and $Cm^R$ on minimal agar plates with incubation at 37°C. Extremely poor growth of C5/pADE261 was observed on this medium when the strain was incubated at 42°C. Removal of the $Ap^R$ determinant from this plasmid would enable cloning of DNA fragments from other $Ap^R$ plasmids without the requirement for fragment purification. Deletion of the 1.0 kb and 0.9 kb *Hinc*II fragments of pADE261 was effected by restriction with *Hinc*II followed by 50-fold dilution of the DNA prior to religation and transformation into S17-1. The 8.9 kb plasmid pADE262 derived in this manner was $Cm^R$, $Ap^S$ and was found to contain a single *Hinc* II site (Fig. 7.1).

### 7.2.2. Construction of a site-specific *rfc* mutation in *S. typhimurium*

Site-directed mutagenesis with the insertion of an antibiotic-resistance determinant enabled the introduction of a precise *rfc* mutation into an otherwise wild-type strain of *S.*

**Figure 7.1: Construction of mobilizable derivatives of pHSG422**

The mobilizable plasmid pADE261 was derived from pHSG422 by addition of the *mob* region of RP4 from pSUP201-1. The *mob* region, as a 1.9 kb *Bam*HI fragment, was end-filled and ligated with end-filled, *Hin*dIII-cut pHSG422, with resultant loss of the $Km^R$ activity. A derivative of pADE261, pADE262, was next constructed, in which a deletion removed the two smaller *Hinc*II fragments, and, as a consequence, the $Ap^R$ determinant was partially removed.


EcoRI
Cm
HindIII
Km
pHSG422
8.76 kb
Ap
PstI
BamHI
Cm
mob
Ap
pSUP201-1
8 kb
BamHI
Cut with HindIII
and end - fill
Purify 1.9 kb BamHI fragment
and end - fill
EcoRI
Cm
mob
pADE261
10.7 kb
Ap
PstI
HincII
HincII
HincII
Cut with HincII, dilute
and religate
EcoRI
Cm
pADE262
8.9 kb
mob
PstI
HincII

*typhimurium*. Earlier (Section 3.2.2), an IS*10* insertion in the *rfc* locus of *S. typhimurium* C5 was characterised. Transposon-induced mutations may be subject to subsequent transpositional events which result in reversion or secondary mutations. The genetic stability of the *rfc*::IS*10* construct during long-term (several weeks) tests of strain virulence in animals could therefore be suspect.

### 7.2.2.1 Insertion of a $Km^R$ cartridge into *rfc*

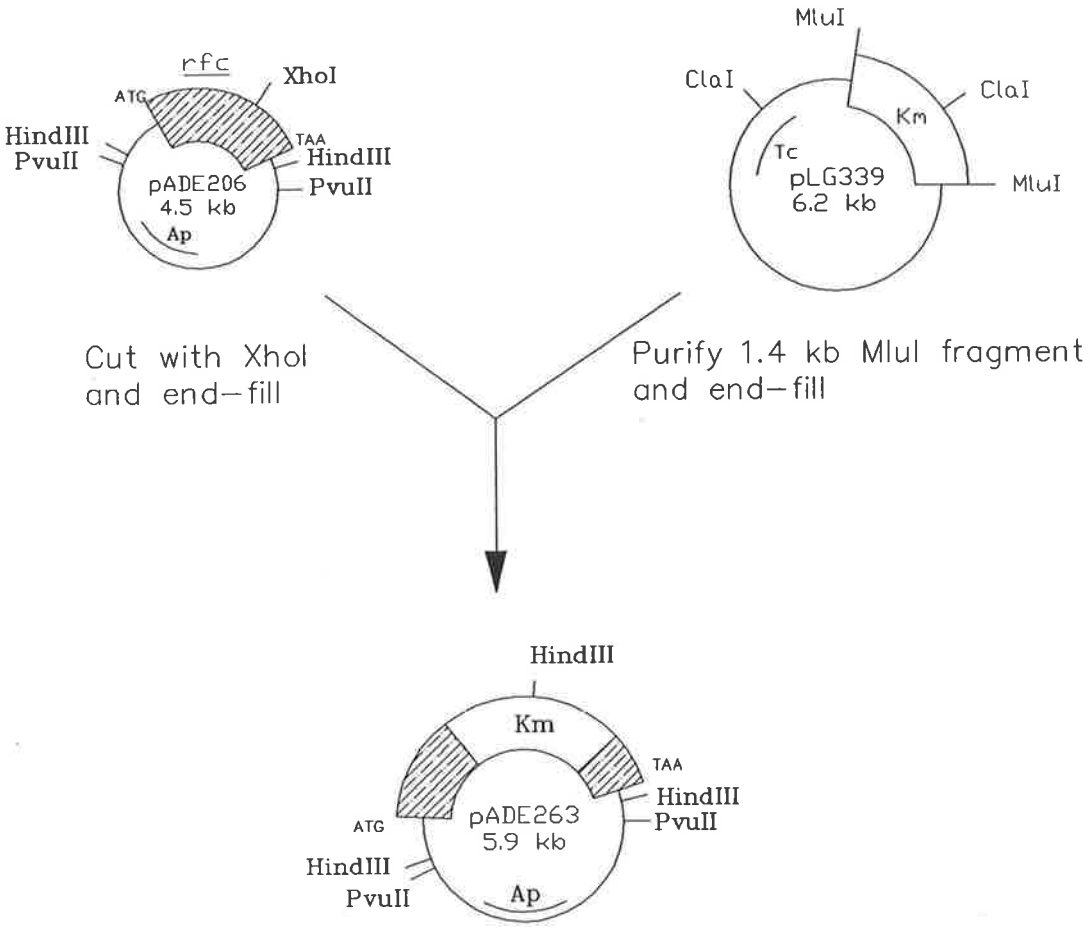
The *rfc* -encoding region was identified, and the DNA sequence of the presumed *rfc* gene established (Chapters 3 and 4). A $Km^R$-encoding 1.4 kb *Mlu* I fragment of pLG339 (Stoker *et al.*, 1982) was purified, end-filled with DNA polymerase I, and ligated into the end-filled *Xho* I site of pADE206 (nt 1282, Fig 4.2). The plasmid pADE263 (Fig. 7.2) effected resistance to Km and Ap. Strain SL1101, transformed with this plasmid, and grown with mannose, had the bacteriophage profile $9NA^R$, $P22^R$, $FO^S$, $P22I^S$, indicating that the cloned *rfc* gene had been inactivated by insertion of the $Km^R$ fragment.

### 7.2.2.2 Sub-cloning *rfc* ::$Km^R$ into pADE262

The 3.4 kb *Pvu* II fragment of pADE263, containing the *rfc* ::$Km^R$ construct (Fig. 7.2), was inserted into the *Hinc*II site of pADE262 (Fig. 7.1). A $Cm^R$, $Km^R$ derivative (pADE264) was selected at 30°C after transformation into S17-1. Poor viability of S17-1/pADE264 on L medium containing Cm and Km at 42°C indicated that the plasmid was lost at the non-permissive temperature.

**Figure 7.2: Construction of the *rfc*$^-$, Km$^R$ plasmid pADE263**

Plasmid pADE206 was cut with *Xho*I and end-filled prior to ligation with the 1.4 kb *Mlu*I fragment of pLG339, which contains a Km$^R$ determinant. The resultant plasmid, pADE263, carried Ap$^R$ and Km$^R$ determinants and an interrupted *rfc* gene.


rfc
XhoI
ATG
TAA
HindIII
PvuII
HindIII
PvuII
pADE206
4.5 kb
Ap
MluI
ClaI
ClaI
Km
Tc
pLG339
6.2 kb
MluI
Cut with XhoI
and end-fill
Purify 1.4 kb MluI fragment
and end-fill
HindIII
Km
TAA
HindIII
PvuII
ATG
pADE263
5.9 kb
HindIII
PvuII
Ap

### 7.2.2.3 Mobilization of pADE264

The chromosomally-integrated RP4 transfer genes (Simon *et al.*, 1983) in S17-1 enabled mobilization of plasmid pADE264 into C5 with selection on minimal agar plates containing Cm and Km, incubated at 30°C. The transconjugant, C5/pADE264, was found to contain a 12.3 kb plasmid and to have the phage phenotype of the smooth parent C5 (i.e. $9NA^S$, $P22^S$, $FO^S$, $P22I^R$) when grown at 30°C on L agar containing Km and Cm.

### 7.2.2.4 Construction and characterization of C5 *rfc*::$Km^R$

The proposed mechanism by which the chromosomal *rfc* gene was replaced by the cloned *rfc* ::$Km^R$ construct is outlined (Fig. 7.3). Strain C5/pADE264 was grown at the non-permissive temperature of 42°C in L broth containing Km. Gene replacement occurred initially by recombination between homologous sequences on pADE264 and on the C5 chromosome, to form a cointegrate. The strain was then plated on L agar containing Km at 30°C, allowing cointegrate resolution and regeneration of a plasmid. The plasmid and the chromosome now contained composite copies of the gene. A plasmid-free derivative, LV386, was subsequently selected by growth overnight in L broth with Km at 37°C and screened $Cm^S$. LV386 had a phage phenotype typical of SR mutants (i.e. $9NA^R$, $P22^R$, $FO^S$, $P22I^S$) (Table 7.1). The phage phenotype of LV386 was unaltered after transformation with pUC18, but was restored to that typical of smooth strains (i.e. $9NA^S$, $P22^S$, $FO^S$, $P22I^R$) upon introduction of the $rfc^+$ clone pADE206 (Table 7.1).

Strains C5 and LV386 were analysed by Southern hybridization to confirm that the wild-type gene on the chromosome had been mutated. Whole genomic DNA was isolated from both C5 and LV386 and digested with either *Eco*RI or *Pst*I, prior to electrophoresis and

**Figure 7.3: Introduction of the defined *rfc*::Km$^R$ mutation into the chromosome of C5**

In the first step, plasmid pADE264 (Cm$^R$, Km$^R$) was mobilized into C5. A cross-over event took place between the chromosomal *rfc* gene (cross-hatched area) and the cloned *rfc* gene (horizontal dashed area), which contained the Km$^R$ element (box labelled "Km"). Growth at 42°C prevented plasmid replication and co-integrate formation occurred.

After growth at 30°C, a second recombination event occurred, which regenerated a plasmid. Both copies of the gene were now composites, and the Km$^R$ element was inserted in the target chromosomal *rfc* gene. The plasmid was lost by subsequent growth at 37°C.

P: *Pst* 1; E: *EcoR* 1

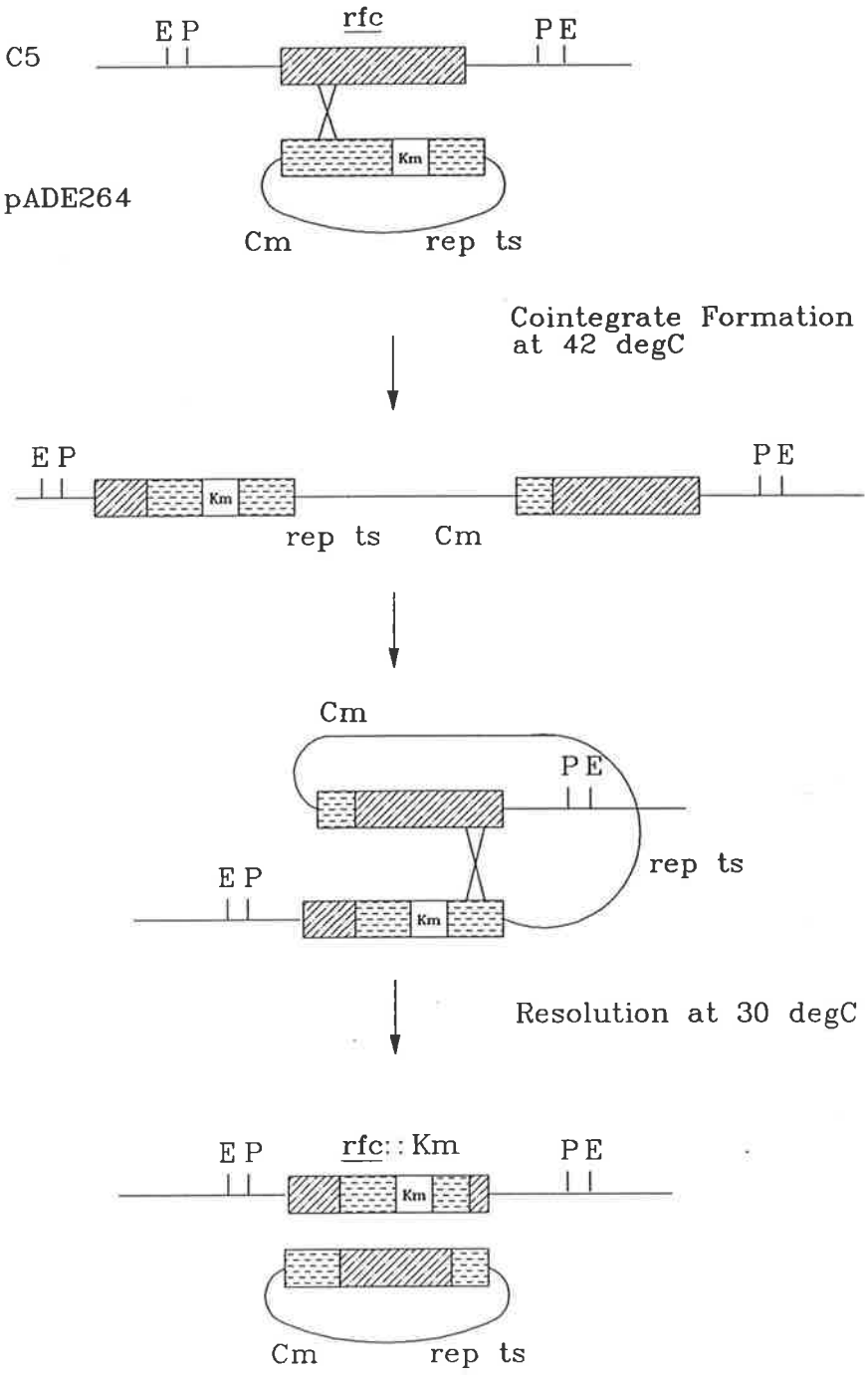

C5
E P
rfc
P E
Km
pADE264
Cm
rep ts
Cointegrate Formation
at 42 degC
E P
P E
Km
rep ts
Cm
Cm
P E
E P
rep ts
Km
Resolution at 30 degC
E P
rfc::Km
P E
Km
Cm
rep ts

**Table 7.1: The efficiency of plaquing of various bacteriophage on various strains**

| Strain | Phage | | | |
|---|---|---|---|---|
| | P22 | 9NA | FO | P221 |
| C5 | 1 | 1 | 1 | $<0.25X10^{-6}$ |
| LV386 | $<0.25X10^{-8}$ | $<0.25X10^{-8}$ | 3 | 1 |
| LV386/ pUC18 | $<0.25X10^{-8}$ | $<0.25X10^{-8}$ | 3 | 1 |
| LV386/ pADE206 | 1 | 1 | 1 | $<0.25X10^{-6}$ |
| LV374, glu | $<0.25X10^{-8}$ | $<0.25X10^{-8}$ | $<0.25X10^{-6}$ | 1 |
| LV374 glu, man | 1 | 1 | 1 | $<0.25X10^{-6}$ |
| LV374/ pBR322, glu | $<0.25X10^{-8}$ | $<0.25X10^{-8}$ | $<0.25X10^{-6}$ | 1 |
| LV374/ pBR322, glu, man | 1 | 1 | 1 | $<0.25X10^{-6}$ |
| LV374/ pADE253, glu | 1 | 1 | 1 | $<0.25X10^{-6}$ |
| LV374/ pADE253, glu, man | 1 | 1 | 1 | $<0.25X10^{-6}$ |

Bacteria, grown either in NB (C5), or in NB with either glucose alone (glu), or glucose and mannose (glu, man) were mixed with phage at various dilutions, and plaques counted after overnight incubation. The efficiencies of plaquing of P22, 9NA, and FO on C5 were taken as unity (underlined above). Similarly, the titre of P221 on LV386 was the reference efficiency of plaquing for this phage (underlined above).

transfer to a nitrocellulose filter. The filter was probed with the $\alpha$ -[$^{32}$P]-dCTP labelled, 0.4 kb *Acc* I fragment, internal to *rfc*, of pADE206 (Chapter 4). The autoradiograph (Fig. 7.4) demonstrated an increase in size of ca. 1.4 kb in the *Eco*RI and *Pst*I hybridizing fragments of LV386, compared with the corresponding fragments of C5.

The effect of the *rfc* ::Km integration in the C5 chromosome on the LPS chemotype was viewed by SDS-PAGE, with LPS-specific silver staining (Fig. 7.5). LPS samples of C5 (lane 1; smooth wild-type), SL901 (lane 2; SR), and TV-119 (lane 3; R) were used as standards. The LPS of LV386 (Fig. 7.5, lane 4) resembled that of SL901 (Fig. 7.5, lane 2) and was unchanged when transformed with pUC18 (Fig. 7.5, lane 5). The introduction of pADE206 into LV386, however, produced smooth LPS (Fig. 7.5, lane 6) resembling that of C5 (Fig. 7.5, lane 1).

These results confirmed that the altered *rfc* gene had replaced its wild-type counterpart on the chromosome (as outlined in Fig. 7.3).

### 7.2.3. Construction of a site-specific *pmi* mutation in *S. typhimurium*

The cloning and sequence data (see Chapters 4 and 5) on $pmi_{stm}$ provided sufficient information about the DNA encoding the Pmi function to enable targetted mutagenesis of the chromosomal *pmi* gene.

#### 7.2.3.1 Insertion of a Km$^R$ cartridge into *pmi*

The 1.4 kb *Mlu* I fragment of pLG339, carrying the Km$^R$ function (Stoker *et al.*, 1982) was purified, end-filled and ligated into the *Dra* I site (nt 686; Fig. 6.2) of pADE250 (Fig. 7.6). The recombinant plasmid, pADE265, was selected Ap$^R$ and Km$^R$ after

**Figure 7.4: Southern hybridization analysis of C5 and the C5 *rfc*::Km$^R$ recombinant LV386**

Total DNA of C5 and LV386 was digested with either *Pst*I or *Eco*RI and electrophoresed on a 0.8% (w/v) agarose gel. Following transfer to nitrocellulose, the filter was probed for homology with the α-[$^{32}$P]-dCTP-labelled 0.4 kb *Acc*I fragment of pADE206, washed, and subjected to autoradiography. The sizes of hybridizing fragments are indicated.

The detected *Pst* 1 and *EcoR*1 fragments of LV386 were 1.4kb larger than the respective fragments from C5.

The numbers are the sizes, in kb, of the indicated fragments.

Lane designations (with restriction enzyme used for cut in brackets): 1: C5(*Pst* 1); 2: LV386 (*Pst* 1); 3: C5(*Eco*R1); 4: LV386(*Eco*R1).

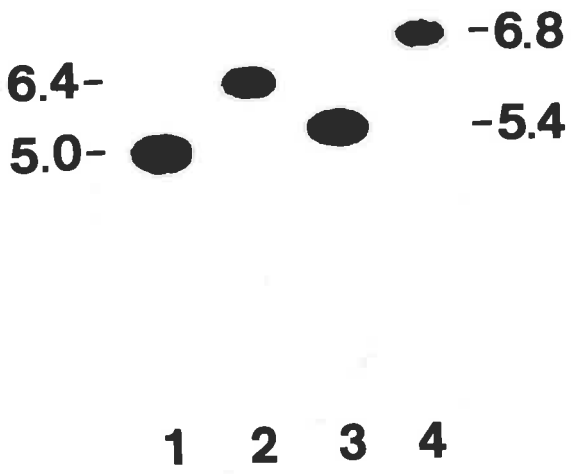

-6.8
6.4-
-5.4
5.0-
1 2 3 4

**Figure 7.5: LPS profiles of various strains in silver-stained SDS-PAGE**

Bacteria were lysed with SDS, treated with Proteinase K, and residual material analysed by SDS-PAGE with LPS-specific silver staining. Lane designations (with relevant genotype in brackets): 1: C5 (wild-type); 2: SL901 (*rfc* ); 3: TV-119 (*rfb* ); 4: LV386 (*rfc* ); 5: LV386/pBR322 (*rfc* ); 6: LV386/pADE206 ($rfc^+$ ). "R" denotes the position of rough (core) LPS; "SR" denotes the position of semi-rough LPS.

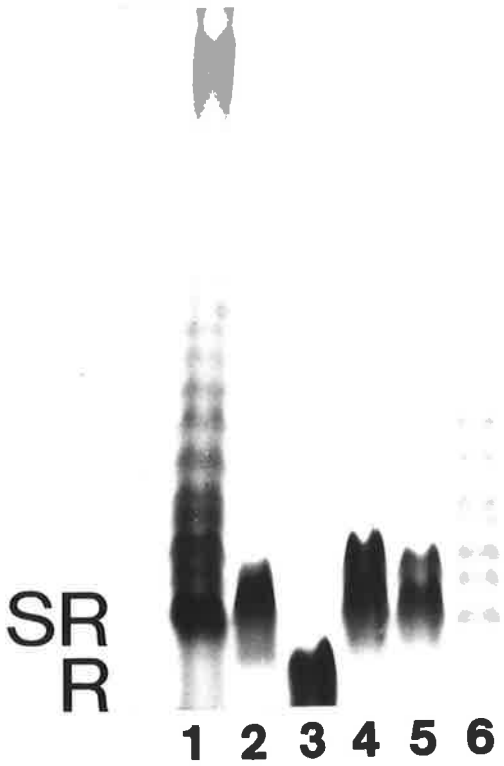
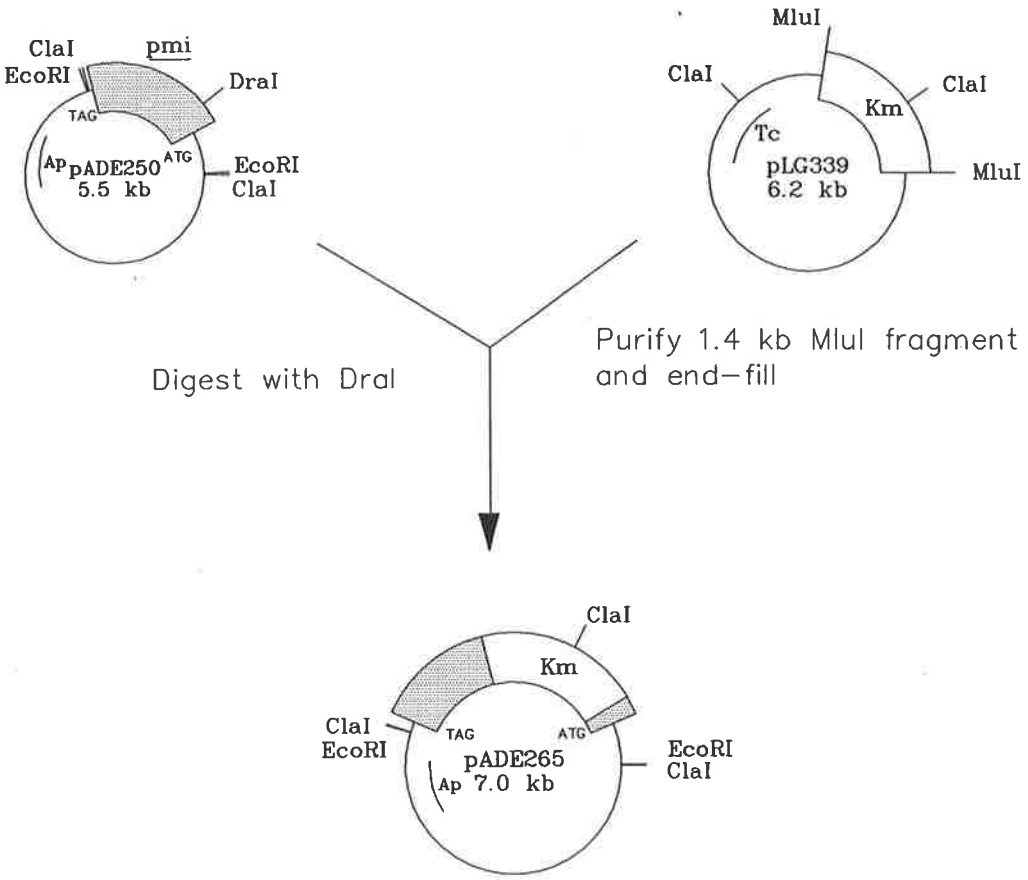

SR
R
1 2 3 4 5 6

**Figure 7.6: Construction of the *pmi* $^{-}$, $Km^R$ plasmid pADE265**

The $Km^R$ element in pLG339 was isolated on a 1.4 kb *Mlu*I fragment, end-filled, and ligated with *Dra*I digested pADE250. The resultant plasmid pADE265 carried $Ap^R$ and $Km^R$ determinants and an interrupted *pmi* gene.


ClaI
EcoRI
pmi
DraI
TAG
Ap
pADE250
ATG
5.5 kb
EcoRI
ClaI
MluI
ClaI
Km
ClaI
Tc
pLG339
6.2 kb
MluI
Digest with DraI
Purify 1.4 kb MluI fragment
and end-fill
ClaI
Km
ClaI
EcoRI
TAG
ATG
pADE265
Ap 7.0 kb
EcoRI
ClaI

transformation into S17-1. After transformation into SL1101 (*S. typhimurium pmi, rfc* ), pADE250 complemented the *pmi* mutation (see Section 5.2.1), but no complementation was observed with pADE265. It was inferred that the plasmid contained an inactivating $Km^R$ determinant in the $pmi_{stm}$ gene (termed *pmi* ::$Km^R$).

### 7.2.3.2 Sub-cloning *pmi*:$_{stm}$:$Km^R$ into pADE261

The 3.25 kb *Eco*RI fragment of pADE265 (Fig. 7.6), containing *pmi*::$Km^R$, was re-cloned into the *Eco*RI site of pADE261 (Fig. 7.1). The recombinant plasmid, pADE266, was transformed into S17-1 at 30°C with selection for $Km^R$ and $Ap^R$, and was found to be $Cm^S$. Poor viability of S17-1/pADE266 on L medium containing Ap and Km at 42°C indicated that the plasmid was lost at the non-permissive temperature.

### 7.2.3.3 Mobilization of pADE266

The chromosomally-integrated RP4 transfer genes (Simon *et al.*, 1983) in S17-1 were used to mobilize pADE266 into C5. Selection of transconjugants at 30°C on minimal agar plates containing Ap and Km, resulted in the isolation of C5/pADE266, which contained a ca. 14 kb plasmid and which had phage phenotype at 30°C on L agar containing Ap and Km characteristic of the smooth LPS chemotypes (i.e. $9NA^S$, $P22^S$, $FO^S$, $P22I^R$).

### 7.2.3.4 Construction and characterization of C5 *pmi*::$Km^R$

The protocol used for recombination of the $pmi_{stm}$::$Km^R$ construct into the C5 chromosome was identical to that used with pADE264 (Section 7.2.2.4). Strain C5/pADE266 was grown at the non-permissive temperature of 42°C in L broth containing Km and susequently plated on L agar containing Km at 30°C, to allow cointegrate

resolution. A Man$^-$ (Pmi$^-$) strain, LV374, carrying a chromosomal Km$^R$ insertion, was selected by growth overnight in L broth with Km at 37°C followed by plating on MTZ containing Km at 37°C. LV374 was Ap$^S$, having lost plasmid pADE266, and had a phage phenotype typical of R mutants (i.e. 9NA$^R$, P22$^R$, FO$^R$, P22I$^S$) (Table 7.1). The phage and Pmi phenotype of LV374 were unaltered after transformation with pBR322, but were restored to smooth phage phenotype (i.e. 9NA$^S$, P22$^S$, FO$^S$, P22I$^R$)(Table 7.1), and Man$^+$, upon introduction of the *pmi*$_{stm}^+$ clone pADE253.

The effect of the integration of *pmi*$_{stm}$::Km on the LPS chemotype of C5 was viewed by SDS-PAGE, with LPS-specific silver staining (Fig. 7.7). LPS samples from TV119 (lane 1; R LPS), SL901 (lane 2; SR LPS), and C5 (lane 3; smooth LPS) were used as standards. The LPS of LV374 grown in a mannose-free glucose-containing medium was of the R form (lane 4); addition of mannose to the growth medium caused the synthesis of smooth LPS (lane 5). Transfer of pHC79 into LV374 did not affect the LPS chemotype of the glucose-grown strain (lane 6). Plasmid pADE253 (*pmi*$_{stm}^+$), however, in LV374 (lane 7) allowed the synthesis of smooth LPS even in mannose-free, glucose-containing medium.

The specific activity of the Pmi enzyme was low in LV374 (Table 7.2), but high levels could be detected in LV374/pADE253.

Total DNA preparations of C5 and LV374 were analysed by Southern hybridization to confirm that the wild-type gene on the chromosome had been mutated. Whole genomic DNA was isolated from both C5 and LV374 and digested with either *Pst* I or *Acc* I, prior to electrophoresis and transfer to a nitrocellulose filter. The filter was probed with the $\alpha$-[$^{32}$P]-dCTP labelled, 1.6 kb *Cla* I fragment of pADE253 (Chapter 5.7.7). The probe hybridised with the two (0.7 kb and 3.6 kb) *Pst* I fragments (Fig. 7.8, lane 2) and a single 3.3 kb *Acc* I fragment of C5 (Fig. 7.8, lane 4), as seen previously (Chapter 5.7.7). In the

**Figure 7.7: LPS profiles of various strains in SDS-PAGE**

Bacteria were lysed with SDS, treated with Proteinase K, and the residual material analysed by SDS-PAGE with LPS-specific silver staining. Lane designations (with relevant genotype in square brackets and growth conditions, where relevant, in round brackets) :1: TV119 [*rfb* ]; 2: SL901 [*rfc* ];3; C5 [wild-type]; 4; LV374 [*pmi* ](grown in glucose); 5: LV374 [*pmi* ] (grown in glucose and mannose); 6: LV374/pHC79 [*pmi* ] (grown in glucose); 7: LV374/pADE253 [wild-type] (grown in glucose). "R" denotes the location of rough (core) LPS; "SR" denotes the location of semi-rough LPS.

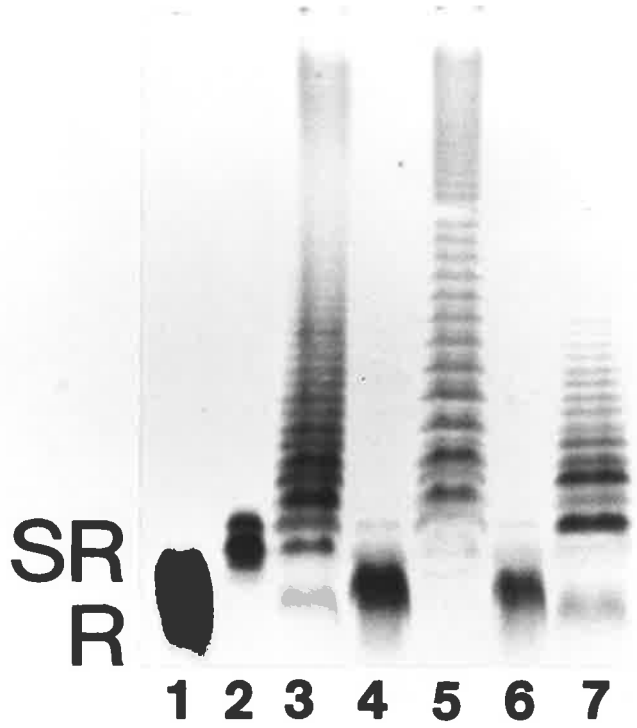

SR
R
1 2 3 4 5 6 7

**Table 7.2: Specific activity of the Pmi enzyme in various strains**

| STRAIN | Pmi enzyme activity* |
|---|---|
| C5 | 134 |
| LV374 | < 5 |
| LV374/pHC79 | < 5 |
| LV374/pADE253 | 248 |

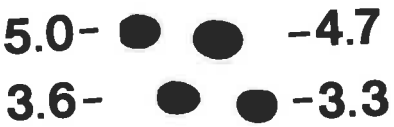
* One unit of activity is that converting one nanomole of mannose-6-phosphate to fructose-6-phosphate per min, per mg protein.

**Figure 7.8: Southern hybridization analysis of C5 and the C5 *pmi*::Km$^R$ recombinant LV374**

Total DNA of C5 and LV374 was digested with either *Acc*I or *Pst*I and electrophoresed on a 1%(w/v) agarose gel. Following transfer to nitrocellulose, the filter was probed for homology with the α-[$^{32}$P]-dCTP-labelled 1.6 kb *Cla*I fragment of pADE253, washed, and subjected to autoradiography. The sizes of hybridizing fragments are indicated by arrows on the left for the *Pst*I digests and on the right for the *Acc*I digests.

Two hybridising *Pst*I fragments (3.6 and 0.7 kb) were observed in C5 DNA. The probe hybridized with 5.0 and 0.7 kb *Pst*I fragments in LV374 DNA. Similarly, in the *Acc*I digests, the hybridizing fragment in LV374 (4.7 kb) was 1.4 kb larger than that in C5 (3.3 kb).

Lane designations (with restriction enzyme used for cut in brackets): 1: LV374 (*Pst* 1); 2; C5 (*Pst* 1); 3: LV374 (*Acc* 1); 4: C5 (*Acc* 1).




1 2 3 4

case of LV374, the probe hybridised with 0.7 kb and 5 kb *Pst* I fragments (Fig. 7.8, lane 1) and with a 4.7 kb *Acc* I fragment (Fig. 7.8, lane 3). In each case, the increase in size of the hybridizing fragment in LV374 is attributable to an insertion of the 1.4 kb $Km^R$ cartridge. Since the probe used contained the entire *pmi* gene it is highly likely that the $Km^R$ chromosomal insertion in C5 is in this gene.

### 7.2.4 The virulence and immunogenicity of the *rfc*::Km and the p$mi_{stm}$::Km constructs in mice

#### 7.2.4.1 The virulence and immunogenicity of the strains in mice

Groups of mice received graded doses of various strains orally, and deaths were enumerated to 28d post-feeding; $LD_{50}$ values for the strains were then calculated (Table 7.3).

The $LD_{50}$ value of C5 ($6X10^4$) in mice was similar to that previously observed (Hone *et al.*, 1987). Strain C5H1, a *galE* mutant of C5, was avirulent by the oral route, again as previously reported (Hone *et al.*, 1987). The *rfc* mutant of C5, LV386, was avirulent by the oral route ($LD_{50}$ of $>10^9$). The *pmi* mutant of C5, LV374, was avirulent when the strain was grown in mannose-free medium prior to feeding, and showed a much redeced virulence ($LD_{50}$ of $2X10^8$), compared with C5, when the strain was fed smooth (grown in mannose-containing medium prior to administration). When the *rfc* and *pmi* mutants were complemented with plasmids expressing the appropriate wild-type genes, reversion to the full wild-type virulence was observed (Table 7.3). This indicated that the attenuation observable in the *rfc* and *pmi* mutants was attributable to lesions in these genes alone.

**Table 7.3: The virulence of various strains of *S. typhimurium* for mice**

| Strain | $LD_{50}$ |
|---|---|
| C5 | $6X10^4$ |
| C5H1 (*galE* ) [glu, gal] | $>10^9$ |
| LV386 (*rfc*) | $>10^9$ |
| LV374 (*pmi* ), [glu] | $>10^9$ |
| LV374 (*pmi* ), [glu, man] | $2X10^8$ |
| LV386/pADE206 | $1X10^5$ |
| LV374/pADE253 | $8X10^4$ |

The genotypes are given in round brackets, and the sugar(s) present in the growth medium, where relevant, in square brackets.

Mice which survived infection with the *rfc* and *pmi* mutants were challenged orally with ca. 25 $LD_{50}$ values of C5, 28d after administration of the attenuated strains. Mice fed saline on d0 served as controls; all were dead by 10d post-challenge. The survival of the challenged mice was monitored for a further 28d.

As previously reported, C5H1 was an effective oral vaccine against subsequent C5 challenge (Hone *et al.*, 1987). Mice immunised with C5H1 at even $10^6$ bacteria /mouse were all resistant to the challenge (7/7 mice). Similarly, mice fed even $10^7$ bacteria of LV374 (grown in mannose-free medium) or LV386, were also all resistant to the challenge (7/7 and 6/6 mice, respectively).

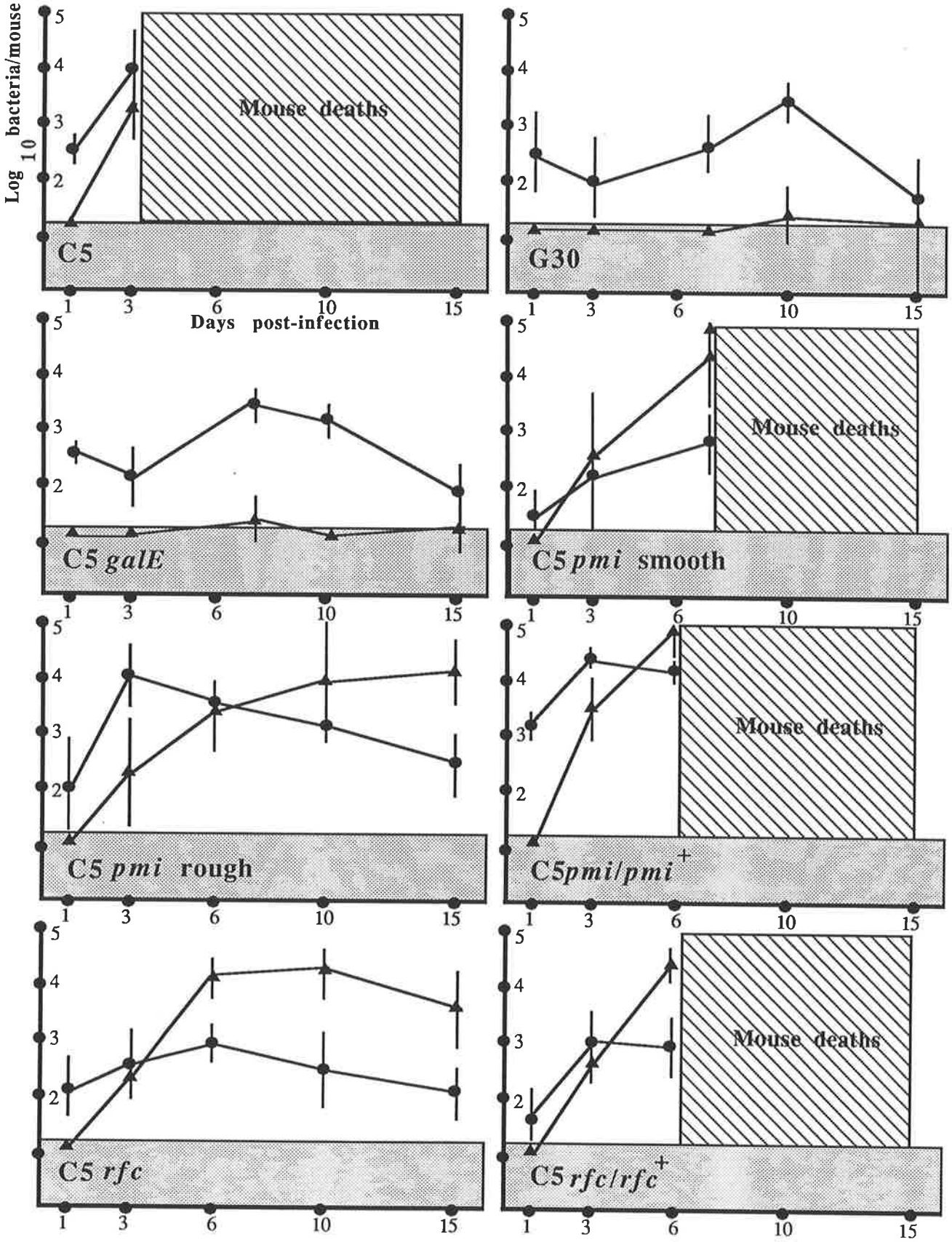
The observation that the *rfc* and *pmi* mutants were avirulent when given orally, yet were capable of immunising mice against later C5 challenge, suggested that the strains retained limited ability to colonise either or both of the intestinal or systemic immune tissues of the mouse. Groups of mice were fed 1.1-2.1X$10^8$ bacteria/mouse of various strains (35 mice/strain), and bacterial numbers in the Peyer's patches and the spleens enumerated at various times post-infection (Fig. 7.9).

The virulent strains C5, LV374/pADE253, and LV386/pADE206, effected rapid colonisation of both Peyer's patches and spleens, and mice succumbed from d3 (C5) or d6 (the other strains) post-infection. The *galE* mutants C5H1 and G30 effected transient colonisation of the Peyer's patches, but did not colonise the spleen, as previously reported (Hone *et al.*, 1987). The *pmi* mutant, LV374, fed after growth in mannose-containing medium (and hence phenotypically smooth), was effective in the colonisation of both Peyer's patches and spleens. By d7 post-infection, mice fed smooth-grown LV374 at this level were visibly ill, and 12 of the 20 mice remaining (the others having been sacrificed for enumeration of bacteria in organs) died to d20 post-infection.

**Figure 7.9: The colonisation of mice by various strains of *S. typhimurium*, given orally**

Groups (30) of mice received 1.1-2.1X$10^8$ bacteria of various strains, and subgroups of 5 mice were sacrificed at the times shown. Bacteria in the Peyer's patches and spleens were enumerated. The counts shown are geometric mean values,with standard errors of the mean. Strain C5*galE* is C5H1. The C5*pmi* strain is LV374. The C5*rfc* strain is LV386. The C5*pmi*/*pmi*$^+$ strain is LV374/pADE253. The C5*rfc*/*rfc*$^+$ strain is LV386/pADE206. Solid circles: bacterial numbers in Peyer's patches; solid triangles: bacterial numbers in spleens.

The limit of detection in these tests was 20 ($10^{1.3}$)bacteria / mouse.


Log 10 bacteria/mouse
Mouse deaths
C5
G30
Days post-infection
C5 galE
C5 pmi smooth
C5 pmi rough
C5pmi/pmi+
C5 rfc
C5 rfc/rfc+

When LV374 was fed after growth in mannose-free medium (which caused the strain to be phenotypically rough), colonisation of the Peyer's patches peaked by d3 post-infection, with bacterial counts in this tissue similar to those seen in C5-infected mice, but the bacterial numbers then declined. Spleen counts rose more slowly than was the case with mice fed C5, or the strains complemented in their attenuating mutation, LV374/pADE253 and LV386/pADE206. The levels of bacteria in the spleens seemed to level at ca. $10^4$ bacteria/mouse between d10 and d15 post-infection; the fact that the strain was essentially avirulent in mice when fed at this or higher levels (Table 7.3) implies that in the longer term, the mice were capable of the complete elimination of the bacteria.

The *rfc* mutant, LV386, colonised the Peyer's patches to a level apparently similar to that seen with *galE* mutants, but, unlike these mutants, was capable of progress from the Peyer's patches to the spleens, where infections corresponding to ca. $10^4$ bacteria/ mouse were established; the bacterial numbers being maintained until about d10 post-infection. Bacterial counts in the spleens were then observed to decline.

## 7.3 Discussion

The molecular cloning, and the DNA sequencing, of both the *rfc* (Chapters 3 and 4) and *pmi* $_{stm}$(Chapters 5 and 6) genes has been described. Construction of stable mutatnts of *S. typhimurium* in either the *rfc* or *pmi* genes, was effected by insertion of a $Km^R$ determinant within the coding region of each gene. A temperature-sensitive "suicide" vector was used to effect replacement of the chromosomal wild-type copy with the mutated gene. The phenotype of the recombinants, by phage-sensitivity tests and by LPS chemotype on SDS-PAGE, was identical to that of previously derived spontaneous mutants. The genetic defects resulting from recombination into the chromosome of either

the *rfc* ::$Km^R$ or the *pmi* $_{stm}$::$Km^R$ constructs could be complemented by introduction of the appropriate cloned gene. The chromosomal insertion in each case was confirmed by Southern hybridization analysis. In each case the results indicated the site-specific integration of the $Km^R$ cartridge into the chromosomal gene copy, resulting in inactivation of either *rfc* or *pmi*.

The strains thus constructed were examined for virulence and persistence in mice, in comparison with the wild-type C5 strain, a previously characterised *galE* mutant of that strain , C5H1 (Hone *et al.*, 1987), and an older *galE* mutant of *S. typhimurium* LT2, G30, which, unlike C5H1, is not sensitive to galactose-induced lysis. The C5 strain, and mutants in *rfc* and *pmi* which were complemented with plasmid-borne wild-type genes, were virulent in mice. The *galE* mutants were avirulent, as previously reported (Hone *et al.*, 1987). The *rfc* mutant of C5 was avirulent in mice, even when given at $10^9$ bacteria/mouse, and mice thus immunised were immune to a later challenge with 25 $LD_{50}$ values of C5. The *rfc* strain effected colonisation of the Peyer's patches and the spleens of mice. Spleen bacterial levels remained at ca. $10^4$ bacteria/mouse for ca. 5d, after which time bacterial numbers began to decline.

The C5 *pmi* mutant, fed rough, colonised the Peyer's patches and spleens of mice, but was avirulent even at ca. $10^9$ bacteria/mouse. In mice fed $1.4X10^8$ bacteria, Peyer's patch bacterial numbers peaked at d3 post-infection, while spleen numbers rose to ca. $10^4$ bacteria/mouse by d10 post-infection, and remained at that level until at least d15 post-infection. When the *pmi* mutant was fed smooth, at $1.2X10^8$ bacteria/ mouse, 12/30 mice died. It may be that the smooth LV374 was more effective than the rough bacteria in the colonisation of an area of the gut wall which was not the Peyer's patches, and that this focus of infection served as the source of sufficient bacteria to later overwhelm the mouse defences. Alternatively, it may simply be that the smooth-grown LV374 colonised the gut

more effectively than the rough bacteria, and that this translated into higher inocula for the infection of spleens. While the proposed differences in gut colonisation ability are not obvious in Fig. 7.9, the standard errors of the mean for a particular point are large enough to conceal small, but possibly critical, differences in the numbers of colonising bacteria.

The most obvious difference between the avirulent *galE* mutants, and the (largely) avirulent *pmi* and *rfc* mutants, was that the *galE* mutants appeared incapable of colonisation of the spleens from foci of infection in the Peyer's patches, while the other strains were capable of such behaviour. This may suggest that mutations at *pmi* or *rfc* are not sufficiently attenuating for use in the construction of *Salmonella*-based vaccine strains destined for human or animal use. It is possible, however, that the mutations may be of use in conjunction with other attenuating mutations (Chapter 1.4.2 ).The fact that a *pmi* mutant of C5 was greatly attenuated in mice suggests that mannose may not be freely available, in a form assimilable by *S. typhimurium*, in mouse tissues. This sugar, were it available, should convert the *pmi* mutant to a phenotypically smooth state, which should result in a strain $LD_{50}$ value identical to that of C5.

It may be reiterated here that a mutation attenuating for the virulence of *S. typhimurium* in mice need not be attenuating for the virulence of *S. typhi* in humans (Hone *et al.*, 1988).

As reviewed earlier (Chapter 1.4.2), a variety of mutations attenuating for *Salmonella* virulence have been described, and some of these (in particular mutations in *aro* loci) are proposed as useful in the construction of live oral human typhoid vaccines. Mutations at *aro* loci or *pur* loci may be rendered ineffective, at least in theory, by the availability in the animal of the chemical(s), the biosynthesis of which is affected by the mutation(s). While animals normally have very low levels of available aromatic chemicals or purines, the fact

that the attenuating *rfc* mutation cannot be neutralised in this manner may be an attractive feature for its use in vaccine construction.

As shown (Figs. 3.9 and 5.8 ), DNA segments of the cloned *rfc* and *pmi* $_{stm}$ genes probed DNA of *Salmonella* strains other than *S. typhimurium*, and (in the case of *pmi* $_{stm}$), DNA of *E. coli* K-12 and *S(higella) flexneri*. It follows that the plasmids constructed in this Chapter may be of use to effect insertion mutations in the chromosomes of these species. The study of the influence of a *pmi* mutation on the virulence of *S. flexneri* would be of interest.

# CHAPTER 8

## Conclusions

1. A 1.75kb *Hin*dIII fragment of *S. typhimurium* DNA complemented the *rfc* lesion in an older *rfc* mutant of *S. typhimurium*, SL901, and in new *rfc* mutants of *S. typhimurium* C5, J208-B1 and J208-B2, here described. The mutations are IS*10* insertions in *rfc*. Transposon insertion mutagenesis of the cloned *rfc*$^+$ fragment delimited the *rfc* DNA to 1.1-1.5kb. The complementation of the *rfc* defect was shown firstly by the restoration to the *rfc* mutants, by the cloned DNA, of the phage sensitivity profiles characteristic of wild-type strains. Secondly, the LPS of the complemented *rfc* mutants, examined by SDS-PAGE, was the smooth LPS characteristic of wild-type strains.

2. It was not possible to visualise a protein product of the *rfc* gene in a variety of systems designed to allow of the detection of plasmid-encoded polypeptides.

3. Total DNA from *Salmonella* strains of groups A, B, and D1 hybridised with a DNA fragment internal to the *rfc* gene. It appeared that 2 restriction sites (*Acc*1) within the *rfc* gene were conserved in these strains, but that the conservation of restriction sites outside, but adjacent to, the *rfc* gene, was not as marked. *Salmonella* strains of groups C1, C2, C3, D2, and E2 did not contain *rfc* -homologous DNA; neither did *E. coli* K-12.

4. DNA sequencing of the 1.75kb *rfc*$^+$ fragment showed the existence therein of an ORF of 1221 bp, with associated promoter and S-D sequences, which could encode a protein of 407 aa, with a $M_r$ of 47,472. A stable stem-and-loop structure could be formed at the translational terminus of this proposed *rfc* gene. Two direct repeats of 10 bp each occurred in the promoter region of the proposed gene.

5. The difficulties experienced in the visualisation of an Rfc protein may be explained by the fact that the putative *rfc* gene contained a very high level of modulating codons (12.5%).

6. The putative Rfc protein was hydrophobic in character, and may contain a large proportion of β-sheet structure, suggestive of the presence of transmembrane domains in the protein.

7. The $pmi_{stm}$ gene, encoding phosphomannose isomerase, was cloned from *S. typhimurium* by selection in a *manA* mutant of *E. coli* K-12, and the DNA region necessary for function defined by transposon insertion mutagenesis. The protein product of the gene was readily visualised as a 42kDa protein , in minicells. The protein was located in the cytoplasmic fraction of the minicells, and did not seem to be subject to post-translational processing. The cloned DNA conferred upon a *manA* mutant of *E. coli* K-12 the ability to synthesise greater than wild-type levels of the Pmi enzyme activity.

8. The cloned $pmi_{stm}$ gene hybridised with DNA from a variety of *Salmonella* strains of groups A, B, and D1. The DNA also probed DNA from *E. coli* K-12 and *S(higella) flexneri.*

9. DNA sequencing of the 1.6kb *Cla*I fragment carrying the $pmi_{stm}$ gene identified the ORF which encoded the Pmi protein, with associated promoter and S-D sequences. The ORF was 1173 bp in length, and encoded a protein of 391aa, with a $M_r$ of 42,600. The codon usage in the $pmi_{stm}$ gene indicated that modulator codons represented only 1.0% of the total codon content.

10. A comparison of the $pmi_{stm}$ sequence with the published sequence of the *E. coli* K-12 *manA* gene showed that both genes were very similar.The DNA homology was 77.5%, while the aa homology was 86.2%.

11. Both of the cloned *rfc* and $pmi_{stm}$ genes were interrupted *in vitro* with a Km-resistance cassette of 1.4kb, and the insertion mutations recombined into the chromosome of *S. typhimurium* C5, which is highly mouse-virulent. Both mutants were markedly attenuated in virulence, by the oral route of infecton, for the mouse, compared with C5. Indeed, the *rfc* mutant appeared to be completely avirulent in this test ($LD_{50}>10^9$). When the mutants were complemented by the appropriate plasmid-borne wild-type genes, mouse-virulence was restored to the C5 wild-type levels. Mice which had received the mutant strains were effectively protected against subsequent challenge with C5.

12. In short-term colonisation tests, it was shown that *rfc* or *pmi* (glucose-grown) mutants of C5, fed to mice, effectively colonised the Peyer's patches of the mouse intestine, and the spleens. This behaviour was in contrast to that of a *galE* mutant of C5, which effected Peyer's patch colonisation on feeding to mice, but which did not colonise the spleens to any noticeable extent. The difference between the *galE* mutant, on the one hand, and the *rfc* and *pmi* mutants, on the other, is that the latter are capable of the constitutive synthesis of LPS core. It may be proposed, then, that derivatives of C5 which maintain this property may transit from the Peyer's patches to the deeper tissues of the mouse post-infection, and that C5 derivatives which are not capable of constitutive LPS core synthesis cannot effect such a transition.

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