REGULATION OF EXPRESSION AND ACTIVITY OF THE LATE GENE ACTIVATOR, B, OF BACTERIOPHAGE 186.

A thesis submitted in fulfilment of the requirements for the degree of Doctor of Philosophy in the School of Molecular and Biomedical Sciences (Biochemistry Discipline) at the University of Adelaide.

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THESIS SUMMARY

Structural gene expression in the temperate double-stranded DNA coliphage 186 shows similarities with other phage – transcription from four promoters late in infection follows phage replication and is activated by B, a small zinc-finger protein in the P2 Ogr family. However, *B* and late gene regulation in 186 shows some novel aspects which were investigated here.

Unusually, the *B* gene is transcribed from early in infection, well before B activates the late promoters. It was shown here that multiple copies of the *B* gene, as provided by phage replication, are necessary to express sufficient protein to stimulate late transcription and complement a 186 B^- phage burst. This was due to non-linear (presumably cooperative) activation of the 186 late promoters by low levels of B protein. However, premature late promoter stimulation by early expression of high B levels, had little effect on the phage infection, consistent with phage replication also being required to amplify 186 late gene dosage.

In a 186 lysogen, *B* transcription is repressed by the CI protein, however, I found that the level of protein produced from a single-copy prophage is insufficient to activate the late promoters and harm the cell. Instead, unrepressed B expression from the phage (or a plasmid) reduced the frequency of lysogen formation – presumably due to B enhancing transcription of lethal 186 late function/s from a partly replicated genome, and killing cells which would otherwise enter lysogeny.

Finally, I characterized four bacterial mutants which it was hoped would reveal novel host proteins required for B activity. Unfortunately in these cells, B function and 186 infection were apparently reduced non-specifically by mutations affecting the copy number of plasmids used and phage adsorption respectively. Re-examination of the *rpoA*109 mutation showed that B interacts with the RNA polymerase α-subunit to enhance transcription, like several related proteins.

Thus, B cooperatively activates the 186 late promoters to allow rapid stimulation of transcription after phage replication increases the *B* and late gene dosage. This sensitivity of the late promoters to B protein means that its expression must be repressed during the establishment of 186 lysogens when some phage replication may occur.

DECLARATION

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

I give consent to this copy of my thesis, when deposited in the University Library, being available for loan and photocopying.

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"Rules for Living by Olivia Joules:

...– Nothing is either as bad or as good as it seems.

– If you start regretting something and thinking 'I should have done...' always add 'but then I might have been run over by a lorry or blown up by a Japanese-manned torpedo.' ..."

'Olivia Joules and the Overactive imagination' by Helen Fielding

"For what do we live, but to make sport for our neighbours, and laugh at them in our turn?"

'Pride and Prejudice' by Jane Austen

ABBREVIATIONS

am – amber mutation
Ap – ampicillin
bp – basepairs
cfu – colony-forming units
Cm – chloramphenicol
CTD – C-terminal domain
DNA – deoxyribonucleic acid
ds – double-stranded
eop – efficiency of plating
IPTG – isopropyl-β-D-thiogalactopyranoside
kb – kilobase (1000 basepairs)
kD – kilodalton
K_d – dissociation constant
Km – kanamycin
LB – Luria Broth
M – molar
MCS – multiple cloning site
μg – microgram
μl – microlitre
μM – micromolar
mg – milligram
ml – millilitre
mM – millimolar
nm – nanometres
ng – nanogram
NTD – N-terminal domain
OD – optical density
PCR – polymerase chain reaction
pfu – plaque-forming units
phage – bacteriophage
^{R} – resistant
RBS – ribosome binding site
RNA – ribonucleic acid
RNAP – RNA polymerase
rpm – revolutions per minute
Sp – spectinomycin
Tet – tetracycline
ts – temperature-sensitive
UV – ultraviolet
X-gal – 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

CHAPTER 1:

INTRODUCTION

1.A. BACTERIOPHAGE DEVELOPMENT.

1.A.1. A GENERAL INTRODUCTION TO BACTERIOPHAGES.

Bacteriophages or simply phages, are viruses that specifically infect bacterial cells. Phage particles consist of a protective protein structure, often icosahedral in shape, surrounding a single or double-stranded DNA or RNA genome, and are by themselves inert in the environment. However, when the virus attaches to a bacterial cell surface and injects its genome into the cell, it is able to use host proteins in order to produce more phage particles or virions. These progeny are then released, usually by phage-induced cell death. Some bacteriophages have an alternative lifecycle where, instead of making new phages following bacterial infection, the viral genome is inserted into the host chromosome and remains dormant in the cell. The inserted genome or prophage may also be stimulated to replicative development by an inductive signal, for example, damage to the host cell.

Bacteriophages are abundant and ubiquitous in the natural environment – as many as 10^8 virions per ml can be found in seawater and it is estimated that more than 10^{30} phage particles are present in total on the earth (Brussow and Hendrix, 2002). They are found everywhere that bacteria are present – in water, soil, the intestines of multicellular organisms (including humans) and even hot springs. The roles of phages in the environment, for example in controlling bacterial numbers and cycling of organic matter, are only just starting to be explored (for example Weinbauer, 2004). Bacteriophages that have been characterized so far show considerable diversity in morphology and genome sequence, which probably reflects the ability of phages to acquire and move DNA sequences (Brussow and Hendrix, 2002). This characteristic may also be important for the transfer of genes between host bacterial cells and the adaptation of these cells to different environments. About half of the bacterial chromosomes sequenced so far contain prophages or prophage-remnants and in a growing number of cases phage-derived sequences have been described which are involved in pathogenesis of the bacteria (Canchaya *et al.*, 2003; Canchaya *et al.*, 2004). However, bacteriophages may also provide a highly specific weapon against diseases caused by bacteria, and have been used as such in Georgia and parts of Russia for many years. The use of phages and phage products for the treatment of bacterial infections, especially by antibiotic-resistant strains, is now being developed also in the US and other Western countries (Summers, 2001). Classically in the West, however, bacteriophages have been used most commonly in molecular biology as a relatively simple and easily manipulated system for the study of gene expression and regulation. A few of

the most extensively studied examples are the double-stranded (ds) DNA coliphages lambda, T4, T7 and P2.

1.A.2. Developmental lifecycles of double-stranded DNA phages.

Most of the classically studied ds DNA bacteriophages have a similar virion morphology (Ackermann, 1998). This consists of an icosahedral-shaped capsid or head surrounding the ds DNA genome, which is connected to a tail structure containing all the proteins required for recognition and attachment of the virion to the host cell, and injection of the phage genome into that cell (see Figure 1.1A). During replicative or lytic development, these phages also show a similar pattern of gene expression for the production of progeny (Ackermann, 1998) (see Figure 1.1B for a stylized diagram). Early after infection, proteins are expressed which initiate replication of the phage genome and may also sequester or depress host cellular functions. Phage replication is followed by the expression of all genes required for the production and release of progeny phage particles. These late-expressed (or simply 'late') morphogenetic functions assemble into a pre-capsid structure, which is then packaged with the phage DNA and attached to a completed tail. Release of the mature virions is achieved by a phage-encoded holin-endolysin system for cell lysis, where the holin protein forms pores in the cell membrane allowing the endolysin protein access to cleave the peptidoglycan layer.

Temperate bacteriophages have an alternative lysogenic lifecycle in which lytic genes are not expressed and the phage genome is integrated into the bacterial chromosome where it is passively replicated with along the host DNA. This lifestyle is often controlled by a phage-encoded protein which directly or indirectly prevents transcription of lytic genes on the prophage. The lysogenic repressor is also able to prevent lytic development of a super-infecting phage, thereby giving the cell immunity to further infection. For coliphage lambda, lysogenic cells are formed more frequently when the host cell is starved of nutrients, suggesting that lysogenic development may allow phage production to be delayed until cells are healthier and a larger burst of progeny can be released (Herskowitz and Hagen, 1980).

The lysogenic state is usually quite stable, with cells spontaneously losing immunity repression and reverting to lytic development only at a low rate. However, many temperate phages also have a mechanism to induce lytic development of the prophage in response to activation of the host SOS-pathway, which allows the phage to escape a damaged and potentially dying cell. A classic example of this is the coliphage lambda in which activated

Figure 1.1. Morphology and developmental lifecycles of classical double-stranded DNA bacteriophages.

A. *Morphology of coliphage P2.*

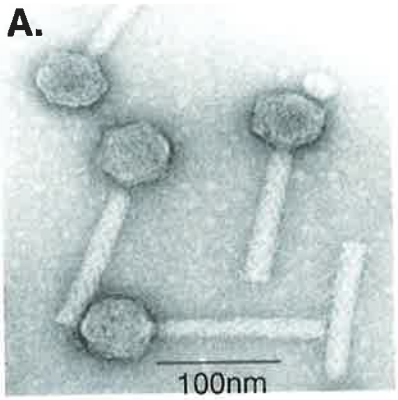
An electron micrograph of the tailed, ds DNA coliphage P2, taken from ICTVdB – The Universal Virus Database, version 3 (http://www.ncbi.nlm.nih.gov/ICTVdb/ICTVdB/).

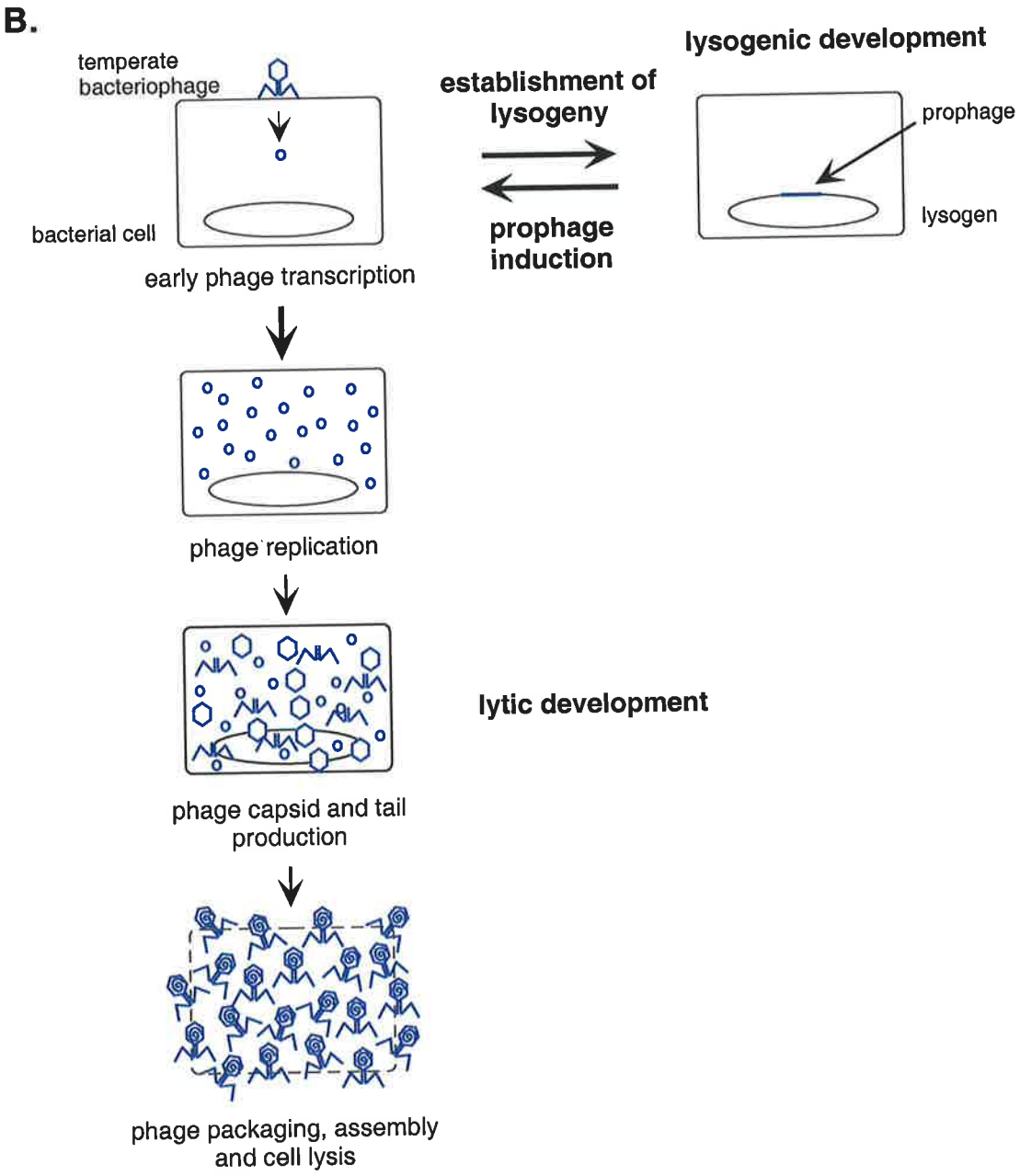
B. *The developmental lifecycles of temperate ds DNA bacteriophages.*

A stylized diagram of the possible modes of development, lytic or lysogenic, of a temperate ds DNA bacteriophage following infection of its bacterial host. The bacteriophage is shown with an icosahedral head and long tail fibres (as seen for coliphages P2 and 186) and with a genome that is circular in its host. An ellipse represents the bacterial chromosome.

After the phage adsorbs to a susceptible bacterial host cell it injects its genome into that cell and early phage genes are transcribed. In most infections, lytic development occurs and new phage particles are produced by an ordered series of events. Replication of the phage genome is followed by the expression of phage structural proteins which are assembled into pre-capsid and tail structures. Completed phage heads, formed by packaging of the phage genomes, are then attached to tails and the resultant mature progeny phages are released by lysis of the cell. Alternatively, lysogenic development may occur (establishment of lysogeny), where lytic gene expression is switched off and the phage genome is (usually) integrated into the host chromosome to form a prophage. A lysogenic cell may also revert to lytic development by excision of the phage genome and expression of lytic genes (prophage induction).

Figure 1.1


A.
100nm


B.
temperate bacteriophage
bacterial cell
early phage transcription
phage replication
phage capsid and tail production
phage packaging, assembly and cell lysis
establishment of lysogeny
prophage induction
lysogenic development
prophage
lysogen
lytic development

RecA stimulates self-cleavage of the lysogenic repressor to induce an otherwise stable lysogen (Roberts and Devoret, 1983; Baek *et al.*, 2003).

1.A.3. CONTROL OF MORPHOGENETIC PHAGE GENE EXPRESSION.

The production of progeny phages depends on the ordered activity of proteins required for phage replication and morphogenesis, which is usually achieved by regulating the expression of the genes involved. Perturbation of the timing or level of lytic gene expression during replicative development may result in a smaller number of phage virions being produced and released – for example, due to premature activity of the phage lysis proteins (Johnson-Boaz *et al.*, 1994; Markov *et al.*, 2004). The expression of phage lytic genes in a lysogenic cell may also be detrimental, even resulting in death of the cell (Dambly *et al.*, 1979; Julien and Calendar, 1996).

Different phages use a variety of mechanisms to regulate the timing of gene expression after phage infection, which so far have been studied in detail for only a few phages. The aim of this work was to investigate apparently unique characteristics of structural gene regulation in a temperate ds DNA coliphage, 186, to gain more understanding about different ways in which late gene expression in particular can be controlled. Structural gene expression in 186 shows some similarities with that of the closely related, temperate coliphage, P2 – transcription is activated by homologous proteins (Kalionis *et al.*, 1986a) and replication is important for late gene expression (Lengyel and Calendar, 1974; Finnegan and Egan, 1981; Hocking and Egan, 1982d; Christie and Calendar, 1983). However, the regulation of late gene expression in 186 also appears to differ from that in P2 in some important aspects, which are the subject of this investigation. Briefly, despite its similarity with the analogous protein of P2, it appeared that the 186 late regulator requires a novel interaction with the host RNA polymerase (RNAP) or other proteins, to facilitate enhanced transcription of the 186 morphogenetic genes (Sauer, 1979). In addition, transcription of the late activator of 186, unlike that of P2, is detectable from early during the phage infection (well before late gene expression is enhanced – Richardson, 1993), and is reduced by the immunity repressor in a lysogenic cell (Dibbens *et al.*, 1992).

This chapter provides a detailed background to this project by reviewing current knowledge about the developmental lifecycles of the 186 phage (Section 1.B.), the mechanism of promoter activation by the 186 late regulator and homologous proteins (Section 1.C.), and the regulation of structural and lysis gene expression in 186 and the closely related phage

P2 (Section 1.D.). The specific aims of the project and experiments used to address them are then given in Section 1.E.

1.B. COLIPHAGE 186.

1.B.1. 186 BELONGS TO A LARGE FAMILY OF P2-LIKE PHAGES.

186 is a temperate ds DNA bacteriophage which infects *Escherichia coli* cells, and was isolated from the sewers of Paris in the 1950s by Jacob and Wollman (Jacob and Wollman, 1961). 186 belongs to the order Caudovirales and *Myoviridae* family and has a ~60nm icosahedral capsid attached to a ~155nm contractile tail sheath and long tail fibres involved in phage adsorption (Dodd and Egan, 1999). The 186 virion structure is identical to that of another well-studied temperate coliphage, P2 (Bertani and Bertani, 1971) (see Figure 1.1), and the ~30kb DNA genomes of these phages show homology in both gene organization and sequence (Dodd and Egan, 1999).

186 and P2 also show varying levels of sequence identity with numerous other bacteriophages, prophages and phage-like elements. These include the homologous phages WPhi and P2 Hy dis from *E. coli* (Bertani and Bertani, 1971; Liu and Haggard-Ljungquist, 1999; Renberg-Eriksson *et al.*, 2001), PSP3 from *Salmonella potsdam* (Bullas *et al.*, 1991; Julien and Calendar, 1996) and L-413C from *Yersinia pestis* (Elliott *et al.*, 2003). Less closely related phages, with identity mainly in the late region, include the cytotoxin-encoding ϕCTX phage from *Pseudomonas aeruginosa* (Nakayama *et al.*, 1999), K139 from *Vibrio cholerae* (Kapfhammer *et al.*, 2002), HP1 from *Haemophilus influenzae* (Esposito *et al.*, 1996), and SopEϕ and Fels-2 from *Salmonella typhimurium* (Pelludat *et al.*, 2003). 186 and P2-related prophages or phage-remnants are also found in the chromosomal sequences of *E. coli* bacteria (Kita *et al.*, 2003; Nilsson *et al.*, 2004) including in the Shiga-toxin encoding O157:H7 strains (Ohnishi *et al.*, 1999); in pathogenic *Salmonella* strains (Thomson *et al.*, 2004); and in a number of other mainly gram-negative hosts (Canchaya *et al.*, 2003). Thus, 186-related sequences are found in numerous phage and bacterial species, most notably in several enteric bacterial pathogens.

1.B.2. THE GENOMIC ORGANIZATION OF 186 AND P2.

The characterization of temperate coliphages 186 and P2 has been reviewed a number of times over the years, including by Bertani and Bertani (1971), Bertani and Six (1988) and Dodd and Egan (1999). As shown in Figure 1.2, the homology between 186 and P2 occurs

Figure 1.2. Comparison of the genomes of coliphages 186 and P2.

A. *Homology in the 186 and P2 genomes.*

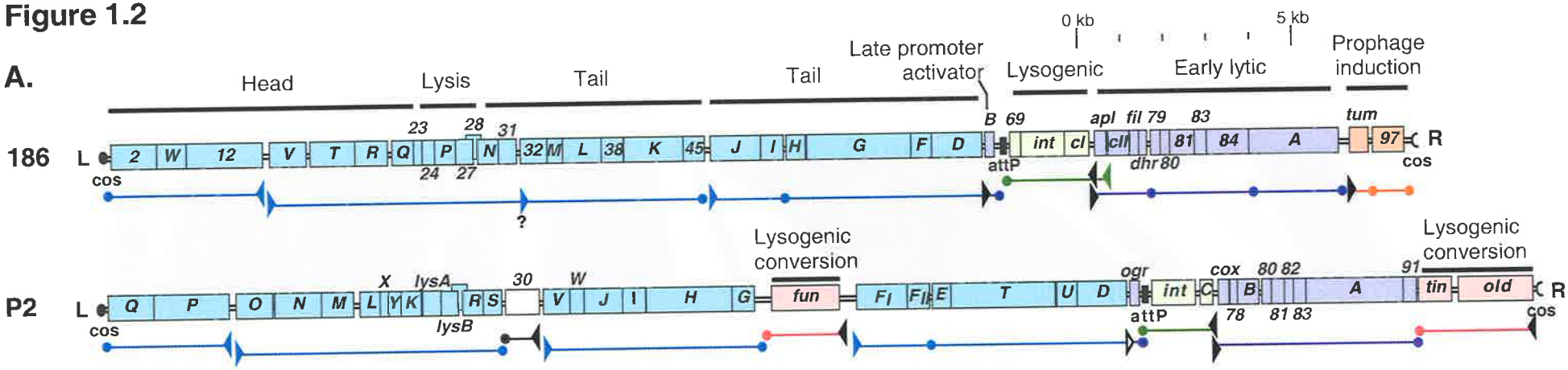
This figure – adapted from Dodd and Egan (1999) – shows the ~30kb double-stranded DNA genomes of coliphages 186 and P2 from left to right, starting from the cohesive ends of the phages. Genes are shown as boxes; promoters as arrows pointing in the direction of transcription; and transcriptional terminators as circles. Black arrows represent constitutive (or independent) promoters; blue arrows indicate promoters requiring the late activator protein, B, for transcription; the green arrow in 186 represents a promoter activated by the 186 CII protein; and the open arrow in P2 is a promoter thought to be activated by an unknown early P2 protein. Genes from the two phages that encode products of significant amino acid identity are indicated by shading. The left two-thirds of each genome consist of genes encoding phage morphogenesis functions (head, tail) and lysis proteins which are expressed late in infection (shown in blue); and the activators of late transcription (shown in purple). Genes expressed early in infection are present on the right end of the genome, where colours represent lysogenic (green) or early lytic (purple) functions as indicated. Genes involved in prophage induction of 186 are shown in orange, and those for lysogenic conversion of P2, in pink.

B. *Comparison of 186 and P2 gene functions.*

This table – adapted from Portelli *et al.* (1998) and Dodd and Egan (1999) – details all known 186 and P2 genes and the functions they encode. Percent amino acid identity for homologous proteins is given, and proteins with analogous functions but no significant sequence identity are also indicated ([a]).

Figure 1.2

A.



B.

Gene 186	Gene P2	Identity	Function (comment)
orf2	*Q*	79	Capsid portal vertex
W	–	–	Unknown (homology to large terminase subunit)
orf12	*P*	89	Large terminase subunit
V	*O*	81	Capsid scaffold
T	*N*	75	Major capsid protein
R	*M*	70	Small terminase subunit
Q	*L*	68	Head completion
orf23	*X*	70	Tail
orf24	*Y*	87	Lysis - holin
P	*K*	95	Lysis - endolysin (homologous to λ R)
–	*lysA*	–	Lysis - timing
orf27	*lysB*	61	Lysis - timing
orf28	*orf*	71	Unknown
N	*R*	69	Tail completion
orf31	*S*	53	Tail completion
–	*orf30*	–	Unknown
orf32	*V*	72	Tail spike
M	*W*	60	Baseplate? (homology to T4 baseplate wedge)
L	*J*	80	Baseplate/tail fiber
orf38	*I*	83	Tail
K	*H*	81	Tail fiber (partial homologies with many phages)
orf45	*G*	43	Tail fiber assembly (partial homologies with many phages)
–	*fun*	–	Makes lysogen resistant to T5
J	*FI*	87	Tail sheath
I	*FII*	74	Tail tube
H	*E*	72	Tail (programmed translational frameshift)

Gene 186	Gene P2	Identity	Function (comment)
G	*T*	78	Tail (ruler protein?)
F	*U*	58	Tail
D	*D*	78	Tail
B	*ogr*	63	Late promoter activator (zinc finger DNA-binding protein)
orf69	–	–	Unknown (not essential)
int	*int*	36	Integrase
cI [a]	*C* [a]	*none*	Immunity repressor
apl [a]	*cox* [a]	*none*	Excision and transcriptional control
cII	–	–	Establishment of lysogeny (transcriptional activator)
–	*orf78*	–	Unknown (overexpression lethal)
fil	–	–	Inhibitor of cell division
dhr	–	–	Inhibitor of host replication (improves burst size)
–	*B*	–	Replication (DnaC analogue?)
orf79	–	–	Unknown (not essential)
–	*orf80*	–	Unknown (overexpression lethal)
–	*orf81*	–	Unknown
orf80	*orf82*	50	Unknown (overexpression of Orf82 lethal)
orf81	–	–	Unknown (not essential)
orf83	*orf83*	54	Unknown (not essential in 186)
orf84	–	–	Unknown (not essential)
A	*A*	36	Replicase (rolling circle replication)
–	*orf91*	–	Unknown
–	*tin*	–	Makes lysogen resistant to T-even phages
–	*old*	–	Makes lysogen resistant to λ
tum	–	–	Prophage induction (antirepressor)
orf97	–	–	Unknown (overexpression inhibits 186 infection)

in both the sequence and organization of the phage genes and extends across most of the genomes. Morphogenesis functions encoded by about 20 genes on the left-hand two-thirds of the chromosomes show the most amino acid sequence identity – between 40 and 90%. The activators of late gene transcription, 186 B and P2 Ogr proteins, are also homologous, with ~63% amino acid identity. In fact, these proteins are functionally interchangeable since viable hybrid phages can be formed which carry the P2 late region and 186 *B* gene and right-hand sequence (Hocking and Egan, 1982a). The 'switch' region of the genome that determines the mode of phage development is also similar in structure for 186 and P2, with convergent promoters controlling the expression of proteins of opposing function. Leftward transcription from 186 *pL* and P2 *pC* results in expression of the immunity repressor (CI for 186 and C for P2) and integrase proteins required for lysogenic development; while the rightward transcripts from *pR* in 186 and *pE* in P2 encode early lytic functions. These include the multifunctional Apl and Cox proteins in 186 and P2 respectively, and *cis*-acting functions required for phage replication (A in 186 and A and B in P2). However, these immunity and early lytic regions of 186 and P2 show almost no sequence identity. In addition, P2 lacks the *cII* and *tum* genes of 186, which, respectively, enhance the establishment of lysogeny and the SOS-induced reversion of a prophage to lytic growth – which does not occur for P2. Instead, P2 carries several non-essential genes not found in 186 that are expressed in a lysogen and interfere with lytic development of unrelated super-infecting phages, such as lambda and T4. P2 phages can also form multiple lysogens by integration at a number of sites within the chromosome, unlike 186 which has one integration site. Thus, while both phages are temperate, the 186 prophage is inducible and is able to escape deleterious conditions facing the lysogen in order to maintain its survival as a free phage particle; and P2 which forms uninducible prophages, appears to have adapted to optimize survival in the lysogenic state.

1.B.3. 186 GENE EXPRESSION DURING DEVELOPMENT.

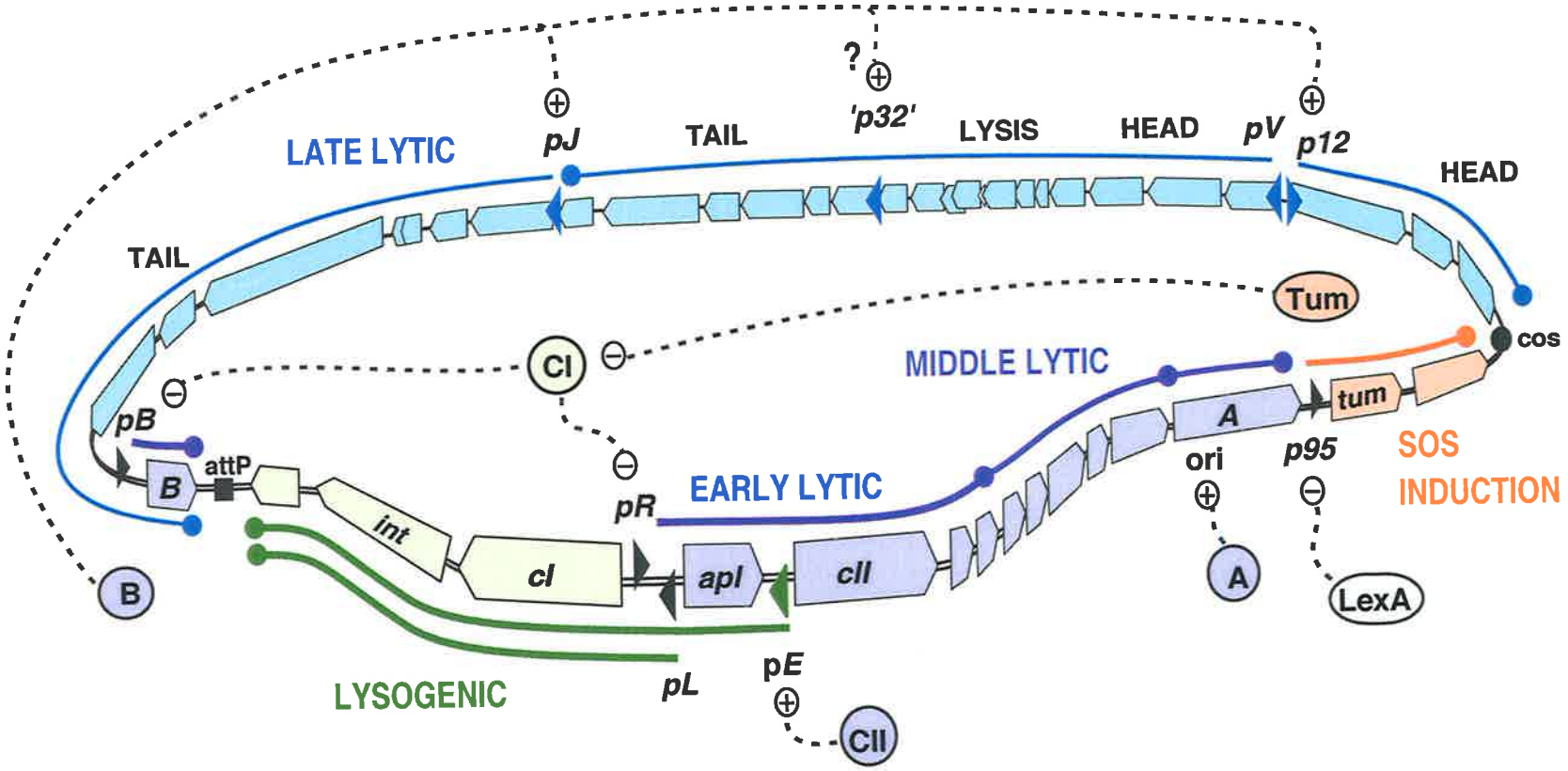
1.B.3.1. 186 lytic development.

As is the case for other bacteriophages, 186 shows an ordered pattern of gene expression during lytic development (Finnegan and Egan, 1981 – see Figure 1.3). The intrinsically strong rightward promoter *pR* is active from early in infection (Finnegan and Egan, 1981; Richardson, 1993), and interferes with convergent transcription from the weaker lysogenic promoter, *pL* (Dodd *et al.*, 1990; Callen *et al.*, 2004). Transcription from *pR* leads to the expression of a number of early lytic proteins including Apl, which can repress transcription from both *pR* and *pL* and acts as an excisionase in prophage induction (Dodd

Figure 1.3. Regulation of gene expression in coliphage 186.

A circular map of the 186 genome showing phage genes, the transcripts that encode them and the actions of regulatory proteins (adapted from a figure designed by Ian Dodd). Genes, promoters and transcriptional terminators are as described for Figure 1.2. Early and middle lytic functions expressed from the intrinsically active promoters, *pR* and *pB* are shown in purple and late lytic functions transcribed from promoters activated by the B protein (*pV*, *p12*, *pJ* and the putative promoter, '*p32*') are in blue. Lysogenic functions expressed from the *pL* promoter are in green and those for SOS-stimulated prophage induction, expressed from the *p95* promoter, are in orange. The CI protein binds over the *pR*/*pL* promoter region to repress *pR* transcription, activating *pL* at low concentrations and repressing its own transcription at high concentrations. CI also binds at *pB* to repress transcription. The CII protein activates a leftward promoter, *pE*, for the establishment of lysogeny; and following SOS induction of the prophage the Tum protein interacts with CI to prevent it binding to the DNA. Transcription of the *tum* gene is repressed by an *E. coli* protein, LexA. The replicase protein, A, initiates phage replication *in cis* by binding to the origin of replication within the *A* gene sequence.

Figure 1.3


LATE LYTIC
pJ
TAIL
?
'p32'
LYSIS
HEAD
pV
p12
HEAD
TAIL
Tum
cos
CI
MIDDLE LYTIC
pB
attP
B
A
tum
ori
p95
SOS
INDUCTION
pR
EARLY LYTIC
int
cI
apI
cII
A
LexA
B
LYSOGENIC
pL
pE
CII

et al., 1993; Reed *et al.*, 1997), and Fil and Dhr proteins which suppress host cell replication and division respectively (Richardson and Egan, 1989). The replicase protein, A, is also expressed from the *pR* promoter, possibly after an antitermination event (Richardson *et al.*, 1989). Of the early lytic genes expressed from *pR*, only the *A* gene is essential for 186 phage production – mutation of all other early genes does not significantly disrupt the phage burst (Dodd and Egan, 1999). Replication of the phage genome is initiated between 5 and 10 minutes after infection (Orsborn, 1986) by the A protein, an endonuclease which acts *in cis* to cause a single-stranded nick in the DNA at the origin of replication within the *A* gene sequence (Sivaprasad *et al.*, 1990). Unidirectional replication then occurs using a rolling circle mechanism (Chattoraj and Inman, 1973) and requiring a number of bacterial replication proteins (Hooper and Egan, 1981). Phage replication probably generates multiple copies of the genome as circular monomers, which are recognized by the phage terminase proteins for packaging into the pre-capsid structure, as seen for P2 (Pruss *et al.*, 1975). About 20-25 minutes after infection, genes on the left-hand two-thirds of the phage genome are transcribed (Finnegan and Egan, 1981; Dibbens and Egan, 1992; Richardson, 1993). The products of these genes, determined by analysis of mutant phenotypes and sequence homology with P2, are required for formation of the 186 capsid and tail structures, packaging of the genome, assembly of mature virions and release from the host by cell death (Hocking and Egan, 1982c, d; Xue and Egan, 1995a,b; Portelli *et al.*, 1998). Bacterial cell lysis occurs using holin and endolysin proteins which show structural or amino acid similarity with those of bacteriophage lambda (Portelli *et al.*, 1998). Phage-induced cell lysis may also be regulated by the protein products of the *orf27/orf28* genes, whose homologues in P2, *lysB/lysC*, appear to be involved in the timing of lysis and may be functionally analogous to the *Rz/Rz1* genes of lambda (Ziermann *et al.*, 1994; Markov *et al.*, 2004). Transcription of these 'late' genes occurs from at least three promoters, *pV*, *p12* and *pJ*, which are activated by the product of the *B* gene, or late activator (Dibbens and Egan, 1992; Xue and Egan, 1995b). In the absence of B protein expression, due to mutation of the *B* gene, little late transcription is seen, cells are not lysed and few progeny phages are formed (Finnegan and Egan, 1981; Hocking and Egan, 1982c, d; Dibbens and Egan, 1992). Instead, presumably because phage templates are not removed by packaging, 186 replication continues and cells become overladen with copies of the phage DNA (perhaps as many as 400-500 copies – Hocking and Egan, 1982d; Orsborn, 1986). Increased transcription of the late promoters during 186 lytic development also requires phage replication (Finnegan and Egan, 1981). Details of the mechanism and regulation of late gene expression will be discussed later in this chapter.

1.B.3.2. 186 lysogenic development.

In around 20-30% of wildtype 186 infections (Ian Dodd, personal communication) the phage enters a lysogenic state due to the action of the lysogenic establishment protein, CII, which is transcribed from the *pR* promoter (Neufing *et al.*, 1996; Shearwin and Egan, 2000; Neufing *et al.*, 2001). CII is an unstable protein which binds to DNA at the 5' end of the *cII* gene to activate a leftward promoter, *pE,* which results in transcription of the *cI* and *int* genes (see Figure 1.3). The CI or lysogenic repressor protein binds to and inhibits transcription from the 186 lytic promoters, *pR* and *pB* (Dodd *et al.*, 1990; Dibbens *et al.*, 1992; Dodd and Egan, 1996, 2002; Shearwin *et al.*, 2002); while expression of the Integrase protein facilitates recombination between attachment sites downstream of the *B* gene on the phage and within an isoleucine tRNA gene of *E. coli*, resulting in insertion of the phage genome into the bacterial chromosome (Reed *et al.*, 1997). CI expression also autoregulates its own promoter *pL* to allow continued repressor transcription in the lysogen (Dodd *et al.*, 1990; Dodd and Egan, 2002). At low concentrations, CI repression of opposing *pR* transcription relieves interference of *pL* transcription, to enhance its own expression; while at high concentrations, , CI negatively regulates *pL* to maintain a constant level of protein in a 186 lysogen.

CI achieves this regulation by binding to two types of inverted-repeat sequence, termed A- and B-type sites, which are found at the switch region and the *B* promoter on the phage DNA (Dodd and Egan, 1996, 2002; Shearwin *et al.*, 2002, see Figure 1.4A). At *pB*, a pair of A-type operators separated by 32bp are situated over the –10 and –35 sequences of the promoter. At *pR*, CI binds with similar affinity to a combination of two B-type sites and an A-type site with a 1bp smaller spacer (B-A'-B), each separated by ~22bp, to repress promoter activity. CI also binds more weakly to two apparently single A-type sites which flank the *pR* sequence and are ~350bp from it (*FL* and *FR*); and to a low affinity operator over the *pL* promoter sequence to facilitate negative autoregulation.

The 186 CI protein consists of two domains, an N-terminal helix-turn-helix domain responsible for DNA binding and a C-terminal self-association domain, joined by a flexible linker region (Shearwin *et al.*, 2002). The structures of CI protein C-terminal domain (CTD) and a dimer-forming mutant of the full-length CI protein have recently been solved by X-ray crystallography (Pinkett *et al.*, 2005). Originally, CI protein was thought to octamerize in solution (Shearwin and Egan, 1996), but in the crystal the CI CTD forms an unusual 14-mer, consisting of seven dimers arranged in a wheel-like structure (see Figure

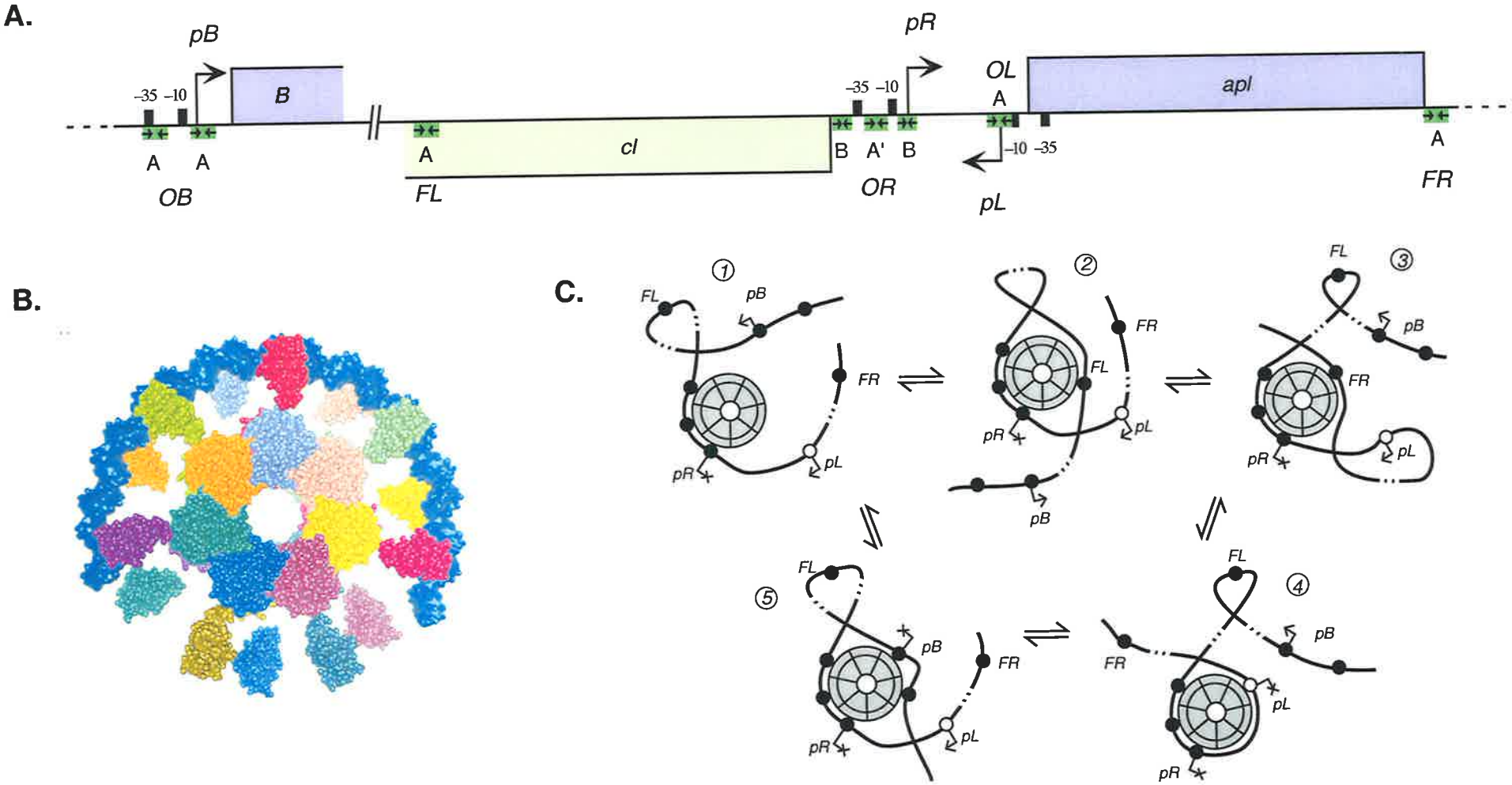
Figure 1.4. DNA binding by the 186 lysogenic repressor.

A. *The positions of CI binding sites on the 186 genome.*
A map of the 186 genome containing the *pB* promoter and part of the *B* gene (position 20 540 to 20 680) and the switch region (22 720 to 23 425) is shown to scale. Filled boxes represent the –10 and –35 RNAP σ^{70} recognition sites of the promoters, with arrows indicating the start of transcription, and genes are shown by coloured boxes. CI binding sites of the A-type (A and A') or B-type (B) inverted-repeats are shown with small arrows in a dark green box. CI binding sites are labelled as *OB* (operator at *pB*), *OR* (operator at *pR*), and *OL* (operator at *pL*); with flanking sites to the left or right of the *pR*/*pL* promoters called *FL* and *FR* respectively.

B. *Model of a 186 CI 14-mer bound to DNA.*
The structures of the 186 CI CTD and a full-length dimerization mutant, solved by X-ray crystallography, suggest that the protein forms a 14-mer of seven dimers arranged in a wheel-like structure (Pinkett *et al.*, 2005). In this structure, self-associating CTDs interact at the centre of the multimer and are attached by flexible linkers to the DNA-binding helix-turn-helix NTDs at the rim of the wheel. Here, a DNA helix is shown wrapped around the 14-mer, such that adjacent dimers in the wheel interact with inverted-repeat sequences separated by two turns of the helix, like those found at *pR* (taken from Pinkett *et al.*, 2005).

C. *Possible interactions of a CI 14-mer with multiple operator sites.*
Looping out of the intervening DNA appears to allow the interaction of DNA-binding domains on a CI 14-mer bound at the pR promoter, with flanking operator sequences to enhance pR repression (Dodd and Egan, 2002). This figure shows some of the alternative complexes that could be formed by interaction of a CI 14-mer (shown as a wheel of 7 dimers) bound at pR operators (closed circles), with additional operator sites (filled or open circles) at *FL* (2), *FR* (3), *OB* (4) or low affinity site *OL* (5). Repression of promoter activity by CI binding is also indicated.

Figure 1.4


A.
pB
pR
OL
B
cl
apl
–35 –10
–35 –10
A A
A
B A' B
–10 –35
A
OB
FL
OR
pL
FR
B.
C.
1
2
3
4
5
FL
pB
FR
pR
pL

1.4B). Genetic analysis appears to confirm the importance of this structure for CI protein activity, and wrapping of the DNA around the CI wheel may explain the protection of sequences extending beyond the operator sites in DNaseI footprinting experiments (Dodd and Egan, 1996). Dodd and Egan (2002) proposed a model to explain the effects of increasing concentrations of CI on *pL* and *pR* activity which was based on CI octamers, but can also be applied to the CI 14-mer (Pinkett *et al.*, 2005). In this model, the CI 14-mer binds to the *pR* operators at low concentrations, to repress lytic transcription from *pR* and allow activity of the *pL* promoter (see Figure 1.4C(1)). *pR* repression is enhanced in the presence of the flanking sites, suggesting that looping out of the intervening DNA allows the CI multimer bound to *OR* to also interact with one of these operator sites, to stabilize the complex (see Figure 1.4C(2), (3)). At higher concentrations, additional CI multimers are expected to independently occupy the weaker *FL* and *FR* sites, and release DNA-binding regions on the *pR*-bound complex to associate with the *pL* sequence, and repress *pL* transcription.

It seems likely that CI also binds as a 14-mer species to operators at the *B* promoter, as retardation of this DNA in gel-shift assays is similar to that seen for fragments containing the *pR*, *FL* or *FR* operators (Dodd and Egan, 1996). CI binding sites at the *B* promoter are separated by 32bp, instead of the 22bp spacing seen at *pR,* which are shown interacting with adjacent CI dimers in the 14-mer, in Figure 1.4B. DNaseI footprinting experiments show large enhancements of cleavage between the CI operators at *pB* when the protein is bound (Dodd and Egan, 1996), suggesting that distortion of the DNA may be required to fit the operators to adjacent DNA-binding domains in the multimer. Interestingly, CI repression of *pB* transcription in reporter assays was around 3-fold weaker than that of *pR* (~30-fold compared to ~100-fold repression of pR, in the absence of the flanking sites – Dodd and Egan, 2002; Shearwin *et al.*, 2002), although the protein bound with similar affinity to promoter fragments in gel-shift assays (Dodd and Egan, 1996).

Wildtype 186 CI has not been tested for looping over very large distances, however a fusion protein with the 186 CI CTD and the lambda CI DNA-binding domain (NTD) can interact with binding sites separated by up to ~3.8kb (Ian Dodd, personal communication). This suggests that a CI 14-mer bound at *pB* could potentially interact with the *pR* operators when they are ~2.5kb away in a circular 186 genome (giving Figure 1.C(4) for example) to enhance *pR* repression. The importance of CI repression of the *B* promoter in the 186 phage is discussed more below (Section 1.D.3.2.) and in Chapter 4.

The 186 lysogenic state is extremely stable, but can be efficiently induced to lytic development via the host SOS response, due to the anti-repressor action of the 186 Tum protein (Lamont *et al.*, 1989; Brumby *et al.*, 1996). Lytic development can also triggered for 186 prophage which carry a temperature-sensitive mutation in the *cI* gene, by transfer of the lysogenic cells to a non-permissive temperature at which the CI protein is inactivated.

1.C. MECHANISM OF LATE GENE ACTIVATION IN 186.

1.C.1. B BELONGS TO A LARGE FAMILY OF ZINC-FINGER PROTEINS.

The late activator or B protein of 186 is a small protein 72 residues in length which contains a high proportion of basic amino acids, and is rich in proline and other hydrophobic residues within the C-terminal sequence (Kalionis *et al.*, 1986a). Although B has no recognizable helix-turn-helix motif for DNA-binding, it contains four essential cysteine residues and requires coordination of a single molecule of zinc or cadmium for activity – suggesting that it contains a zinc-finger motif (Pountney *et al.*, 1997).

A BLAST search with the B amino acid sequence shows that it belongs to a growing family of homologous proteins or putative proteins, which includes several transcriptional activators (Figure 1.5). These are found mainly in 186-related bacteriophages and phage-like elements in the chromosomes of gram-negative bacteria including *Serratia marcescens* (Ferrer *et al.*, 1996; Jin *et al.*, 1996). The satellite phage P4, which requires structural proteins from a helper phage such as P2 or 186 for the production of progeny (reviewed by Deho and Ghisotti, 1999) also contains a protein with homology to B – as do some P4-related elements, such as the retronphage ϕR73 (Inouye *et al.*, 1991; Sun *et al.*, 1991; Canchaya *et al.*, 2003). These B-related proteins are all less than 100 residues in length, with the exception of the P4 Delta protein which appears to have two B-like sequences joined covalently head-to-tail (Julien *et al.*, 1997). They show varying levels of sequence identity with B, however almost all contain a $CX_2CX_{22}CX_4C$ motif which is reminiscent of eukaryotic Cys4 zinc-fingers. The four cysteine residues of this motif are indeed required for zinc binding and transcriptional activity of the P2 Ogr and ϕR73 δ proteins (Gebhardt, 1993; Julien *et al.*, 1998), and so far the B homologues are the only examples of prokaryotic zinc-finger transcriptional activators. It has also been suggested that the redox state of the cell or zinc-binding proteins such as metallothioneins may affect coordination of the zinc ion and protein activity, and may be manipulated to regulate the activity of Zn-finger proteins such as B (Pountney *et al.*, 1997).

Figure 1.5. Alignment of B-related sequences from a BLAST search.

The 186 B amino acid sequence was used in a BLASTp search and proteins or putative proteins which gave an E value <5 were aligned by the ClustalW program (version 1.8, GenomeNet website) and processed by the BoxShade 3.21 program (on the Swiss EMB net server). Identical conserved residues are shown with a black background, while conserved amino acids with similar properties are shown with grey background shading. A consensus of residues found in more than half of the aligned sequences is shown underneath the alignment, where letters in capitals are found in 75% or more of the sequences. The organisms (bacteriophages or bacterial strains) in which the sequences are found are shown, as are the names of the proteins and the Genbank protein identification tags. The two halves of the P4 Delta protein are each aligned, where half I is residues 1-83 and half II is residues 84-166, as indicated.

Abbreviations of bacterial strains are as follows:

Bu.ps. = *Burkholderia pseudomallei* K96243

Ch.vi. = *Chromobacterium violaceum* ATCC12472

E.coli K-12 = *Escherichia coli* K-12 MG1655

E.coli CF = *Escherichia coli* CFT073

E.coli O = *Escherichia coli* O157:H7

E.coli OE = *Escherichia coli* O157:H7 EDL933

Er.car. = *Erwinia carotovora* subspecies *atroseptica* SCRI1043

Er.amy. = *Erwinia amylovora*

Ph.lum. = *Photorhabdus luminescens* subsp. *laumondii* TTO1

Rah.aq. = *Rahnella aquatilis*

Ral.s. = *Ralstonia solanacearum* GMI1000

S.ty. T = *Salmonella enterica* subspecies *enterica* serovar Typhi Ty2

S.ty. C = *Salmonella enterica* subspecies *enterica* serovar Typhi CT18

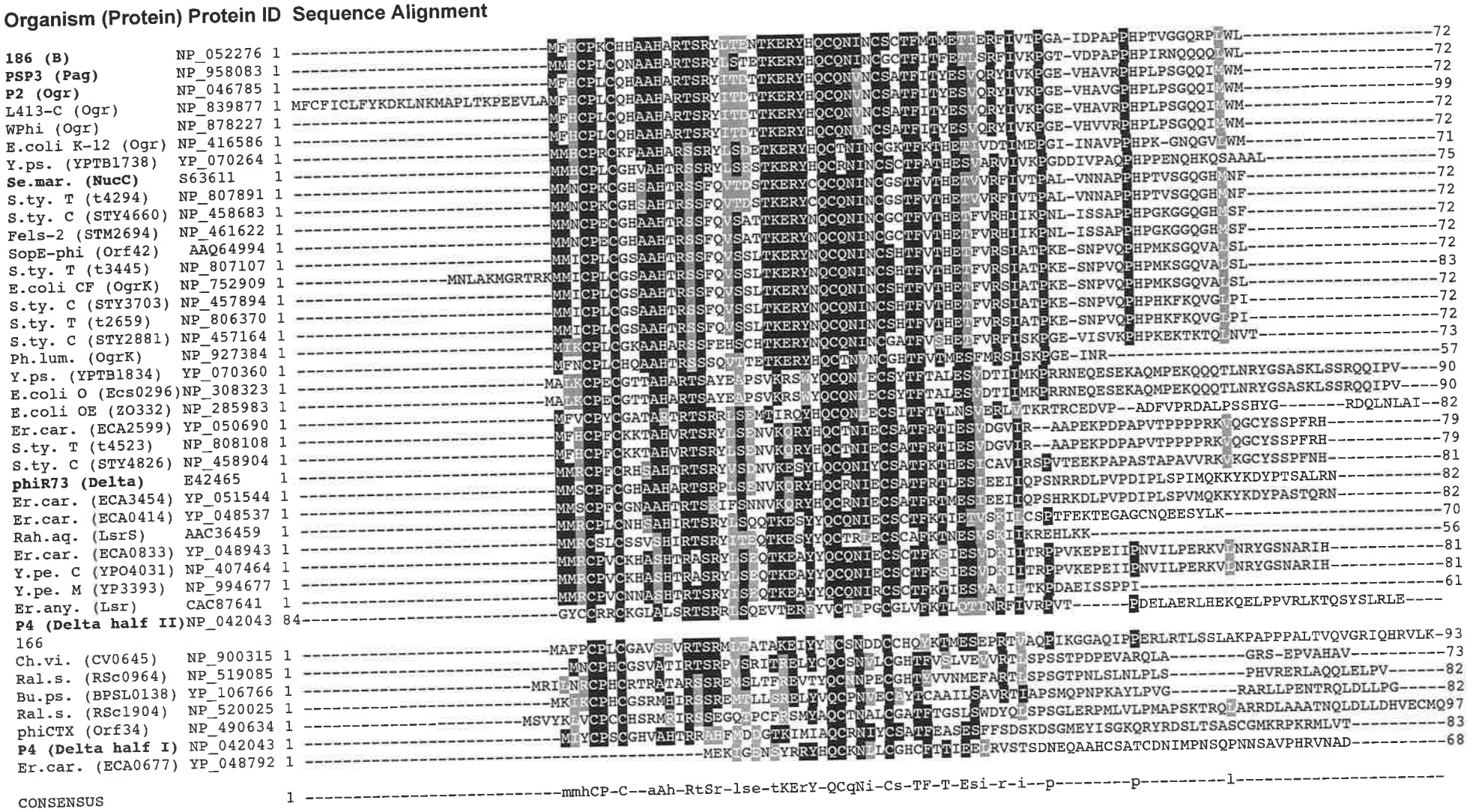
Se.mar. = *Serratia marcescens*

Y.pe. C = *Yersinia pestis* biovar Orientalis CO92

Y.pe. M = *Yersinia pestis* biovar Medievalis 91001

Y.ps. = *Yersinia pseudotuberculosis* IP 32953

Figure 1.5


Organism (Protein) Protein ID Sequence Alignment
186 (B) NP_052276
PSP3 (Pag) NP_958083
P2 (Ogr) NP_046785
L413-C (Ogr) NP_839877
WPhi (Ogr) NP_878227
E.coli K-12 (Ogr) NP_416586
Y.ps. (YPTB1738) YP_070264
Se.mar. (NucC) S63611
S.ty. T (t4294) NP_807891
S.ty. C (STY4660) NP_458683
Fels-2 (STM2694) NP_461622
SopE-phi (Orf42) AAQ64994
S.ty. T (t3445) NP_807107
E.coli CF (OgrK) NP_752909
S.ty. C (STY3703) NP_457894
S.ty. T (t2659) NP_806370
S.ty. C (STY2881) NP_457164
Ph.lum. (OgrK) NP_927384
Y.ps. (YPTB1834) YP_070360
E.coli O (Ecs0296) NP_308323
E.coli OE (ZO332) NP_285983
Er.car. (ECA2599) YP_050690
S.ty. T (t4523) NP_808108
S.ty. C (STY4826) NP_458904
phiR73 (Delta) E42465
Er.car. (ECA3454) YP_051544
Er.car. (ECA0414) YP_048537
Rah.aq. (LsrS) AAC36459
Er.car. (ECA0833) YP_048943
Y.pe. C (YPO4031) NP_407464
Y.pe. M (YP3393) NP_994677
Er.any. (Lsr) CAC87641
P4 (Delta half II) NP_042043
Ch.vi. (CV0645) NP_900315
Ral.s. (RSc0964) NP_519085
Bu.ps. (BPSL0138) YP_106766
Ral.s. (RSc1904) NP_520025
phiCTX (Orf34) NP_490634
P4 (Delta half I) NP_042043
Er.car. (ECA0677) YP_048792
CONSENSUS

1.C.2. CHARACTERIZED B HOMOLOGUES ARE FUNCTIONALLY INTERCHANGEABLE TRANSCRIPTIONAL ACTIVATORS.

Biochemical characterization of 186 B-related proteins, including B itself, has been hampered by their tendancy to form insoluble aggregates at high concentrations, and initial solutions of purified P2 Ogr protein, achieved by denaturation and refolding, showed quite low specific activity (Lee and Christie, 1990). This problem has been overcome to some extent for several proteins, including B, which have subsequently been characterized in more detail, although no structures are as yet available. These characterized B-homologues, namely the late activators of the 186-related phages, P2 and PSP3 (Ogr and Pag, respectively), the late activators of 186- or P2-dependent satellite phages, P4 and ϕR73 (Delta proteins of each), and a chromosomal activator from *Serratia marcescens* (NucC), are aligned in Figure 1.6. Improved protein solubility of 186 B was achieved by the addition of cadmium ions to the growth medium which allowed purification of a cadmium-derivative of the protein, CdB (Pountney *et al.*, 1997). The Delta proteins from phages P4 and ϕR73, and the Pag protein from PSP3, have also been successfully purified by fusion to a more soluble protein, the maltose-binding-protein (MBP) (Julien and Calendar, 1995, 1996). However, in this case MBP-activator fusions alone were inactive, and a mixture of fused and unfused activators was required to see DNA-binding and transcriptional activation *in vitro*. More recently, soluble wildtype NucC protein, from *S. marscescens*, was purified successfully by using a high pH buffer, and the resultant protein appeared to retain a high level of specific activity (McAlister *et al.*, 2003).

186 B and these characterized homologues are activators of transcription which are also able to substitute for each other in promoter activation. 186 B protein expressed from a plasmid can complement a P2 Δ*ogr* phage (Gebhardt, 1993), and P2-186 hybrid phages carrying the P2 late genes and 186 late activator gene are viable (Bradley *et al.*, 1975; Hocking and Egan, 1982a), indicating that the B protein is able to activate the P2 late promoters. A 186 lytic infection is also able to act as a helper for the satellite phage, P4, even when P4 carries a mutated delta gene (Sauer *et al.*, 1982), demonstrating that 186 B can enhance expression of P4 late genes. The P4 δ, ϕR73 δ, Pag and NucC proteins are all also able to complement infection by a P2 Δ*ogr* phage (Halling and Calendar, 1990; Jin *et al.*, 1996; Julien and Calendar, 1996), and the P4 δ gene can be replaced by that of ϕR73 δ or P2 *ogr*, and still give a normal P4 phage burst (Julien *et al.*, 1998). In addition, transcriptional reporters of the P2 and P4 late promoters can be activated by each of the B homologues expressed from a plasmid (Julien and Calendar, 1996), and NucC, Ogr and P4

Figure 1.6. Alignment of functionally interchangeable B homologues.

Amino acid sequences of the 186 B, PSP3 Pag, P2 Ogr, *Serratia marcescens* NucC, ϕR73 Delta, and P4 Delta (shown as two halves – residues 1-83 and residues 84-166) proteins were aligned using ClustalW (version 1.8, GenomeNet website) and BoxShade 3.21 programs (on the Swiss EMB net server). Identical conserved residues are shown with a black background, while conserved amino acids with similar properties are shown with grey background shading. A consensus of residues found in at least 50% of the proteins is shown at the bottom, where letters in capitals are found in 75% or more of the sequences, and those with a black background are found in all proteins..

Coloured letters indicate residues whose alteration gives the protein a particular property:
Blue – mutations found which overcome the block to activity seen in a *rpo*A109 host
Cyan – mutations found which overcome the block to activity seen in a *rpo*A155 host
Pink – most missense mutations inactivate the protein
Olive – missense mutations investigated do not inactivate the protein
Red – mutation to a stop codon (i.e. truncation) inactivates the protein
Green – mutation to a stop codon (i.e. truncation) does not inactivate the protein
* above the sequences indicates residues of the P2 Ogr protein which are proposed to interact with the DNA.

Figure 1.6


Organism (Protein)
Sequence Alignment
186 (B)
PSP3 (Pag)
P2 (Ogr)
Se.mar. (NucC)
phiR73 (Delta)
P4 (Delta half II)
P4 (Delta half I)
CONSENSUS :
(>50% – U>75%, l<75%)

δ proteins are also able to activate a reporter of a NucC-stimulated promoter from *S. marcescens* (Winslow *et al.*, 1998). Thus, these activators are functionally interchangeable – that is, they are all able to activate the same promoter sequences. Two other B-related proteins included in the alignment of Figure 1.5, LsrS from *Rahnella aquatilis* (Seo *et al.*, 2002) and the Orf34 protein from the ϕCTX phage (Alber *et al.*, 2002), are also activators of transcription. However, it is not known if these proteins are able to activate the same promoters or bind the same DNA sequences as 186 B.

1.C.3. NATIVE PROMOTER TARGETS ACTIVATED BY 186 B PROTEIN AND ITS HOMOLOGUES.

1.C.3.1. Promoters activated by P2 Ogr and other B homologues.

The Ogr protein of coliphage P2 controls expression of the genes required for the assembly and release of P2 progeny phages by activating transcription from four phage promoters *pP*, *pO*, *pV* and *pF* late after a P2 phage infection (see Figure 1.2, Christie and Calendar, 1983, 1985).

The Delta protein of satellite phage P4 is also an activator of phage transcription late in infection. It increases expression of the P4 capsid and replication proteins from the intrinsically weak P4 promoters *psid* and *pLL*, as well as activating the P2 late promoters *in trans*, for the expression of the P2 structural and lysis functions required for P4 progeny phage production (Lindqvist *et al.*, 1978; Dale *et al.*, 1986; Deho *et al.*, 1988).

The NucC protein was identified by its ability to activate expression of two chromosomal *Serratia marcescens* genes which encode extracellular proteins thought to be involved in pathogenicity of the bacteria. These are the *nucA* extracellular nuclease gene, expressed from *pnucA* (Jin *et al.*, 1996), and the bacteriocin 28b gene transcribed from the *bss* promoter (Ferrer *et al.*, 1996). The presence of phage-like endolysin and holin genes upstream of the *nuc*C gene suggests that this is part of a phage-remnant in the chromosome (Ferrer *et al.*, 1996; Jin *et al.*, 1996).

Purification and characterization has also been carried out for the putative late transcription activator proteins of the *Salmonella potsdam* phage, PSP3 (Pag) and *E. coli* retronelement ϕR73 (ϕR73 Delta protein) (Julien and Calendar, 1996). The native phage promoters targeted by these proteins are not known.

1.C.3.2. B-activated promoters of phage 186.

As mentioned, the 186 B protein activates transcription of the 186 structural and lysis genes 20-25 minutes after infection, from at least three phage promoters (see Section 1.B.3.1. and Figure 1.2). At the left end of the chromosome, 186 late genes are expressed from back-to-back promoters, *p12* and *pV*, which transcribe left- and rightward respectively (Dibbens and Egan, 1992). The large terminase subunit and DNA packaging accessory proteins are transcribed from *p12*; while the *pV* transcript encodes all phage genes required for capsid formation and cell lysis, and some involved in tail assembly (Portelli *et al.*, 1998). A third 186 late promoter, *pJ*, is situated to the right of *pV* and transcribes rightward across additional genes required for formation of the tail structure (Xue and Egan, 1995b; Portelli *et al.*, 1998).

Interestingly, despite the different DNA sequences of the 186 *pV*, *p12* and *pJ* promoters, transcripts from these promoters appear with similar kinetics after temperature induction of a 186 *cI*ts lysogen (Finnegan and Egan, 1981; Dibbens, 1990; Dibbens and Egan, 1992; Richardson, 1993). Transcriptional fusions of these promoters on *gal*K reporter plasmids have no activity in the absence of B protein or at the start of phage lytic development, but show increased expression late after induction of a 186 *cI*ts lysogen, or in the presence of a B-producing plasmid (Dibbens, 1990; Dibbens and Egan, 1992; Xue and Egan, 1995b). B protein expressed from a plasmid can, in fact, induce higher promoter activity from *pV* than that seen during phage development; and *pV* reaches a much higher level of activity compared to *p12* in the presence of similar levels of B protein (Dibbens, 1990). Although B protein alone is able to activate a transcriptional reporter of the 186 *pV* promoter, downstream sequences of the promoter may also play a role in regulating the activity of this promoter – possibly by binding of another phage or host protein. This idea is based on the finding that a DNA fragment ending 230 basepairs downstream of the transcriptional start site is 10-20 times more active than one which ends at +100 of the promoter (Dibbens and Egan, 1992, Tina Rathjen, unpublished results). The implications of this possible downstream enhancer element for regulation of the 186 *pV* promoter is addressed experimentally in Chapter 2.

The late regions from phages 186 and P2 show homology in both sequence and structure, and the 186 late promoters, *p12*, *pV* and *pJ*, show sequence identity with and occur in analogous positions to the P2 late promoters, *pP*, *pO* and *pF* respectively (see Figures 1.7 and 1.2). Based on this homology, it was originally proposed that 186 had a fourth late

promoter, *p32*, analogous to the P2 *pV* promoter and preceding the P2 *V* gene homologue, *orf32* (Portelli, 1995). However, 186 is lacking an equivalent to the *orf30* gene which lies upstream of and in the opposite direction to the *V* operon in P2, and which may stop transcription from the upstream *pO* promoter in this phage (see Figure 1.2). Thus, in 186, the upstream late promoter (equivalent to P2 *pO*) could continue transcribing rightward across *orf32* and downstream genes for the expression of the tail structural genes present there. In addition, this intergenic region of 186 shows little sequence identity with the P2 *pV* promoter sequence (see Figure 1.7), and no match was found to a weight matrix of B recognition sequences in previous analysis (Portelli *et al.*, 1998). Therefore, it was surprising to see a transcript which originated in this region (from position 9727 or 9728) in a preliminary primer extension experiment on temperature-induced 186 *cI*ts lysogenic cells (data not shown). Further experiments were performed to confirm the presence of a B-dependent promoter in this region (in Chapter 3) and the presence of a B binding site is discussed more below.

1.C.4. DNA BINDING BY 186 B AND B-HOMOLOGOUS PROTEINS.

1.C.4.1. Activated promoters are bound by the B homologues.

Figure 1.7 shows an alignment of promoters activated by the functionally interchangeable B-homologues: the 186 late promoters, P2 and P4 late promoters and the *S. marcescens* promoter, *pnucA*. Putative RNAP –10 and –35 recognition sequences are shown, as are bases that were protected in DNaseI footprinting experiments with either 186 B (186 *pV* and *p12*), or P4 Delta protein (other promoters). (Other promoters known to be activated by B-homologous proteins are not included in this alignment since the transcriptional start sites and/or activator binding sites are not known.) No basepair gaps were included upstream of the –35 sequence since this spacing appears to be important for promoter activation (see below). Gaps of up to 3bp were included between the other promoter sequences to align the +1 sites and putative –10 and –35 sequences, reflecting flexibility in the spacing of RNAP sigma70 recognition sequences. For the *pnucA* promoter, 11bp between the putative –35 sequence and upstream region has been removed in the alignment (as indicated) since promoter activation by NucC is unaffected by this deletion (Winslow *et al.*, 1998).

Promoters shown to be activated by B and homologous proteins have little similarity to the –10 and –35 consensus sequences required for sigma70 recognition and RNAP binding in *E. coli* and *S. marcescens* (see Figure 1.7), which is consistent with their low intrinsic

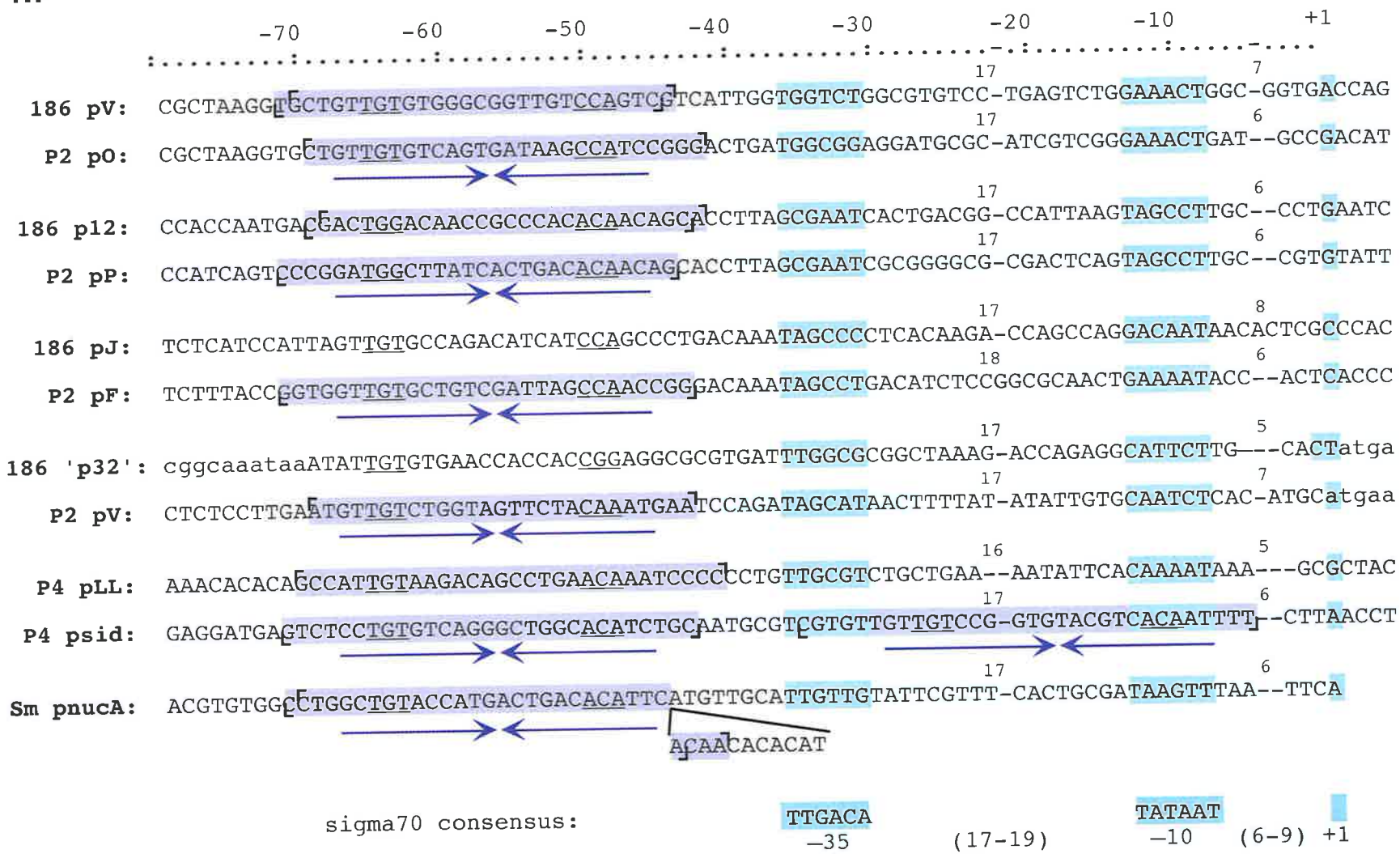
Figure 1.7. Promoters bound and activated by the interchangeable B-homologues.

Promoter sequences from 186 (*pV*, *p12*, *pJ* and putative promoter '*p32*') are aligned with the analogous promoters of P2 (*pO*, *pP*, *pF* and *pV* respectively), the late promoters of satellite phage P4 (*pLL* and *psid*) and the *Serratia marcescens* promoter *pnucA*. The transcriptional start site (+1) and putative –10 and –35 regions of the promoters are shaded in blue, and the number of nucleotides separating these sequences are indicated. Gaps of up to 3 basepairs are included between the promoter sequences in order to align them. *E. coli* and *S. marcescens* RNAP σ^{70} consensus –10 and –35 recognition sequences and optimal separation distances are given below the alignment. Eleven nucleotides from the *pnucA* promoter (between –44 and –54) are shown below the sequence in order to align the promoter and upstream activator-bound sequences. Numbering of nucleotides above the alignment is shown relative to the 186 *pV* +1 position. Sequencing of the 186 '*p32*' promoter showed deletion of a T at position 9723 of the full 186 sequence (Genbank accession number NC_001317), or –5 of the promoter, which was included here. The P2 *pP* sequence from Genbank (accession number NC_001895) which is shown here has an additional A at position –31 of the promoter, compared to the original sequence reported (Christie and Calendar, 1983).

The promoter regions protected in DNaseI footprinting experiments with purified 186 B protein (for 186 *pV* and *p12* promoters) or with purified P4 Delta protein (for other promoters) are shown with purple shading. Corners at the top or bottom of the sequence indicate the limits of protection from DNaseI cleavage when the top or bottom strand of the DNA respectively, was analyzed. Two regions bound by P4 Delta at the P4 *psid* promoter are each indicated – site I is upstream of the promoter, site II overlaps the putative promoter sequences. Imperfectly conserved 22bp inverted-repeat sequences within each of the protected regions are indicated by arrows, and three of the most highly conserved bases within these repeats (TGT/ACA) are underlined.

Promoter sequences (with Genbank accession numbers) are:
186 (NC_001317): *pV* 3786–3869; *p12* 3832–3748; *pJ* 14079–14163; '*p32*' 9650–9725
P2 (NC_001895): *pO* 3049–3131; *pP* 3094–3012; *pF* 17547-17630; *pV* 10229–10312
P4 (NC_001609): *pLL* – 9164-9084; *psid* – 9479-9561
Serratia marcescens (M19495): *pnucA* – 1-79 (–74 to +5) (plus –89 to –75 sequence from Chen *et al.*, 1992)

Figure 1.7


-70
-60
-50
-40
-30
-20
-10
+1
186 pV: CGCTAAGGTGCTGTTGTGTGGGCGGTTGTCCAGTCGTCATTGGTGGTCTGGCGTGTCC-TGAGTCTGGAAACTGGC-GGTGACCAG
17
7
P2 pO: CGCTAAGGTGCTGTTGTGTCAGTGATAAGCCATCCGGGACTGATGGCGGAGGATGCGC-ATCGTCGGGAAACTGAT--GCCGACAT
17
6
186 p12: CCACCAATGACGACTGGACAACCGCCCACACAACAGCACCTTAGCGAATCACTGACGG-CCATTAAGTAGCCTTGC--CCTGAATC
17
6
P2 pP: CCATCAGTCCCGGATGGCTTATCACTGACACAACAGCACCTTAGCGAATCGCGGGGCG-CGACTCAGTAGCCTTGC--CGTGTATT
17
6
186 pJ: TCTCATCCATTAGTTGTGCCAGACATCATCCAGCCCTGACAAATAGCCCCTCACAAGA-CCAGCCAGGACAATAACACTCGCCCAC
17
8
P2 pF: TCTTTACCGGTGGTTGTGCTGTCGATTAGCCAACCGGGACAAATAGCCTGACATCTCCGGCGCAACTGAAAATACC--ACTCACCC
18
6
186 'p32': cggcaaataaATATTGTGTGAACCACCACCGGAGGCGCGTGATTTGGCGCGGCTAAAG-ACCAGAGGCATTCTTG---CACTatga
17
5
P2 pV: CTCTCCTTGAATGTTGTCTGGTAGTTCTACAAATGAATCCAGATAGCATAACTTTTAT-ATATTGTGCAATCTCAC-ATGCatgaa
17
7
P4 pLL: AAACACACAGCCATTGTAAGACAGCCTGAACAAATCCCCCCTGTTGCGTCTGCTGAA--AATATTCACAAAATAAA---GCGCTAC
16
5
P4 psid: GAGGATGAGTCTCCTGTGTCAGGGCTGGCACATCTGCAATGCGTCGTGTTGTTGTCCG-GTGTACGTCACAATTTT--CTTAACCT
17
6
Sm pnucA: ACGTGTGGCCTGGCTGTACCATGACTGACACATTCATGTTGCATTGTTGTATTCGTTT-CACTGCGATAAGTTTAA--TTCA
17
6
ACAACACACAT
sigma70 consensus:
TTGACA
−35
(17-19)
TATAAT
−10
(6-9)
+1

activity (for example, Dibbens and Egan, 1992; Jin *et al.*, 1996; Julien and Calendar, 1996). However, alignment of the promoter sequences reveals some identity in the upstream regions that resembles a poorly conserved inverted-repeat, and DNaseI protection assays with purified activator proteins confirm that these sequences are bound by the B homologues.

Cadmium-complexed B protein, CdB, bound to a DNA fragment from –35 to –76 of the 186 *pV* promoter with a K_d of ~4μM in gel-shift experiments, giving a single retarded species (Pountney *et al.*, 1997). No improvement in binding was seen with a larger fragment of the promoter, implying that this region contains the only sequence recognized by B protein upstream of the *pV* or *p12* promoters. Binding of the B protein resulted in protection of nucleotides between –44 to –71 of *pV* (or –41 to –68 of *p12*) from DNaseI cleavage. Several enhancements of DNaseI cleavage in the presence of CdB also occurred at either end of the protected region, and in the inverted-repeat halfsite closest to *pV*, on either side of the most highly conserved basepairs (see Figure 1.7). These results suggest that B protein binds to a single site between the *p12* and *pV* promoters to enhance transcription from both of these promoters.

The purified B homologues (apart from Ogr) were also able to bind promoter sequences *in vitro* using DNA fragments spanning as little as –43 to –72 of the activated promoter (Julien and Calendar, 1995; Reiter *et al.*, 1998; McAlister *et al.*, 2003). DNaseI footprinting experiments on the P2 and P4 late promoters showed a similar pattern of protection for binding of each activator protein, with sequences between approximately –40 and –70 fully protected from DNaseI cleavage (Julien and Calendar, 1995, 1996; Reiter *et al.*, 1998; Christie *et al.*, 2003; McAlister *et al.*, 2003). The exact ends of the protection varied by about 1-3bp for different proteins or different promoters and in some cases weak enhancements of cleavage were seen at one or two bases at one end of the protected sequence. Binding of purified NucC protein to the P2 *pF* or *pnucA* promoters causes a significant bend (~89°) in the DNA (McAlister *et al.*, 2003). Similar DNA distortion by each of the activators would explain the enhanced access of DNaseI enzyme to the minor groove seen at the ends of the other activator binding sites. For the P2 *pO* and *pP* promoters a single protected area was seen which covered from –41 to –68 of *pO* or –43 to –70 of *pP*, indicating a single activator binding site between the promoters. Binding of the P4 δ protein to the *pnucA* sequence from *S. marcescens* also reduced DNaseI cleavage of a region upstream of the promoter, but in this case bases between –51 and –80 were protected

(Winslow *et al.*, 1998). In addition, a second region of the P4 *psid* promoter, between –5 and –35, was protected from DNaseI cleavage in the presence of purified P4 δ, ϕR73 δ or Pag protein (Julien and Calendar, 1995, 1996; Reiter *et al.*, 1998). Protection of ~30 basepairs at each binding site suggests that the activator proteins contact the DNA across approximately three turns of the helix.

The distance between the activator and RNAP binding sites has been shown to be important for promoter activation in reporter studies. The addition of 1bp at the P2 *pF* promoter abolished activation (but not binding) by the P4 δ protein (Christie *et al.*, 2003), and the removal of 2 to 9bp, but not 11bp, prevented activation of the *pnucA* promoter (where the activator binding site is further upstream) by the NucC protein (Winslow *et al.*, 1998). These results suggest that activator and RNAP binding sites must be on the same face of the DNA helix in order to facilitate an interaction between the proteins and allow activation of promoter transcription.

Several missense mutations in the B protein, which reduce the ability of B to activate the 186 late promoters, occur within the zinc-finger region of the protein. These changes, namely S16N, R17C, E25K and R26C, have been proposed to affect amino acids involved in DNA binding, though this has not been tested directly (Pountney *et al.*, 1997). Several residues within the zinc-finger of the Ogr protein have also been suggested to be involved in DNA binding, based on the importance of particular side chain groups for DNA binding by other proteins (Gebhardt, 1993). These residues are highlighted on the alignment of interchangeable B homologues in Figure 1.6.

Therefore, B and homologous proteins bind to their target promoters to protect ~30 basepairs of sequence centred mainly at –56 (or 23 basepairs upstream of the –35 sequence) from DNaseI cleavage. The protected region and promoter sequences must be on the same face of the DNA helix for activation to occur, suggesting that the activator proteins interact with the RNAP complex.

1.C.4.2. DNA recognition by the 186 B and homologous proteins.

An alignment of the two putative halfsites of the inverted-repeat sequences that are bound by B protein and its homologues in DNaseI footprinting experiments is shown in Figure 1.8. Sequences from the P2 and P4 late promoters, the *S. marcescens* promoter, *pnucA* and the 186 *pV/p12* promoters are shown, and a consensus recognition sequence (consensus 1) is also given. This sequence resembles a 22bp inverted-repeat with halfsites of:

GTTGTg(c/t)c(A/g)(g/t)C – where bases in capital letters are found in at least 8 of the 16 halfsites, and lower case letters indicate bases in at least 6 halfsites. Sequences centred at –56 of the 186 *pJ* and *p32* promoters which have homology with consensus 1 are also given, and a second consensus (consensus 2) including these sequences is not much altered: GTTGTG(T/c)(c/g)(A/g)(g/t)C (where bases in capitals are found in at least 10 of the 20 halfsites and those in lower case in at least 7 halfsites).

Mutational analysis of the *pF* and *psid* promoters in reporter assays suggest that three of the most highly conserved bases (TGT in each halfsite) are the most important for protein binding and promoter activation by the P4 δ protein (Van Bokkelen *et al.*, 1991; Reiter *et al.*, 1998; Christie *et al.*, 2003). However, it seems likely that recognition of the promoter DNA also involves other basepairs of the inverted-repeat and perhaps the surrounding sequence. Mutation of several other bases in the inverted-repeat sequence, apart from the conserved TGT, were found to affect activation of the *pF* and *psid* promoters by the P4 δ protein (Van Bokkelen *et al.*, 1991; Christie *et al.*, 2003). In addition, the two sites of *psid*, which each contain the conserved TGT sequence in both halfsites, showed an approximately 8-fold difference in affinity for the P4 δ protein (Reiter *et al.*, 1998). It is also likely that the different B-homologous activators have slightly different requirements for sequence recognition. As mentioned, P4 δ showed preferential binding to site II of *psid*, compared to site I, but the Pag protein appeared to bind to site I and II DNA equally well (Julien and Calendar, 1996; Reiter *et al.*, 1998). Purified proteins also showed different affinities for the *pF* promoter *in vitro* – NucC had a K_d of ~0.5μM in gel-shifts (McAlister *et al.*, 2003), while approximately 2μM and 75μM solutions of ϕR73 Delta and Pag, respectively, were required for ~50% DNA-binding in footprinting experiments (Julien and Calendar, 1996). However, this may also reflect the relative levels of active protein present in the preparations.

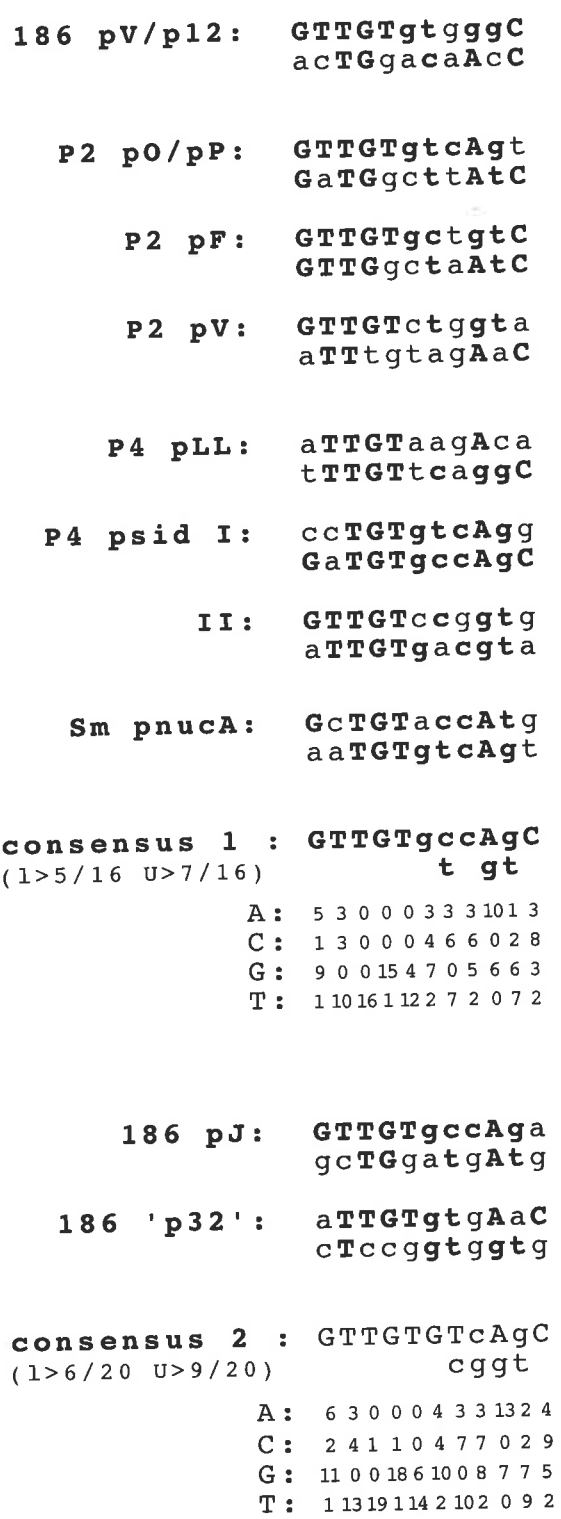
B-like activators have been divided into two classes – 'helper' or 'satellite' – based on the ability of the proteins to activate reporters of the P2 and P4 late promoters, and to cause the lethal expression of P2 late genes from a P2 prophage (McAlister *et al.*, 2003). Helper activators (Ogr and Pag) activated the P4 *sid* promoter more strongly than the satellite activators (P4 δ, ϕR73 δ and NucC), while the reverse was true for the P2 late promoters. Satellite activators expressed from a plasmid were also able to cause the lysis of P2 lysogenic cells, while helper activators were not, even though similar levels of helper or satellite activator protein were present (Julien and Calendar, 1996; McAlister *et al.*, 2003).

Figure 1.8. Alignment of recognition sequences bound by B-homologous proteins.

Each half of the 22bp inverted-repeat sequences highlighted in Figure 1.7. are aligned for the indicated promoters. For promoters shown to be bound by B or a B-related protein a consensus of the protein recognition sequences was determined using the frequency of each base at each position (consensus 1). Uppercase letters in the consensus indicate bases found in at least 8 of 16 sites and lower case letters represent bases found in 6 or more sites. Bases in the promoter sequences which are identical to consensus 1 are shown in bold, and in upper or lower case as for the consensus.

The 186 *pJ* and *'p32'* promoter sequences centred at –56 which show homology with the other promoters but have not been shown to be bound by a B-related protein, are also aligned and bases found in consensus 1 are indicated as for the other promoters. A second consensus (consensus 2) which includes these sequences was derived as for consensus 1, except that bases shown in upper case letters are found in at least 10 out of the 20 sites, and lower case letters in 7 or more sites.

Figure 1.8


186 pV/p12: GTTGTgtgggC acTGgacaAcC
P2 pO/pP: GTTGTgtcAgt GaTGgcttAtC
P2 pF: GTTGTgctgtC GTTGgctaAtC
P2 pV: GTTGTctggta aTTtgtagAaC
P4 pLL: aTTGTaagAca tTTGTtcaggC
P4 psid I: ccTGTgtcAgg GaTGTgccAgC
II: GTTGTccggtg aTTGTgacgta
Sm pnucA: GcTGTaccAtg aaTGTgtcAgt
consensus 1 : GTTGTgccAgC
(1>5/16 U>7/16) t gt
A: 5 3 0 0 0 3 3 3 1 0 1 3
C: 1 3 0 0 0 4 6 6 0 2 8
G: 9 0 0 15 4 7 0 5 6 6 3
T: 1 10 16 1 12 2 7 2 0 7 2
186 pJ: GTTGTgccAga gcTGgatgAtg
186 'p32': aTTGTgtgAaC cTccggtggtg
consensus 2 : GTTGTGTcAgC
(1>6/20 U>9/20) cggt
A: 6 3 0 0 0 4 3 3 13 2 4
C: 2 4 1 1 0 4 7 7 0 2 9
G: 11 0 0 18 6 10 0 8 7 7 5
T: 1 13 19 1 14 2 10 2 0 9 2

Presumably, these differences are at least partly due to different affinities of the proteins for the promoter sequences.

The activator-bound regions of 186 *p12/pV* and *pJ*, and the P2 and P4 late promoters, were previously used to construct a weight matrix of B recognition sequences (Portelli *et al.*, 1998). When these promoter sequences were scored with the weight matrix in comparison to random DNA, the worst scoring was the P4 *pLL* recognition sequence, which would be expected to occur by chance once every 4.6kb. No high-scoring site was found upstream of *orf32* of 186, and as Figure 1.8 shows, only one halfsite of the putative *p32* recognition site has much identity with the consensus sequence. However, this region gave a transcript initiating just downstream of the putative p32 promoter sequence in a preliminary primer extension assay (see earlier) and B activation of transcription from this region was investigated further here (see Chapter 3).

Thus, the B protein and its functionally interchangeable homologues bind to a 22bp poorly conserved inverted-repeat sequence usually centred at –56 on the promoter, with halfsites of GTTGTg(c/t)c(A/g)(g/t)C. Three of the most highly conserved basepairs, TGT, appear to be most important for activator binding, although other sequences may specify the affinity of the individual sites for different activator proteins.

1.C.4.3. The B homologues appear to bind the DNA as multimeric species.

A number of lines of evidence suggest that B-homologous proteins bind to the DNA as dimers or higher order multimers. Firstly, the inverted-repeat structure of the conserved sequence bound by the activator proteins suggests binding of at least two protein monomers to this sequence. Secondly, the P4 Delta protein – which is able to bind and activate promoters as well as, or better than, other B-homologous activators – appears to be a covalently joined head-to-tail dimer of B-like sequences (Julien *et al.*, 1997). Single halves of P4 expressed alone also show no protein activity, suggesting that the covalently joined halves have evolved to work together. Thirdly, P4 δ, ϕR73 δ and Pag proteins were purified as an active mixture of wildtype protein and an MBP-fusion protein (Julien and Calendar, 1995, 1996). Although the MBP-fused protein alone was inactive, DNA binding and promoter activation were seen in combination with the wildtype protein (and the fusion protein was required to keep the wildtype protein soluble). When used in gel-retardation assays with DNA fragments of the P2 and P4 late promoters, these mixed preparations of P4 δ protein gave multiple retarded bands of different mobilities (Julien and Calendar, 1995). Presumably these bands were the result of binding of increasingly larger multimers

of the P4 δ protein, perhaps including MBP-fused protein as well as wildtype. The formation of dimers of the P4 Delta protein, which is itself like a B dimer, suggests that higher order complexes of B and other shorter homologues may also form.

In vitro analyses of the purified B protein also suggest that it forms multimers in solution, though these experiments have been hampered by the insolubility of the protein at very high concentrations (Pountney *et al.*, 1997). Full-length B protein eluted from a gel chromatography column with an apparent molecular weight of 75kD, suggesting a large protein complex; while a 53-residue truncated CdB (molecular weight of ~6.4kD) eluted as a 13kD protein, and appeared to form dimers weakly in preliminary sedimentation equilibrium experiments. The truncated B53 protein was able to complement a phage lacking wildtype B expression *in vivo*, and *in vitro* appeared to bind a *pV* promoter fragment with approximately the same affinity as full-length protein. These results suggest that at least dimerization of the B protein is required for DNA binding and activation of promoter transcription. The C-terminal region appears to be required to form multimers larger than dimers, but is not absolutely necessary for protein activity. Further truncation of the B protein abolished protein activity *in vivo* – possibly due to the removal of residues important for protein dimerization or function, or due to a loss of protein structure and/or stability. Similar results have been found with the P2 Ogr and ϕR73 Delta proteins – protein activity is retained with removal of the C-terminal 15-20 amino acids, but further truncation abolishes activity (Gebhardt, 1993; Julien *et al.*, 1997).

Thus, 186 B and its homologues form dimeric or higher order multimers which appear to be required for DNA binding by the proteins.

1.C.4.4. Cooperativity in promoter activation by B-homologous proteins.

The P2 *pF* and P4 *psid* promoters show a sigmoidal response to increasing levels of purified Ogr or P4 Delta protein supplied to a transcription/translation coupled reporter assay *in vitro* (Lee and Christie, 1990; Julien and Calendar, 1995). In addition, when one halfsite of an inverted-repeat recognition sequence was mutated to remove activator binding, no P4 δ protein binding was seen to either halfsite and activation of the promoter was reduced (Reiter *et al.*, 1998; Christie *et al.*, 2003). These results suggest that the proteins may bind cooperatively to adjacent halfsites of the recognition sequence, although the result for P4 δ is complicated by the fact that it appears to naturally have a dimeric structure. Interestingly, no interaction was seen between P4 Delta protein bound to the two recognition sites at *psid*. Mutation of site II did not affect binding to site I, and these

sequences showed a difference in P4 Delta affinity that would not be expected if the sites were occupied simultaneously by a single protein complex (Julien and Calendar, 1996; Reiter *et al.*, 1998). Therefore, the P4 δ protein was unable to bind cooperatively to more than one inverted-repeat sequence when these binding sites were separated by 40 basepairs.

Therefore, the Ogr and P4 Delta proteins appear to show cooperativity in the activation of promoter targets. This cooperativity may occur in the formation of an active multimeric species in solution, which is able to bind the DNA; or in the binding and subsequent multimerization of the protein on the DNA. In either case, this apparent cooperativity may be important in enhancing the sensitivity of activated promoters to the regulator protein, which may be important in the regulation of late promoter transcription from the phage. This concept is discussed for the regulation of P2 Ogr and 186 B protein activity during phage infection in more detail later (see Chapter 5.D.).

1.C.5. THE B HOMOLOGUES APPEAR TO INTERACT WITH RNAP ALPHA-SUBUNIT IN ORDER TO ACTIVATE TRANSCRIPTION

1.C.5.1. Interaction with the RNAP α-subunit is required for activation by P2 Ogr, P4 Delta and φR73 Delta proteins.

Transcription in *E. coli* is catalyzed by the RNA polymerase (RNAP). The minimal protein complex required for transcription initiation consists of α, α, β, β' and σ subunits, and many transcriptional activators enhance polymerase activity by interacting with one of these, most commonly α and σ which contact the promoter DNA.

An interaction between the B-related proteins, P2 Ogr, P4 δ and φR73 δ, and the RNAP α-subunit, has been demonstrated by the isolation of alpha mutations which reduce promoter activation and which can be overcome by suppressor mutations in the activator protein. In fact, the first RNAP mutation found to affect transcriptional activation by a positive regulatory protein was the *rpoA*109 mutation, which prevented P2 phage plating by reducing Ogr activation of the P2 late promoters (Sunshine and Sauer, 1975). The *rpoA*109 mutation results in an L290H substitution in the C-terminal region of the RNAP α-subunit (Ayers *et al.*, 1994). The block to Ogr activity caused by this mutation can be overcome by a tyrosine-to-cysteine substitution at residue 42 of the activator (Christie *et al.*, 1986; Ayers *et al.*, 1994), and several other small, hydrophobic residues at this position restore Ogr activity in the *rpoA* strain (King *et al.*, 1992). This suggests that the *rpoA*109 block to Ogr

activity is caused primarily by steric hinderance of the protein-protein contact rather than by removal of a specific interaction. P4 plating and late promoter activation by the P4 δ protein is also reduced in the *rpoA*109 strain, unless Delta carries a mutation causing a threonine to alanine change at residue 127 of δ (equivalent to residue 44 of Ogr, when the second-half of Delta is aligned to the Ogr amino acid sequence – Halling *et al.*, 1990).

Another RNAP α-subunit mutation, *rpoA*155, which results in a leucine-to-phenylalanine substitution at residue 289, also prevents plating by P2 phages and P4 carrying the φR73 δ gene (Ayers *et al.*, 1994; Julien *et al.*, 1998). Compensatory mutations result in replacement of an alanine with valine at residue 13 in Ogr (Ayers *et al.*, 1994) or a valine to methionine change at residue 19 in φR73 δ protein (Julien *et al.*, 1998) respectively. These mutations seemed to give a more general increase in phage plaque formation and are therefore thought to enhance a specific side-chain contact between B homologues and alpha that is required for activation. In addition, mutation of residues 20 or 42 and 44 in φR73 δ results in reduced activation of a late promoter reporter but not DNA binding in an *in vivo* assay (Julien *et al.*, 1998). It has therefore been suggested that residues 19, 20, 42 and 44 of the φR73 δ protein form a surface patch that is involved in a direct interaction with the RNAP α-subunit to increase promoter transcription.

Alanine-scanning mutagenesis of the C-terminal domain of the α-subunit suggests that residues E286, V287, L289 and L290 form the contact site in alpha for the Ogr protein interaction (Wood *et al.*, 1997). Activation of transcription by Ogr was also reduced by mutations which appear to affect the general structure of the α-subunit, and some which have previously been implicated in binding of alpha to UP elements on the promoter. UP-elements are AT-rich sequences found between –40 and –60 on certain promoters that are thought to enhance transcription by increasing the affinity of RNAP for the promoter sequence (Gourse *et al.*, 2000). Although the P2 late promoters do not have obvious UP-element sequences, these mutants suggest that the RNAP α-subunit may also make contacts with the DNA at these promoters.

How does this protein contact between the RNAP α-subunit and the B-homologous proteins enhance transcription? As mentioned above, promoters activated by B-like proteins generally have a poor match to the –10 and –35 consensus sequences recognized by RNAP (Figure 1.7). In fact, RNAP alone was unable to bind to the P2 *pF* late promoter sequence (McAlister *et al.*, 2003). *In vitro* footprinting experiments showed enhanced

RNAP binding to the *pF* promoter DNA in the presence of purified NucC protein (McAlister *et al.*, 2003), suggesting that activation may be via recruitment of the polymerase. However, when the –35 sequence of *pF* was replaced by the consensus sequence for sigma70 recognition (TTGACA) only a small increase in intrinsic transcription from the promoter was seen, which could still be significantly activated by P4 Delta (Christie *et al.*, 2003). Therefore, other steps in the initiation of transcription at the activated promoters may also be limiting and may be enhanced by the B-homologous proteins.

1.C.5.2. Does 186 B interact with the RNAP α-subunit?

It was initially reported that the RNAP α-subunit mutation *rpoA*109 did not disrupt the formation of plaques of the Hy5 phage, which carries the late activator and early lytic genes of 186 and the late region of P2 (Sauer, 1979). This implied that B activation of the late promoters was unaffected by the mutation and therefore that B may make a completely different interaction with RNAP or another host protein, despite having a very similar amino acid sequence compared to P2 Ogr. The potential enhancer element downstream of *pV* (see Section 1.C.3.2.) also suggested that other host proteins may be involved in stimulation of transcription from this 186 late promoter. This was a novel idea at the time and was the incentive for the selection of bacterial mutants which reduced B activation of the 186 late promoters and were therefore hoped to reveal unique interactions required for B activity (Ravi Tiwari, unpublished results). Four of these mutants which had not been fully characterized were analyzed here, as described in Chapter 2.

More recently, in preliminary experiments B activation of a *lacZ* reporter of the 186 late promoter, *pV*, was somewhat reduced (to ~32% of wildtype) by the *rpoA*109 mutation (see Section 2.A.2.1. – Tina Rathjen, unpublished). In addition, several missense mutations which inactivate B protein (Pountney *et al.*, 1997) may be explained if B does contact the RNAP α-subunit in a similar way to Ogr and other homologous proteins. T41I and E43K mutations of B are found adjacent to residues 42 and 44 which have been implicated in activator-alpha interactions, and a T20I substitution occurs in the same region as residues thought to be important for the ϕR73 δ protein contact with alpha. In the light of these results, the effect of the *rpoA*109 mutation on 186 phage plating and B protein activation of a transcriptional reporter was more thoroughly reexamined here (see Chapter 2).

Thus, 186 B and homologous proteins which are functionally interchangeable are transcriptional activators which bind upstream of certain promoter sequences, and interact

with the RNAP α-subunit to enhance transcription from the promoters. They appear to recognize a poorly conserved 22bp inverted-repeat sequence, which they probably bind as a dimer or higher order multimer. 186 B may differ from the other activators in the interaction it makes with the RNAP α-subunit, and by possibly requiring novel host functions for activity. Both of these possibilities are investigated in Chapter 2 of this thesis. A putative fourth 186 late promoter is also investigated in Chapter 3, and the implications of this promoter for recognition of promoter sequences by B protein are further discussed in Chapter 5.

1.D. REGULATION OF LATE GENE EXPRESSION IN 186.

1.D.1. NECESSITY FOR AND EXAMPLES OF LATE GENE REGULATION IN PHAGES.

For optimal virion production following phage infection, the expression of multiple copies of the structural proteins and their assembly must be delayed until after replication of the phage genome has occurred, and the cell must be lysed only after large numbers of mature progeny phages have been formed. Expression of the structural and lysis genes earlier in the infection could result in virions being formed with empty heads (lacking the phage genome) and/or lysis of the cell before progeny have had a chance to assemble. Therefore, the expression of phage structural and lysis genes is usually regulated so that it only occurs later in the phage infection after replication has taken place.

Expression of structural genes is often coordinated by their co-location in the genome and the use of a common means of regulation. Expression is usually broadly regulated at the level of transcription – allowing the phage to both save energy by reducing unnecessary expression when these functions are not required, and to avoid premature activity of the morphogenetic proteins during lytic development. Finer control of protein activity for individual functions may occur at later stages of gene expression, such as protein translation (for example, in lambda – Sampson *et al.*, 1988) or through interaction with other phage proteins. Therefore, regulation of phage structural gene expression is often achieved by controlling the expression or activity of a regulatory protein that is required to enhance transcription of these genes from one or more promoters. Examples of this are seen in several well-characterized phages. For the T7 phage, structural gene transcription requires a phage-encoded RNAP and entry of the relevant part of the genome into the cell, which is delayed until after early and middle phage gene expression (Hausmann, 1988). While for the T4 phage, late promoter transcription only occurs when the sliding clamp

protein, which is part of the phage replisome, passes across the promoters and interacts with phage proteins attached to RNAP to stimulate transcription (reviewed by Miller *et al.*, 2003). This also provides a mechanism to directly link late gene expression to replication of the genome and avoid premature expression of the structural or lysis genes.

For other bacteriophages which have been studied in some detail, it is still not completely clear how late gene expression is delayed during lytic development. An example of this is lambda. Lambda late genes are transcribed when the phage-encoded Q protein antiterminates transcription of a constitutive phage promoter, *pR'* at the *tR'* terminator (reviewed by Roberts *et al.*, 1998). The *Q* gene is transcribed at the end of the ~5kb transcript from the lytic promoter, lambda *pR*, and requires the antitermination activity of the early lytic protein N, for full expression (Herskowitz and Hagen, 1980). Kobiler *et al.* (2005) predict that it would take ~3 minutes for the *Q* gene to be transcribed, however, Q-enhanced late transcription and late protein activity can only be detected from 10-15 minutes after infection (Murialdo and Siminovitch, 1972; Chang *et al.*, 1995; Kobiler *et al.*, 2005). Q-dependent antitermination is enhanced in a non-linear fashion by increasing levels of protein *in vitro* and *in vivo*, and it has been suggested that Q protein must reach a threshold concentration to function during phage development (Yang *et al.*, 1987; Kobiler *et al.*, 2005). Expression of these levels of Q protein (and therefore late gene transcription) may be delayed by several mechanisms. Earlier expression and activity of the late proteins is seen in the absence of the lambda CII protein (which is reponsible for establishment of lysogeny) due to its activation of an oppositely orientated promoter within the *Q* gene, *paQ* (McMacken *et al.*, 1970; Ho and Rosenberg, 1985; Hoopes and McClure, 1985; Stephenson, 1985; Kobiler *et al.*, 2005). In addition, increased Q expression may require phage replication to provide a greater number of copies of the *Q* gene – Q expression from a non-replicating phage gives little increase in late protein expression from the phage or a Q^- prophage (Dambly *et al.*, 1968).

Replication of the late genes may also be required to supply the necessary levels of structural and lysis functions for phage production, since infection of a lysogenic cell by a hetero-immune phage with a mutated late gene generally results in <1 phage per cell being released (Thomas, 1970). Therefore, *Q* and late gene expression appears to be delayed by CII-dependent and CII-independent mechanisms, and full expression of the late functions may require the increased *Q* and late gene dosage provided by replication of the phage genome.

During lysogenic development by temperate bacteriophages, expression of the phage morphogenesis and lysis genes is not necessary and may be detrimental to the growth of the cell – perhaps by interfering with normal bacterial functions, or even causing cell lysis. Where transcription of these genes is positively regulated, their expression in the lysogen can be avoided simply by preventing production of the activator protein. Lambda provides a well-characterized example of this. In a lambda lysogen, *Q* (and therefore late gene) expression is prevented by the CI protein repressing lytic transcription from *pR* and expression of the N protein, both of which are required for *Q* transcription (Herskowitz and Hagen, 1980). If Q is expressed from a constitutive promoter on the phage which is not under the control of CI (as for the λ *qin*101 mutant) stable lysogens cannot be formed (Dambly *et al.*, 1979). In this case, mutation of the lysis genes, *S* and *R*, and the *A* gene which encodes the large terminase subunit, restores lysogen formation, implying that expression of these genes is lethal to the lambda lysogenic cell.

1.D.2. LATE GENE REGULATION IN THE 186-HOMOLOGOUS PHAGE P2.

1.D.2.1. Regulation of P2 morphogenetic gene expression during lytic development.

Late gene transcription in the 186-homologous phage P2 is activated by the Ogr protein. Increasing levels of purified Ogr protein *in vitro* give non-linear activation of a P4 promoter, *psid*, suggesting that there may be some cooperativity in promoter activation by this protein (Lee and Christie, 1990). It also suggests that P2 late promoter activity may increase rapidly with a small increase in Ogr expression *in vivo*, and that Ogr protein levels may be regulated around this threshold concentration in order to control the timing of late promoter transcription from the phage.

Ogr is expressed from a promoter which shows some identity with the –10 and –35 RNAP consensus recognition sequences (5/6 and 3/6 respectively – Christie *et al.*, 1986; Kalionis *et al.*, 1986a) but has little constitutive activity *in vitro* with purified RNAP (Pritchard and Egan, 1985) or fused to a chloramphenicol acetyltransferase (*cat*) gene reporter *in vivo* (Birkeland *et al.*, 1991). Transcripts from the *ogr* promoter during a wildtype P2 phage infection are detectable after ~10 minutes (slightly later than those of the early lytic gene, *cox*) and increase rapidly between 15-20 minutes after infection (Birkeland *et al.*, 1991). Ogr-activated expression of structural genes can be detected at least 10 minutes later during wildtype lytic development by the phage (Christie and Calendar, 1983; Birkeland *et al.*, 1991). It has been proposed that increased *pogr* transcription at a middle time after

infection is due to activation of the promoter by an early expressed P2 protein, although no candidates have been identified. Ogr itself does not appear to affect transcription from its own promoter, since a similar increase in *pogr* transcripts was seen when the *ogr* gene was mutated. A similar pattern of *ogr* transcription was also seen in a non-replicating phage (mutated in the P2 *A* gene), although a lower maximal level of message was reached due to the lower number of copies of the genome present. In addition, the level of protein produced by this non-replicating P2 phage was sufficient to complement a co-infecting phage defective in *ogr* gene expression, though the P2 *ogr*$^-$ burst was slightly smaller than that of wildtype P2 (Halling and Calendar, 1990; Birkeland *et al.*, 1991). These results suggest that increased Ogr expression is not dependent on the P2 A protein or phage replication.

From about 15-20 minutes after a wildtype P2 phage infection, the *ogr* gene could also be detected on a larger transcript which probably originated at the upstream late promoter, *pF*, since there are no transcriptional terminators separating the *FETUD* operon and *ogr* gene (Birkeland *et al.*, 1991). It is not clear how important this positive autoregulation of *ogr* transcription is for activator expression, since a P2 phage carrying a polar amber mutation in the *F* gene was still able to complement a co-infecting phage deleted in the *ogr* gene. However, when Ogr activated the late promoters, including *pF*, transcription from *pogr* was inhibited (Birkeland, 1992). It was suggested that this was due to negative regulation of *pogr* by a late gene product, but it seems equally likely that the upstream late promoter interferes with *pogr* transcription. Activity of a strong upstream promoter has been shown in several cases to inhibit transcription from a weaker downstream promoter sequence, and a number of mechanisms have been proposed to explain this interference (Callen *et al.*, 2004; Sneppen *et al.*, 2005).

Increased expression of the P2 late functions during a P2 infection also requires replication of the phage, since a non-replicating P2 is unable to express enhanced levels of late transcripts or to complement a co-infecting phage mutated in one of the essential late genes (Lindahl, 1970; Geisselsoder *et al.*, 1973; Christie and Calendar, 1983). Phage replication proteins (A and B) do not act as co-activators of late transcription since Ogr is the only phage protein required for expression of plasmid-encoded reporters of the P2 late promoters *in vivo* (Grambow *et al.*, 1990; Ayers *et al.*, 1994; Wood *et al.*, 1997). Instead, based on similar findings for the T4 phage, it was originally suggested that the Ogr protein required the replication machinery or a replicating DNA template in order to activate these

promoters (Bertani and Six, 1988; Birkeland *et al.*, 1991). In support of this idea, high levels of Ogr protein from a plasmid were unable to induce the lethal expression of lytic genes from a P2 prophage, which would be replicated only once per cell generation (Julien and Calendar, 1996; McAlister *et al.*, 2003). However, in other experiments a chromosomal reporter of the P4 promoter, *psid*, was significantly activated by plasmid-expressed Ogr protein (Reiter *et al.*, 1998). It is therefore likely that replication of the P2 late genes is required simply to supply an increased number of copies of these genes, in order to provide sufficient levels of late functions for the phage burst.

1.D.2.2. Control of P2 late gene expression during lysogeny.

Ogr expression also appears to be under the control of the immunity repressor of P2, as very little *ogr* transcription is seen during P2 infection of a cell expressing the P2 C protein (Birkeland, 1992). However, repressor control of the P2 *ogr* promoter may be indirect, since the promoter sequence does not appear to contain potential C binding sites. Experiments with the P2 Hy dis phage instead support the idea that repressor control is due to the C protein repressing expression of an early P2 phage protein required for *pogr* activation (Birkeland *et al.*, 1991). The P2 Hy dis phage is a hybrid phage which is thought to carry the *ogr* and late genes of wildtype P2 and the immunity region and part of the early gene sequence from a defective hetero-immune P2-like prophage in *E. coli* B. P2 Δ*ogr* infection of a P2 Hy dis lysogen gives P2 Δ*ogr* plaques and shows increased P2 Hy dis *ogr* transcription – suggesting that Ogr expression from the P2 Hy dis lysogen (which is not induced) is stimulated by a P2 early lytic protein. However, the entire P2 Hy dis sequence has not been determined and it is possible that the *ogr* gene present on the hybrid phage is regulated differently to the wildtype P2 *ogr* gene.

When transcript levels are measured directly in a P2 lysogen, a low level of *pogr* mRNA can be seen (Birkeland *et al.*, 1991). These transcripts are most likely due to spontaneous reversion of the prophage to lytic development in a small number of lysogenic cells, and the accompanying lytic transcription that occurs. This explanation was dismissed by Birkland *et al.* (1991), since similar levels of *pogr* transcript were seen when the *cox* gene was mutated and the prophage could not excise. However, it seems that induced P2 prophages excise at a very low rate normally, and that P2 replication and Ogr expression can occur *in situ* (Bertani and Six, 1988).

Thus, it appears that the *ogr* gene is expressed from a weakly constitutive promoter that is activated by an unidentified early expressed phage protein at a middle time after P2 phage

infection, in order to produce sufficient levels of Ogr protein for activation of the P2 late promoters. Repression of early lytic gene expression by the P2 lysogenic repressor protein, C, probably prevents expression of the *pogr* activator and therefore also of significant Ogr expression and activity in a P2 lysogen. Later during infection, the *ogr* gene is expressed at high levels from the upstream late promoter, *pF*, whose transcription may also interfere with the activity of the *ogr* promoter. In addition, replication of the phage late genes may be important to express sufficient levels of late functions for a normal P2 phage burst.

1.D.3. LATE GENE REGULATION IN 186.

1.D.3.1. Regulation of structural gene expression during 186 lytic development.

Like many other characterized phages, 186 regulates the expression of phage structural and lysis genes by using a phage-encoded activator, B, to enhance transcription of these genes late in infection. However, 186 *B* has an unusual transcription pattern for an activator of late gene expression. *B* transcripts can be detected from as early as 2.5 minutes after the start of lytic development (Richardson, 1993) – at the same time as mRNA from the early lytic promoter, *pR* and much earlier than late gene transcripts (Finnegan and Egan, 1981; Kalionis *et al.*, 1986a; Dibbens and Egan, 1992; Richardson, 1993). The *B* promoter has near-consensus –10 and –35 RNAP σ^{70} recognition sequences (each with 5/6bp identical to the consensus) and the isolated promoter is intrinsically quite strong *in vitro* (Pritchard and Egan, 1985) and *in vivo* (Shearwin *et al.*, 2002). A transcriptional fusion of *pB* to the *lacZ* reporter gene, inserted into the host chromosome, gave ~140 units of LacZ activity (Shearwin *et al.*, 2002). (This is compared to ~1000 LacZ units from the unrepressed lambda *pR* promoter using a similar single-copy *lacZ* reporter – Dodd *et al.*, 2004.) Although B protein expression also requires that the message be translated, and the polypeptide folded correctly, and each of these may be less than ideal, it seems likely that at least some B protein is present early during the 186 infection. However, such early *B* transcription does not result in premature expression of the late genes, so there must be additional mechanisms to restrict B protein activity to later in infection.

Delayed expression of the late genes does not seem to be due to the requirement for an additional factor, such as a co-activator of late transcription, which is only present late in the phage infection. The B protein alone can activate transcriptional reporter fusions of the late promoters *in vivo* (Dibbens, 1990; Dibbens and Egan, 1992; Xue and Egan, 1995b) and mutation of most early lytic genes does not significantly affect phage production (Dodd and Egan, 1999). However, Finnegan and Egan (1981) found that increased transcription of the

186 late genes during temperature induction of a 186 *cI*ts lysogen required expression of a wildtype replicase protein, as well as B. It was originally suggested that phage replication could provide the correct topology for B protein binding and activation, as a similar theory had been put forward for Ogr activation of the P2 late genes (Kalionis *et al.*, 1986a). However, Dibbens and Egan (1992) showed that B protein could give a similar level of late gene transcription from a 186 prophage as from a replicating 186 genome, taking into account the difference in copy number; and B protein could also activate chromosomal *lacZ* reporters of the 186 late promoters to a high level (Tina Rathjen, unpublished). It was therefore concluded that template topology played no role in B stimulation of the 186 late promoters.

Alternatively, B protein activity could be enhanced late in phage development by the increase in protein expression provided by the larger number of *B* genes present after phage replication. This would provide a simple mechanism for delaying late gene expression so that it occurred only after the phage genome had been replicated. In fact, higher levels of the B message were seen ~20-25 minutes after induction of a 186 *cI*ts lysogen, presumably due to 186 replication, at about the same time as transcripts of the phage structural genes could be detected (Richardson, 1993). This idea, originally proposed by Dibbens and Egan (1992), has been directly investigated here by determining the effect of an increasing number of copies of the *B* gene on 186 late promoter activity and on production of phages in a 186 B^- infection (see Chapter 3).

It is also possible that the level of B protein expression (and therefore activity) is enhanced late in 186 infection by other means, such as positive regulation of the B promoter, though this seems unlikely. As mentioned, no 186 early lytic proteins except the replicase (A) are essential for the phage burst, suggesting that none of these proteins are required to stimulate *B* transcription. In addition, previous experiments suggest that the B protein does not positively regulate its own promoter. Little difference in the level of transcription from *pB* was seen whether it controlled expression of a wildtype or mutated *B* gene on a plasmid; and the activity of a transcriptional reporter of *pB* was not affected by B protein expressed from a compatible plasmid, although only one concentration of B was tested (Dibbens, 1990). Late in infection, the *B* gene also appears on a longer transcript that probably originates at the upstream late promoter, *pJ*, since no intervening transcriptional terminators have been found between the late operon and B gene (Kalionis *et al.*, 1986b; Kalionis *et al.*, 1986a; Portelli *et al.*, 1998). This indirect positive autoregulation by B does not appear to

be essential, however, as a polar amber mutation in the *J* operon does not prevent B protein activity (Portelli *et al.*, 1998). In order to confirm that B does not regulate its own promoter, transcription from *pB* was also assayed here in response to a range of B protein concentrations (see Chapter 3).

Previous experiments by Hocking and Egan (1982a, b, c, d) suggest another role for phage replication in 186 late gene expression – to directly increase the level of late protein production. In these experiments, 186 late gene expression from a prophage was unable to complement a 186 *vir* phage mutated in an essential late gene (for example, 186 *vir* V^-), with recombination occurring instead. Similarly, the late functions expressed from a non-replicating 186 phage (186 A^-) were unable to complement a co-infecting 186 late gene mutant (A protein is *cis*-acting). These results suggest that a single copy of the late genes provides insufficient late functions for the formation of progeny phages, and therefore that replication may be required to increase the late gene dosage and enhance late protein expression. If correct, early expression of active levels of B protein may not affect the overall timing of significant late protein expression (which would still require replication of the late genes) and may not affect the phage yield. This idea was tested directly here, in Chapter 3.

1.D.3.2. Regulation of late gene expression during 186 lysogenic development.

Transcription from the 186 *B* promoter is repressed ~30-fold in a 186 lysogen by binding of the CI protein to the promoter sequence (Dibbens *et al.*, 1992; Dodd and Egan, 1996; Shearwin *et al.*, 2002). Although this repression is not complete (a chromosomal *pB.lacZ* reporter still gives 10-20 units of LacZ activity in a 186 lysogen), it appears to be sufficient to prevent detectable levels of 186 late transcripts being expressed in a lysogenic cell (Finnegan and Egan, 1981; Dibbens and Egan, 1992; Xue and Egan, 1995b). A low level of B message was measured in a 186 lysogenic culture, but this may have been due to a small proportion of the cells having spontaneously reverted to lytic development (Kalionis *et al.*, 1986a; Dibbens *et al.*, 1992). In a 186 B^- lysogen, higher levels of *pB* (and pR – Gregory, 1991) transcripts were measured, probably due to the excessive phage replication that is seen in 186 B^- phage development resulting in increased phage transcription in these lytic revertants (Finnegan and Egan, 1981; Hocking and Egan, 1982d; Orsborn, 1986).

As might be expected, B protein expression can be lethal to a 186 lysogenic cell – transformation of a wildtype 186 lysogen by a plasmid expressing high levels of B protein gave <1 transformant per microgram of plasmid DNA (Dibbens and Egan, 1992).

Transformation was restored if B expression from the plasmid was reduced or abolished, or if the host was a non-lysogen, implying that the B protein was activating expression of lethal levels of 186 late genes from the prophage. Preliminary experiments performed by Ravi Tiwari (unpublished) suggested that cell death in these assays was due to the expression of a 186 phage gene or genes in the *V* operon. Amber mutations in this operon, which inactivate the gene product in a non-suppressor host and show polar effects on downstream genes, allowed the 186 lysogen to be transformed by the B plasmid, when present in the *R* or upstream genes, but not in the *P* (endolysin) gene. This region includes the *orf24* gene (see Figure 1.2) which encodes the holin protein, and would be a good candidate for the lethal function.

These experiments provide an obvious explanation for the repression of *B* transcription by the 186 CI protein, to prevent lethal late gene expression in a 186 lysogen. However, in the above experiments, reducing B protein expression from the plasmid allowed transformation of a 186 lysogen (Dibbens and Egan, 1992), and lower B expression would be expected from the single-copy prophage genome. In addition, when a 186 *cI*ts prophage which is unable to excise is derepressed by inactivation of CI protein at high temperature, no lysogenic cells are killed if the early lytic protein Dhr (which represses host cell replication) is inactive (Richardson and Egan, 1989). This implies that derepressed *B* and late gene expression from the prophage is not lethal to the cell.

Why then B expression under CI control in a 186 lysogen? It is possible that unrepressed expression of *B* (and the late genes) from the prophage, although not lethal, may still be detrimental to the cell. Even affecting the growth of the lysogen by a small amount may give the cell a disadvantage compared to non-lysogenic cells. Alternatively, B or late gene expression may affect another stage of lysogenic development – for example, reducing the formation of lysogens or assisting prophage induction to reduce lysogenic stability – in an as yet uncharacterized (and unexpected) way. These possibilities are discussed more in Chapter 4, and are then examined directly by removing CI repression of *pB* on the phage, and assaying the effect on lysogenic development.

It is also possible that CI repression of the B promoter in a 186 lysogenic cell has a protective effect against infection by 186-related phages with B-responsive promoters (for example, satellite phages P4 and ϕR73). In this case, B expression in the 186 lysogen could enhance lytic development by the super-infecting phage and potentially kill the cell without

release of the 186 prophage. This explanation is not exclusive of other possibilities, but was not considered further here.

Lastly, it also plausible that repression of *B* gene expression is incidental to another role of CI binding to this region – namely, to interact with a 14-mer of the lysogenic repressor bound to *pR*, in order to increase *pR* repression and enhance the formation of 186 lysogens. This possibility (outlined in Section 1.B.3.2.) is discussed more in Chapter 4, where it is also addressed experimentally by measuring the response to CI protein of a *pR* reporter which includes the *pB* operator sequences.

1.E. THESIS AIMS.

The aims of this thesis were to investigate potentially novel aspects of the regulation of *B* and morphogenetic gene expression in coliphage 186, in order to understand more fully how late gene expression is controlled in this phage, and how gene expression may be regulated in general.

Three specific aims were pursued in this project:

(1) to characterize *E. coli* mutants that appear to abolish 186 B protein activity;

(2) to determine the role of replication for the provision of late functions during the phage lytic cycle; and

(3) to determine the role of CI repression of the 186 *B* promoter.

The results of these investigations are described in Chapters 2, 3 and 4 respectively.

CHAPTER 2 :

CHARACTERIZATION OF *E. COLI* MUTANTS WHICH APPEAR TO ABOLISH 186 B PROTEIN ACTIVITY

2.A. INTRODUCTION.

2.A.1. THE SELECTION OF *E. COLI* MUTANTS WITH REDUCED B ACTIVITY.

2.A.1.1. The rationale for Host Mutant selection.

The first *Escherichia coli* mutant to demonstrate that regulation by a transcriptional activator protein could be due to interaction with the RNA polymerase complex, was the *rpoA*109 strain (Sunshine and Sauer, 1975). This mutation results in a leucine-to-histidine change at residue 290 of the C-terminal domain of the α-subunit and prevents P2 phage production by reducing Ogr activation of the P2 late promoters (Grambow *et al.*, 1990; Ayers *et al.*, 1994). Other *rpoA* mutations affecting Ogr function have since been characterized (Wood *et al.*, 1997), and the requirement for an interaction between the Ogr protein and RNA polymerase α-subunit to facilitate P2 late promoter activation, is well established. Similarly, two other B homologues, the late activator proteins of satellite phages P4 and φR73 show reduced activity in the *rpoA*109 mutant strain.

Since Ogr and B are so similar (~63% identity – Kalionis *et al.*, 1986a) it was expected that B would make the same interaction with the RNA polymerase α-subunit. However initial experiments found that the Hy5 phage, which carries the 186 *B* and early genes and P2 late genes (Hocking and Egan, 1982a), could plate on the *rpoA*109 strain (Sauer, 1979), implying that B activity was not significantly reduced by this substitution. It was concluded that transcriptional activation by B is mediated either by contact with other residues in the α-subunit, or by interaction with a different RNA polymerase subunit altogether.

With the expectation of discovering a novel interaction between B and RNA polymerase, or other *E. coli* proteins required for normal B activity (such as chaperones or regulatory proteins), a selection system was developed based on B-mediated expression of a lethal function.

2.A.1.2. Selection of Host Mutants affecting B activity (by Ravi Tiwari).

Dibbens and Egan (1992) found that a 186 lysogen could not be transformed with a plasmid expressing high levels of B protein, presumably due to B-activated expression of a lethal 186 late protein or proteins. This result could be reproduced using a p15A-origin pACYC184 plasmid carrying the 10-22% BamHI fragment of 186 (pKill), which extends from within *orf12* to within the *Q* gene of the 186 genome (Ravi Tiwari, unpublished – see

Figure 1.2). Thus, E251 cells were transformed with the pKill plasmid with ~10^7-fold lower efficiency in the presence of a ColE1-based B expression plasmid, pKO2-B, than in its absence (when about 10^8 transformants per microgram of plasmid were obtained).

These plasmids were therefore used by Ravi Tiwari (unpublished) to select for host mutants which showed reduced expression of the lethal function from the B-activated 186 late promoter. In this protocol, E251 cells carrying the pKill plasmid were mutagenized by treatment with nitrosoguanidine for 40 minutes and cultured overnight before transformation with the pKO2-B plasmid. Surviving transformants appeared at a frequency of ~10^4 colonies per microgram of plasmid DNA.

It was considered unlikely that the transformants carried a mutation in the 186 lethal gene itself, since this was present on a multiple copy plasmid. The survivors were therefore expected to have a host mutation that interfered with B-dependent expression of the lethal gene. Such a mutation could affect a host function required for normal B activation of the 186 late promoters, or for maintenance of the selection plasmids. To distinguish between these possibilities, Ravi Tiwari (unpublished) screened survivors for sensitivity to 186 *vir1* phage infection by cross streaking against this phage. Most were found to be 186 sensitive and were presumed to have mutations which disrupted maintenance of one or both of the selection plasmids, or which decreased but didn't eliminate B function. However, ~1 in 200 survivors proved resistant to 186 *vir1*. As resistance to 186 replication *per se* had never been used in the selection procedure, these surviving colonies were expected to be mutated in a host function or functions whose loss completely and specifically prevented B activation of 186 late promoters and thereby abolished 186 infection.

Four of these 186 resistant survivors, Host Mutants (HM) 2, 3, 4 and 6 were then cured of the selection plasmids by growth without antibiotic selection (Ravi Tiwari, unpublished).

2.A.2. Initial characterization of *E. coli* mutants with reduced B activity.

2.A.2.1. Initial characterization of the Host Mutants (by Tina Rathjen).

These B-affecting bacterial mutants were initially characterized by Tina Rathjen (unpublished) who assayed the efficiency of 186 plating and B-stimulated transcription of a *lacZ* reporter from the 186 late promoter, *pV*, in these strains. E251 cells carrying the *rpoA*109 mutation (E251 *rpoA*109) of the RNA polymerase α-subunit were also assayed,

and the results of these experiments are shown in Figure 2.1A. In these experiments, 186 phages plated with an efficiency of $<10^{-8}$ on each of the Host Mutants compared to wildtype E251, indicating that 186 infection was completely abolished in these strains. In contrast, 186 plated on the *rpoA*109 strain with an efficiency of plating of 0.98, implying that 186 phage production was not dramatically affected by this mutation.

In order to assay B function, a 186 *pV* promoter fragment (from –260 to +86) was cloned into the pMRR9 plasmid to give a transcriptional fusion to the *lacZ* reporter gene. B expression was induced with 0.1mM IPTG from the pTMC10-B plasmid, which carries the *B* gene under control of the *lac* promoter and also encodes the LacI repressor protein. Control cells lacked the pMRR9-*pV.lacZ* reporter plasmid, and LacZ activity was determined by the method of Miller (1972). Unlike the wildtype E251 host, the Host Mutants showed no increase in LacZ expression in the presence of the reporter plasmid, suggesting that these strains carry mutations which prevent B stimulation of *pV* transcription. In all strains, the addition of IPTG resulted in a constant level of LacZ expression due to induction of the host wildtype *lac* operon. This background level was not significantly changed in the Host Mutants compared to E251, implying that the lack of *pV* activation in these cells was not due to a general effect on transcription or LacZ production. In contrast, ~35% of wildtype *pV* activity (above background) was measured in the E251 *rpoA*109 strain, suggesting that this substitution in the RNA polymerase α-subunit has only a moderate effect on B activity.

Thus, unlike the *rpoA*109 strain, the Host Mutant strains carried mutations which appeared to prevent 186 lytic development by abolishing B activation of the 186 late promoters. The phenotype of resistance to 186 infection was then used to map this mutation (186^R) for each of the Host Mutants.

2.A.2.2. Initial mapping of the 186^R mutation of the Host Mutants (by Tina Rathjen).

To determine the site of the 186^R mutation in the Host Mutants, these strains were initially used as recipients in conjugational mapping using the Hfr::Tn10(Tc^R) kit from the *E. coli* Stock Centre (Tina Rathjen, unpublished). The Hfr strains KL14, PK3 and J4 cotransferred the Tn10 marker and 186^S locus at a high frequency (20-50%) for each Host Mutant strains, indicating the probable location of the 186^R mutation in the 75-85' region of the chromosome.

Figure 2.1. Initial characterization of the Host Mutants (by Tina Rathjen).

A. *186 plating and B activity in the Host Mutant and* rpoA*109 strains.*

The efficiency of plating (eop) of 186 phage stocks on the strains HM2, HM3, HM4, HM6 and E251 *rpoA*109 was determined relative to the wildtype E251 host. B activation of a *pV.lacZ* reporter plasmid in the same strains was measured using a –262 to +86 promoter fragment cloned into pMRR9, pMRR9-pV_{AH}, with B protein expression induced from the pTMC10-B plasmid by 0.1mM IPTG. Background LacZ expression was determined in the presence of the pTMC10-B plasmid only and 0.1mM IPTG, and LacZ activity was assayed using the method of Miller (1972). Average LacZ units ± 95% confidence limits are shown for assays on 4 colonies of each strain. *pV* activity (above background) is the difference in LacZ activity measured in the presence or absence of the pMRR9-pV_{AH} plasmid, and is given in LacZ units and as a percentage of *pV* activity (above background) in the wildtype E251 host.

B. *Transduction linkage for the* 186^R *mutation of the Host Mutants.*

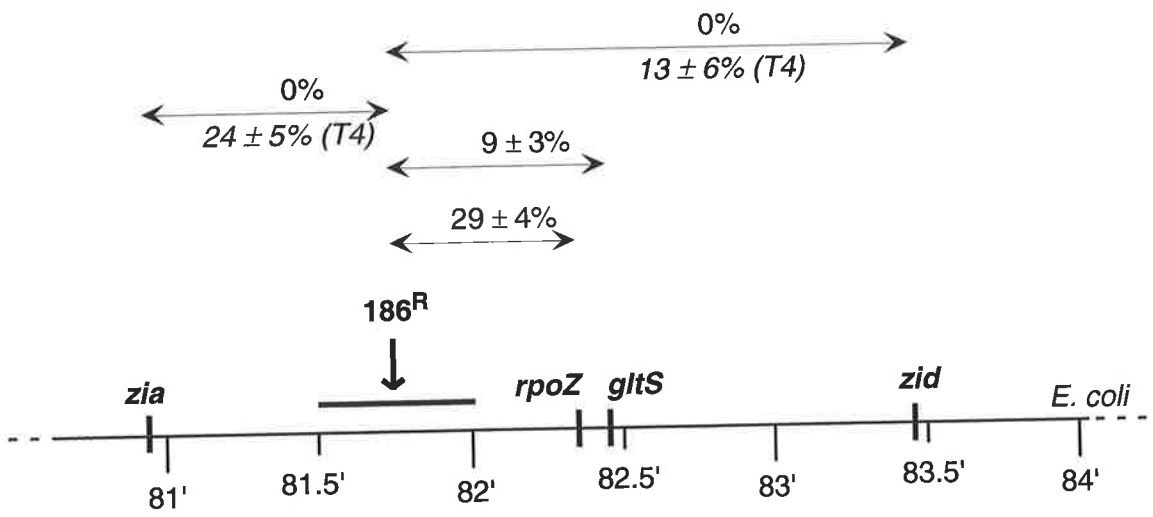
P1 or T4 phage transductions were performed on each of the Host Mutants using donor strains carrying an antibiotic-resistance marker at the indicated position. Linkage between the 186^R mutation of each HM strain and the marker was calculated as the percentage of antibiotic-resistant transductants in which the 186^R allele had been replaced by wildtype sequence. The average percent linkage determined for all Host Mutant strains is given (± standard deviations), for transductions using the P1 phage (above the line) or T4 phage (below the line and in italics). The positions of the markers and the approximate site of the 186^R allele deduced from these experiments (arrow) are shown on a diagram of the *E. coli* K-12 chromosome between 81 and 84 minutes. Donor strains used for transductions were: E4412 (*zia* at 80.96'), E4419 (*zid* at 83.48'), E4465 (*gltS* at ~82.45') and E4466 (*rpoZ* at 82.34').

Figure 2.1

A. Tina Rathjen

host	186 eop	LacZ units		*pV* activity	
		+ IPTG	+ pMRR9-*pV.lacZYA* + IPTG	LacZ units	% E251
E251	**1.0**	411 ± 53	**2310** ± 138	1899	**100**
HM2	**$<10^{-8}$**	483 ± 67	**444** ± 72	–39	**–2**
HM3	**$<10^{-8}$**	450 ± 52	**386** ± 38	–64	**–3**
HM4	**$<10^{-8}$**	528 ± 56	**467** ± 42	–61	**–3**
HM6	**$<10^{-8}$**	480 ± 37	**454** ± 43	–26	**–1**
E251 ***rpoA*109**	**0.98**	372 ± 61	**972** ± 76	600	**32**

B. Tina Rathjen



The 186^R mutation was mapped more precisely by P1 and T4 transductional analyses of each of the Host Mutants, using strains from the *E. coli* Stock Centre with Tn10(Tc^R), Tn5(Km^R) and other markers in this region as donors (Tina Rathjen, unpublished). No linkage was found between the 186^R mutation of any of the Host Mutants with markers outside ~81-84' on the *E. coli* chromosome when either of the transducing phages were used. Within this region, markers at ~81' or 83.5' gave an average of 24% and 13% linkage respectively to the 186^R mutation by T4 transduction, though no linkage was seen using P1 phages (see Figure 2.1B). In further P1 transduction assays the *rpoZ*::Cm^R insertion at ~82.3' showed average linkage (± standard deviation) of 29 ± 4% with the 186^R mutations of the Host Mutant strains (from 30% for HM2, 27% for HM3, 24% for HM4 and 33% for HM6). Similarly, the Host Mutants gave an average of ~9% linkage to a Km^R cassette within the *gltS* gene (at ~82.4') in P1 transductions. The similarity of results with each of the Host Mutants suggested that the 186^R mutations of each strain was found in a similar location, which mapped to between approximately 81.5' and 82' on the *E. coli* chromosome.

Therefore, from the unpublished work of Ravi Tiwari and Tina Rathjen, *E. coli* mutants were available that had apparently simultaneously lost the ability to support functioning of the 186 late activator and had acquired resistance to 186 infection. Therefore, my initial project was to accurately map these mutations, as an approach to identifying the host function(s) involved in B activation of the 186 late promoters. In this chapter I describe my further mapping of the 186 resistance mutation of a Host Mutant strain (in Section 2.B.1.), followed by characterization of the two phenotypes of the Host Mutant cells: resistance to 186 infection (Section 2.B.2.) and the inability to host B function (Section 2.B.3.). I finally re-examine the effect of the *rpoA*109 mutation on 186 infection and B activity in Section 2.B.4.

2.B. RESULTS.

2.B.1. THE 186^R MUTATION OF HM6 MAPS TO AN OPERON FOR LPS BIOSYNTHESIS.

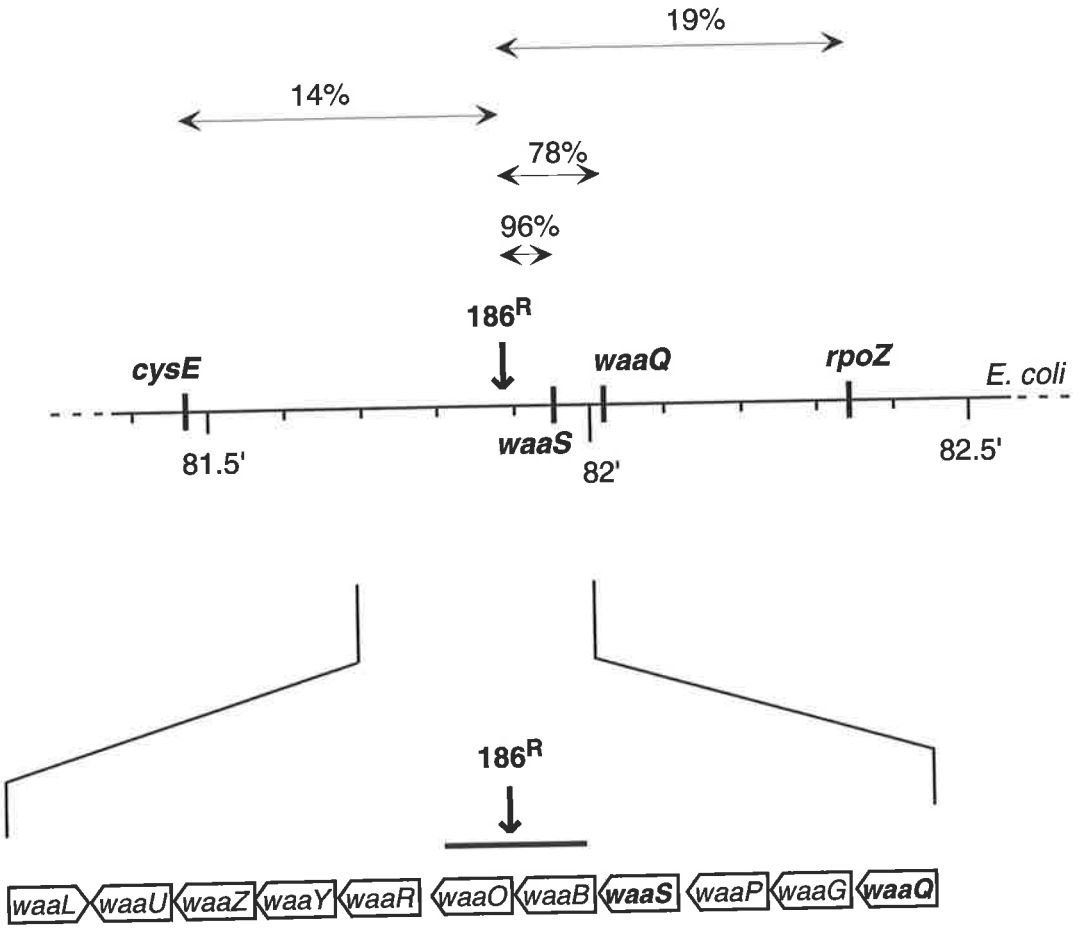
The site of the mutation conferring resistance to 186 infection in the HM6 strain was mapped using P1 transduction of Tn10(Tc^R), Tn5(Km^R) and other markers around 81-82' on the *E. coli* chromosome. Figure 2.2 summarizes the frequencies of cotransduction of the given marker and the 186^S (wildtype) allele into the HM6 recipient. Here, the *rpoZ*::Cm^R

Figure 2.2. Further P1 transduction mapping of the 186^R mutation of HM6.

The upper half of the figure shows the percent linkage between the 186^R mutation of HM6 and the given markers, as determined in P1 transduction assays. The positions of the markers and approximate site of the 186^R mutation (arrow) are shown on a map from ~81.5 to 82.5 minutes on the *E. coli* K-12 chromosome. Donor strains used for transductions were: E4479 (*cysE* at ~81.47'), E4493 (*waaS* at ~81.95'), E4492 (*waaQ* at ~82.01') and E4466 (*rpoZ* at ~82.34').

The lower half of the figure shows the *waa* genes found between ~81.8 and ~82.0 minutes on the *E. coli* K-12 chromosome. Antibiotic-resistance insertions of the *waaS* and *waaQ* genes (shown in bold) were used to map the 186^R mutation. The probable location of the 186^R mutation is within the *waaO* or *waaB* genes as indicated.

Figure 2.2


19%
14%
78%
96%
186R
cysE
waaQ
rpoZ
E. coli
waaS
81.5'
82'
82.5'
186R
waaL
waaU
waaZ
waaY
waaR
waaO
waaB
waaS
waaP
waaG
waaQ

marker at 82.34' was found to be 19% linked to 186 resistance in HM6 – slightly less than the 29% average linkage seen for all the Host Mutants by Tina Rathjen (see Figure 2.1B). The 186^R mutation of HM6 was also found to give 14% linkage to the *cysE*::Tn10 insertion at 81.47', 78% linkage to the *waaQ*::Tn5 marker at 82.01' and 96% linkage to the *waaS*::Tn5 mutation at 81.95'. (Note that the *waa* genes were previously known as the *rfa* genes and that the nomenclature described by Heinrichs *et al.* (1998) is used here.) These results placed the mutation conferring resistance to 186 infection in HM6 to the left of the *waaS* marker and within ~2kb of this gene – probably within the *waaO* or *waaB* gene.

It was surprising to find this mutation, which was originally thought to prevent 186 infection by abolishing B protein function, mapping within the *waaQ* locus. The products of these genes are involved in biosynthesis of the lipopolysaccharide (LPS) structure which is found in the outer membrane of the *E. coli* cell (reviewed by Heinrichs *et al.*, 1998). Various components of the LPS are used as receptors by a number of other bacteriophages (Lindberg, 1973; Pradel *et al.*, 1992; Yokota *et al.*, 1994; Traurig and Misra, 1999), and the involvement of Waa proteins in transcriptional activation by the B protein would certainly be novel. It was therefore important to determine whether these mutations in the *waaQ* operon really prevented 186 infection by reducing B activation of the 186 late promoters, rather than by removing a receptor for 186 phage adsorption to the cell.

2.B.2. Characterization of the 186^R phenotype of the Host Mutants.

To analyze the 186^R mutation of the Host Mutant cells in isolation, these mutations and the nearby *rpoZ*::Cm^R marker were cotransduced into the original parental strain (E251) using P1 phage transduction. The *rpoZ* gene encodes the ω subunit of RNA polymerase which is seemingly not required for transcription *in vitro* or *in vivo* (Gentry *et al.*, 1991), and is not known to interact with any activator proteins (Dove and Hochschild, 1998). 186 plating and *pV.lacZ* reporter assays confirmed that the *rpoZ*::Cm^R insertion had no significant effect on 186 infection or B activity (see Figure 2.3B).

The *rpoZ*::Cm^R mutation was transduced into the Host Mutant cells and chloramphenicol-resistant transductants were screened for resistance to 186 infection, by cross-streaking against 186 *vir* phages. Transductants of each Host Mutant that were 186 sensitive (HM *rpoZ*::Cm^R 186^S) were retained and used in Section 2.B.3.1. Isolates which remained 186 resistant (HM *rpoZ*::Cm^R 186^R) were used as donor strains to cotransduce the 186^R mutation and Cm^R marker into an E251 host. In these experiments the average linkage of

the *rpoZ*::Cm^R marker and the 186^R mutation of HM2 was 26%, similar to that found previously by Tina Rathjen (see Section 2.A.2.2.).

2.B.2.1. 186 lytic development is not prevented by the 186^R mutation of HM2.

As an initial test, the effect of the 186^R mutation from HM2 on 186 lytic development was assayed. This mutation was cotransduced into a 186$^+$ lysogen of E251, and spontaneous phage production was measured. Lysogens of 186 spontaneously revert to lytic development with a low frequency to give a small number of virus particles in the supernatant of a lysogenic culture. The rate of prophage induction and the size of the phage burst determines the overall number of virions released, but the level of free phages present in the culture may be reduced by adsorption to bacterial cells and cell debris. It was expected that if the 186^R mutation from HM2 affected the 186 lytic cycle, fewer phages would be produced by spontaneous induction of lysogenic cells carrying the mutation.

Overnight cultures of 186$^+$ lysogens of E251 carrying or lacking the 186^R mutation from HM2 were grown to approximately the same cell density, and the level of free phages in the supernatant of each was determined. The number of free phages present was not reduced when the 186^R mutation of HM2 was present in the 186 lysogenic cell. In fact, there were actually ~460-fold more free phages in the culture of a 186 lysogen carrying the HM2 186^R mutation ($3.9x10^7$ pfu/ml), compared to a 186 lysogen of wildtype E251 ($8.5x10^4$ pfu/ml).

Thus, 186 lytic development was <u>not</u> prevented by the mutation that conferred resistance to 186 infection in HM2 – implying that this mutation did not affect B protein activity. Instead, the higher level of free phages in the supernatant of the mutant lysogenic cell was consistent with the 186^R mutation preventing readsorption of free phages to the bacterial cells.

2.B.2.2. B activity is not affected by the 186^R mutation of HM2.

To determine whether B protein function was affected by the 186^R mutation of the Host Mutants, it was necessary to first modify the *lacZ* reporter assay used to measure B activation of the late promoters. The 186 late promoter *pV* had previously been used in our laboratory to assay B activity, but the reporter constructs studied showed a marked discrepancy in activated transcription with different sized promoter fragments. Dibbens and Egan (1992) had noted a 10-fold higher level of maximal activation of a *pV.galK* reporter plasmid when the promoter sequence ended 229 basepairs, rather than 19 basepairs, downstream of the +1 site. A similar discrepancy was found by Tina Rathjen (unpublished)

when measuring B-stimulated expression of a *pV.lacZ* reporter (see Figure 2.3A). In this case, LacZ expression from the chromosomal *pV* reporter in the presence of B protein was improved ~13-fold when the promoter fragment extended to +229 rather than +125 on the promoter. These results suggested that *pV* has a downstream enhancer element between +125 and +229, which could be bound by a bacterial co-activator protein to stimulate B activation of the promoter. However, others (Linn and St Pierre, 1990) have also noticed variation in the level of expression from a transcriptional *lacZ* reporter when different sized fragments of a given promoter were used. They noted that this difference in LacZ protein expression could have been due to alteration of the *lacZ* mRNA leader sequence affecting the stability or translation efficiency of the *lacZ* message. In support of their proposal they found that insertion of a RNaseIII cleavage site between the promoter and *lacZ* gene (which would give a consistent 5' end to the *lacZ* mRNA) removed the effect.

To determine whether the results seen with different length *pV* promoter fragments were due to an enhancer sequence, or were simply an effect of the differences in *lacZ* mRNA sequence on reporter production, these DNA fragments were cloned into the pMRR9R reporter plasmid. This plasmid is identical to the pMRR9 plasmid used by Tina Rathjen, except for the addition of a RNaseIII cleavage site from pTL61T (Linn and St Pierre, 1990) cloned between the polylinker and *lacZ* gene (Dodd and Egan, 2002). The alternate *pV* promoter fragments ending at +103 or +229 were cloned into pMRR9R, transferred to the λRS45 phage (Simons *et al.*, 1987) and recombinant phage used to make mono-lysogens of the Lac$^-$ host, NK7049. LacZ activity was determined in the presence of B protein induced from the pTMC10-B plasmid with 0.1mM IPTG.

As shown in Figure 2.3A, B activation of the *pV* promoter in the pMRR9R-based reporter was not significantly different whether the DNA fragment ended 103 or 229 basepairs downstream of the +1 site. No increase in *pV* activation was seen with the longer promoter fragment, implying that the previous results with different length fragments were due to differences in the *lacZ* leader sequences, and not due to binding of a co-activator protein. For consistency in all following reporter assays, transcription from *pV* (or other 186 late promoters) was assayed using a promoter fragment from approximately –260 to +230, and the reporter plasmids (pMRR9R or pTL61T) included an RNaseIII site between the polylinker and *lacZ* gene. In addition, LacZ activity was measured using a modified, kinetic assay performed in microtitre plates (Dodd *et al.*, 2001 – see Section 6.B.4.2). LacZ units

Figure 2.3. Effect of promoter fragment length and the 186^R mutation of HM2 on B activation of a *pV.lacZ* reporter.

A. *Investigation of a potential downstream enhancer element in the* pV *promoter sequence.* 186 *pV* promoter sequences from –262 to +125, or –262 to+229 were cloned by Tina Rathjen into the *lacZ* reporter plasmid pMRR9 (which lacks an RNaseIII site). *pV.lacZ* fustions were transferred to λRS45, and the resultant reporter phage, λpV_{AHa}.*lacZYA* and λpV_{AA}.*lacZYA* respectively, were lysogenized in the Lac^- host MC1061. Similarly, I cloned *pV* fragments from –260 to +103, or –260 to +229 into the pMRR9R plasmid, in which an RNaseIII site is inserted between the polylinker and *lacZ* gene. *pV.lacZ* fusions were transferred to λRS45, and the resultant reporter phage λpV_{103}.*lacZYA* and λ*pV.lacZYA* respectively, were lysogenized in the Lac^- host NK7049. LacZ activity was assayed by the method of Miller (1972 – see Section 6.B.4.1), in the presence of B protein induced from pTMC10-B by 0.1mM IPTG. Background reporter expression was measured in the presence of the pTMC10 plasmid. For the pMRR9-based assay (performed by Tina Rathjen, unpublished) four colonies were tested, and average LacZ units ± 95% confidence limits are shown. For the pMRR9R-based assay, 2-6 colonies were tested and average LacZ units ± standard deviation errors are given. nd indicates that LacZ activity was not determined.

B. *The effect of the 186^R mutation of HM2 on 186 plating and B activity.*
Plating and LacZ assays were performed on wildtype E251, E251 *rpoZ*::Cm^R, E251 *rpoZ*::Cm^R 186^R(HM2) and HM2 strains. The efficiency of plating (eop) of a 186^+ phage stock was determined in comparison to wildtype E251, in one or two assays. LacZ expression from the pTL61T-pV reporter plasmid was determined in the presence of the constitutive B expression plasmid, pZA-B, or control plasmid, pZA-Bam (which carries the *B*am17 gene). Average LacZ units ± 95% confidence limits are shown for microtitre plate assays performed on 6-11 colonies of each strain. *pV* activity (above background) is the difference in LacZ units seen in the presence and absence of wildtype B protein expression and is also given as a percentage of *pV* activity (above background) in the E251 host.

Figure 2.3

A.

host	λ*pV.lacZYA* reporter: RNaseIII site	λ*pV.lacZYA* reporter: *pV* fragment	LacZ units: + pTMC10 + IPTG	LacZ units: **+ pTMC10-B** + IPTG
Tina Rathjen				
MC1061	–	**+125**	nd	**69** ± 7
		+229	nd	**904** ± 16
here				
NK7049	+	**+103**	2.9 ± 1.5	**205** ± 49
		+229	3.3 ± 1.6	**243** ± 36

B.

host	186 eop	pTL61T-*pV.lacZ* LacZ units: + pZA-Bam	pTL61T-*pV.lacZ* LacZ units: **+ pZA-B**	*pV* activity: LacZ units	*pV* activity: **% E251**
E251	**1.0**	171 ± 12	**1748** ± 234	1577	**100**
E251 ***rpoZ***	**1.07**	174 ± 8	**2085** ± 285	1911	**121**
E251 ***rpoZ*** **186^{R}(HM2)**	**<10^{-7}**	156 ± 20	**2390** ± 193	2234	**142**
HM2	**<10^{-8}**	18 ± 2	**15** ± 3	-2.5	**-0.2**

determined from this assay are approximately equivalent to those determined by the method of Miller (Dodd *et al.*, 2001).

This improved *pV.lacZ* reporter assay was used to determine the effect of the 186^R mutation from HM2 in isolation on B protein activity. E251 cells, cotransduced with the *rpoZ*::Cm^R marker and 186^R mutation from HM2 *rpoZ*::Cm^R were therefore transformed with the ColE1-origin pTL61T-*pV.lacZ* reporter plasmid. LacZ activity was assayed in the presence of a compatible (p15A-origin) plasmid, pZA-B, which constitutively expresses the B protein (see Section 3.B.2.1 and Figure 3.3). A control expression plasmid was identical except that the *B* gene carried the amber17 mutation, which results in a truncated and inactive protein being expressed in a non-suppressor host, such as E251. An E251 host transduced with the *rpoZ*::Cm^R mutation only, was also assayed to confirm that the *rpoZ* insertion did not affect B activity, and wildtype E251 and the original HM2 strain were included for comparison. The average LacZ activity determined for each strain is shown in Figure 2.3B. *pV* promoter activity (above background) was calculated as the difference in LacZ units with and without B protein expression, and was also expressed as a percentage of *pV* activity (above background) in wildtype E251.

In this system, B expression from the pZA-B plasmid activated the plasmid-encoded *pV.lacZ* reporter about 10-fold (to ~1700 LacZ units) in the wildtype E251 host, and activated *pV* expression was slightly increased by the *rpoZ*::Cm^R mutation, to ~120% of wildtype. Some background LacZ activity (between 17 and 170 units) was seen in all strains carrying the pZA-Bam control plasmid, probably due to a low level of intrinsic *pV* transcription on the high copy number reporter plasmid, pTL61T. This LacZ expression was not from the host *lac* operon, since in the absence of IPTG and plasmids, the E251 and Host Mutant cells gave between 0 and 4 LacZ units (data not shown). Consistent with earlier results, B-dependent transcription from the *pV* reporter was completely abolished in the original HM2 strain. In contrast, B activation of *pV* in E251 cells transduced with the 186^R mutation of HM2 was not reduced, but increased somewhat to ~2400 LacZ units, ~40% higher than in wildtype E251.

Therefore, the 186^R mutation of HM2 in isolation did not reduce B protein activation of the 186 *pV* promoter. It seemed likely then that mutation of the *waa* genes in the Host Mutant strains prevented 186 infection, not by reducing B activation of the late promoters, but by removing the receptor required for phage adsorption to the bacterial cell.

2.B.2.3. Lipopolysaccharide structure requirements for 186 infection.

Lipopolysaccharide structures in *E. coli* consist of a membrane-embedded lipidA moiety attached via a conserved core oligosaccharide to a highly variable polysaccharide repeat structure (or O-Antigen). In *E. coli* K-12 strains, the core oligosaccharide (shown in Figure 2.4A) is made up of an inner heptose and outer hexose region, and laboratory strains, which lack the O-Antigen repeat, appear to terminate in an N-acetyl-D-glucosamine, or GlcNAc, molecule (Feldman *et al.*, 1999). Genes of the *waaQ* and nearby operons encode the proteins required for assembly of the core region of the LPS, and the functions of these genes, as indicated in Figure 2.4A, are fairly well-characterized (Raetz, 1996; Heinrichs *et al.*, 1998). The *waaO* and *waaB* genes, which were implicated in the 186^R phenotype of the Host Mutant strains (see Section 2.B.1.), are involved in formation of the outer core region – specifically in the addition of the second glucose (Glc II) and first galactose (Gal I) residues respectively (Pradel *et al.*, 1992).

Therefore, as an initial investigation into the lipopolysaccharide structure required for 186 infection, I measured the efficiency of 186 plating on strains mutated in different *waa* genes required for LPS synthesis. Mutations were either the result of apparently non-polar Tn5 insertions (Pradel *et al.*, 1992 – obtained from John Klena, University of Auckland) or in-frame deletions (kindly given by Renato Morona, University of Adelaide). Sensitivity to infection by P2 *vir24* phages (which had been passaged on *E. coli* K-12) was also determined for some *waa* strains and the Host Mutants; and the results of these assays are summarized in Figure 2.4B. Mutants of the *waaQ* and *waaS* genes – which are expected to lack sidechain molecules in the inner core – proved to be sensitive to 186 infection; as did *waaL* and *waaU* gene mutants – which lack the terminal GlcNAc, and GlcNAc and fourth heptose (Hep IV) molecules respectively. However, removal of the third or second glucose of the backbone or sidechain galactose of the outer core region, by *waaR*, *waaO* or *waaB* mutations, respectively, prevented plaque formation by even a high titre of 186 phages. These results agree with previous observations (Ian Dodd, personal communication) that intact *galE* and *galU* genes, which are involved in galactose metabolism and required for its inclusion in the LPS (Lin, 1996), are necessary for infection by 186 phages.

Therefore, the minimal structure required for 186 infection includes the second and third glucose and sidechain galactose molecules in the outer core of the LPS. Presumably, these residues form the receptor recognized by 186 tail proteins for phage adsorption.

Figure 2.4. The effect of LPS outer core mutations on sensitivity to 186 infection.

A. *The LPS core structure of* E. coli *K-12.*

The inner and outer regions of the core oligosaccharide of the lipopolysaccharide structure of *E. coli* K-12 are shown, along with the *waa* genes implicated in their assembly (reviewed by Raetz, 1996 and Heinrichs *et al.*, 1998). Lipid A is imbedded in the outer cell membrane and the LPS terminates at a N-acetyl-D-glucosamine (GlcNAc) residue (Feldman *et al.*, 1999). The following abbreviations are used: Gal – D-galactose; Glc – D-glucose; GlcNAc – N-acetyl-D-glucosamine; Hep – L-*glycero*-D-*manno*-heptose; Kdo – 3-deoxy-D-*manno*-octulosonic acid; P –phosphate and PPEA – ethanolamine pyrophosphate. The *waa* gene mutants tested for 186 phage sensitivity are shown in bold, and those showing resistance to 186 infection are also underlined. The minimum LPS structure required for 186 infection implied from these results is shown in blue.

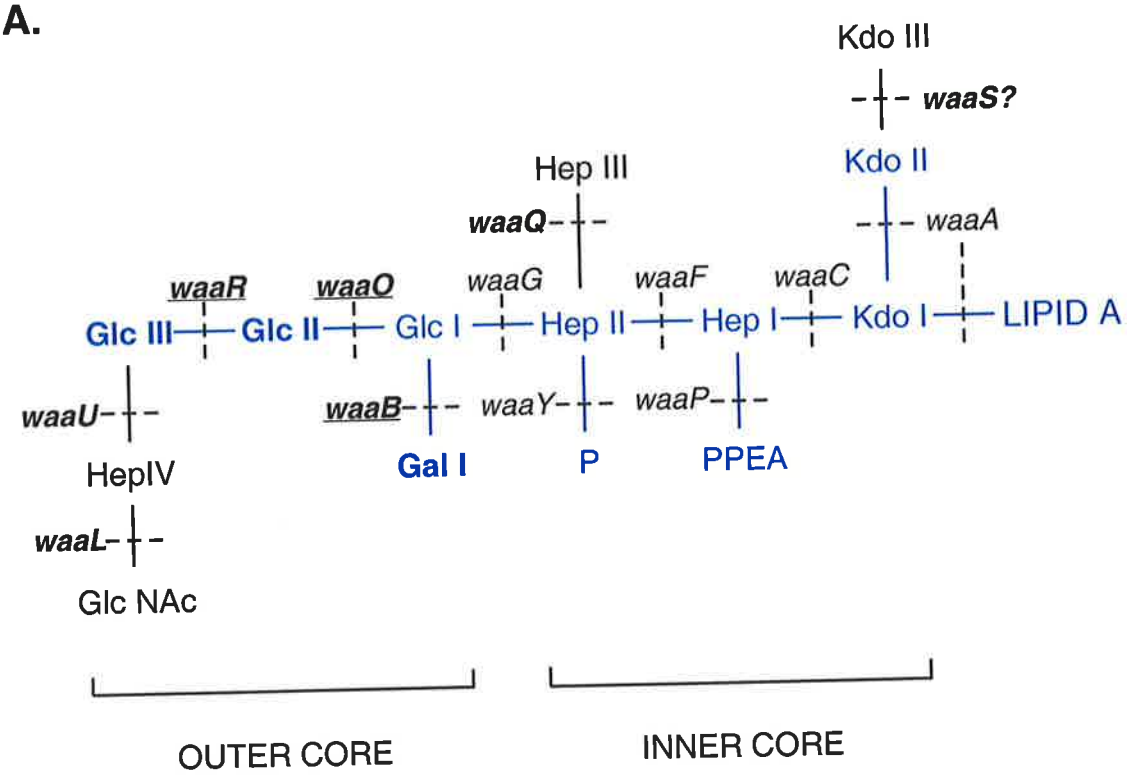
B. *186 phage sensitivity of several* E. coli *K-12* waa *gene mutants.*

The efficiency of plating (eop) of 186 phage was determined on strains mutated in the indicated *waa* gene, either by a Tn5 insertion or by an inframe deletion mutation (Δ*waa*), relative to a wildtype host strain (E251 for Tn5 mutants or C600 for deletion mutants). Either 186^{+} or 186 *cI*10 phage stocks were used in the assay. The efficiency of plating of a P2 *vir*24 phage stock (passaged on *E. coli* K-12) was measured in the same way as for 186. For 186 and P2 plating:

+ indicates an eop >0.1, – indicates an eop $<10^{-3}$, and nd is not determined.

Figure 2.4

A.



B.

host	186 eop	P2 eop
wildtype	+	+
***waaQ*::Tn5**	+	+
***waaS*::Tn5**	+	+
***waaB*::Tn5**	–	+
***waaO*::Tn5**	–	–
***waaL*::Tn5**	+	+
Δ*waaOB*	–	nd
Δ*waaR*	–	nd
Δ*waaU*	+	nd
Δ*waaL*	+	nd
HM2-4	–	+
HM6	–	–

It was interesting to note that the P2 phage appears to have a similar receptor on the surface of *E. coli* K-12 cells. *Waa* strains showed similar efficency of plating of P2 and 186 phages, with the exception that P2 could plate on the *waaB* strain. This suggests that the sidechain galactose sugar enhances but is not essential for P2 phage adsorption, whereas it is essential for 186.

Plating on the Host Mutant strains showed that HM2, HM3 and HM4 cells are sensitive to P2 but not 186 plating, and that the HM6 strain is insensitive to both P2 and 186 infection (see Figure 2.4B). Linkage frequencies (Sections 2.A.2.2 and 2.B.1) mapped the 186^R mutations of these cells to the *waaO* or *waaB* genes. These results strongly suggest that strains HM2, HM3 and HM4 carry mutations in the *waaB* gene which confer resistance to 186 infection, while the 186^R mutation of HM6 is found in the *waaO* gene.

In summary, it appears that the 186^R phenotype of each of the Host Mutant cells is conferred by a mutation within the *waa* genes, which does not affect B protein function but instead abolishes 186 phage adsorption. Therefore, the loss of B-activated transcription from a 186 late promoter seen previously in the Host Mutants must have been due to an additional mutation in these cells.

2.B.3. Analysis of reduced B function in the Host Mutants.

2.B.3.1 Characterizing the B-affecting mutation of the Host Mutants

The above results show that the Host Mutant strains carry two mutations – one in the *waa* operon which inhibits 186 infection by reducing phage adsorption (Figures 2.2 and 2.4); and a second at an unknown location, which apparently abolishes 186 B protein function (Figure 2.3B).

Homologues of B have been shown to interact with the RNAP α-subunit to stimulate transcription from target promoters. Although B protein activity was apparently not affected by one *rpoA* mutation, *rpoA*109, it seems possible that B could contact a different region of alpha, and that the Host Mutants could carry alternative *rpoA* mutations which are more deleterious to B function. Therefore, the *rpoA* gene of each of the Host Mutants was sequenced. No changes were found compared to the wildtype *rpoA* gene of E251 (or E. coli K-12 – Blattner *et al.*, 1997), indicating that the lack of B function in the Host Mutant cells was not due to mutation of the RNAP α-subunit.

As a first step to characterizing the B-affecting mutations of the Host Mutant strains, the effect of these mutations on 186 plating was examined in the absence of the 186^R mutation in the *waa* operon. Transduction of the *rpoZ*::Cm^R insertion into the Host Mutants resulted in some cells which were sensitive to 186 infection (see Section 2.B.2. preamble). These strains, HM *rpoZ*::Cm^R 186^S, which presumably retained the B-affecting mutation, were first compared to wildtype E251 cells for their ability to host 186^+ infection in plating experiments. Average efficiencies of plating (with standard deviation errors) of 0.97 ± 0.02, 0.93 ± 0.18, 0.89 ± 0.04 and 1.00 ± 0.12, were found for the 186^S derivatives of HM2, HM3, HM4 and HM6 respectively (from two experiments). 186^+ plaques on these strains were normal in size and appearance.

These were surprisingly high efficiency of plating values since previous measurement of B-dependent transcription from a 186 late promoter reporter by Tina Rathjen showed that B function was abolished in these strains (see Figure 2.1A). When these reporter assays were initially repeated for HM2 using a modified system, similar results were found – a *pV.lacZ* reporter plasmid was not activated by B protein expression (see Figure 2.3B). However, closer examination of these assays showed a strange result: background transcription from the 186 *pV* reporter plasmid, in the absence of B protein, was reduced about 10-fold in the Host Mutant strain compared to E251. This suggested that perhaps LacZ activity or transcription from the reporter plasmid could be non-specifically reduced in the HM2 cells, which may affect the measurement of B protein activity in these assays.

Therefore, to test for a reduction in intrinsic 186 *pV* expression or LacZ activity in HM2, the reporter construct was also assayed in the host chromosome. The *pV.lacZ* transcriptional fusion from pTL61T was transferred to the λRS45ΔYA phage and the recombinant phage used to make mono-lysogens of E251 and HM2. To remove background expression from the host *lac* operon, a *lacZ*::Km^R insertion mutation was also transduced into each strain. (E251 *lacZ*::Km^R and HM2 *lacZ*::Km^R colonies were white on X-gal indicator plates, and gave ~0 LacZ units – data not shown.) In the E251 host, intrinsic LacZ expression from the λ*pV.lacZ* reporter was 1.2 ± 0.5 units (with 95% confidence limits from assays on 19 colonies). The chromosomal *pV* reporter in the HM2 *lacZ*::Km^R host gave a similar level of LacZ expression, 2.2 ± 0.4 units (with 95% confidence limits from assays on 18 colonies).

Thus, intrinsic (un-activated) transcription from the *pV* promoter was reduced ~10-fold in HM2 compared to E251 when present on the pTL61T plasmid, but was not significantly different when inserted into the bacterial chromosome. This suggested that the HM2 strain contained a mutation which reduced transcription from the pTL61T plasmid in general, possibly by affecting the copy number of the plasmid in this cell. Such a mutation could potentially explain the reduction in B activation of the 186 late promoters seen in previous *lacZ* reporter assays and in the original selection system, without specifically affecting B function. I therefore wished to confirm this result for HM2 and to determine whether the other Host Mutants showed a similar effect.

2.B.3.2. Transcription from the pTL61T plasmid is reduced in all Host Mutants.

To determine if HM2 and other Host Mutants possessed a mutation which in some way affected transcription from the pTL61T plasmid, the activity of a constitutive, B-independent promoter expressed either from this plasmid or from the bacterial chromosome, was compared in each of these strains.

The 186 p*L* promoter is constitutively active and gives a moderate intrinsic level of expression from a chromosomal reporter (~30-50 units – Dodd and Egan, 2002). A λp*L.lacZ* reporter phage (supplied by Ian Dodd) carrying a promoter-*lacZ* transcriptional fusion from pTL61T, was used to lysogenize E251 and Host Mutant cells carrying the *lacZ*::Km^R insertion, and LacZ activity was assayed. E251 *lacZ*::Km^R and HM *lacZ*::Km^R strains were also transformed with the pTL61T-p*L.lacZ* plasmid (also from Ian Dodd), and LacZ activity determined. Results are shown in Figure 2.5A. Intrinsic p*L* promoter activity from the chromosomal reporter in the Host Mutant strains varied between 56 and 59 LacZ units, but was not significantly different to that seen in E251 cells. In contrast, LacZ expression from the pTL61T-p*L.lacZ* reporter plasmid in each of the Host Mutant cells was significantly reduced compared to that seen in the wildtype strain. HM2-, HM3- and HM4-based strains gave only ~10% of wildtype p*L* activity, while in the HM6-derived strain the p*L* reporter gave 18% of the activity seen in E251.

Thus, the level of LacZ protein produced from the constitutive 186 p*L* promoter was reduced to ~10-20% of wildtype levels in each of the Host Mutant strains when expressed from the pTL61T plasmid, but not from the bacterial chromosome. The Host Mutant cells had not lost the reporter plasmid completely since transformed cells remained antibiotic-resistant, therefore, it seemed likely that these cells carried mutations which reduced the copy number of this plasmid.

Figure 2.5. Reduced transcription and copy number of ColE1 plasmids in the Host Mutants.

A. *Constitutive promoter expression from the chromosome or pTL61T plasmid in the Host Mutant strains.*

Wildtype E251 or Host Mutant strains transduced with the *lacZ*::Km^R insertion mutation were transformed with the pTL61T-pL reporter plasmid, or lysogenized with the λRS45Δ*YA* phage carrying this 186 *pL-lacZ* transcriptional fusion (λ*pL.lacZ*). Microtitre plate LacZ assays were performed on 8-22 colonies of each strain and average LacZ activity ± 95% confidence limits is shown. LacZ units and errors are also expressed as a percentage of the average LacZ activity measured in the wildtype E251 host strain.

B. *Activation of a chromosomal* pV *reporter by pKO2-B in the Host Mutants.*

E251 or Host Mutant cells transduced with the *lacZ*::Km^R mutation were lysogenized with the λ*pV.lacZ* reporter phage and transformed with the pKO2-B (or pKO2-Bam control) plasmid. Average LacZ units ± 95% confidence limits were determined for microtitre plate LacZ assays performed on at least 4 colonies of each strain. *pV* activity (above background) is the difference in LacZ units measured with the pKO2-B and pKO2-Bam plasmids in each strain, and is also shown as a percentage of the *pV* activity (above background) measured in E251.

C. *DNA minipreps of the pKO2-B plasmid in the Host Mutants.*

DNA minipreps were performed on cultures of E251 and strains HM2, HM3, HM4 and HM6, as indicated, carrying the ~4.5kb pKO2-B plasmid. Plasmid DNA was extracted from 2ml overnight cultures grown to an OD_{600} ~4, using a Qiagen miniprep kit, and resuspended in 50μl of elution buffer. 5μl samples of each miniprep were run on an agarose gel against 500ng of SPP1 DNA markers, and DNA was visualized with ethidium bromide. The sizes (in kb) of some of the markers are shown.

Figure 2.5

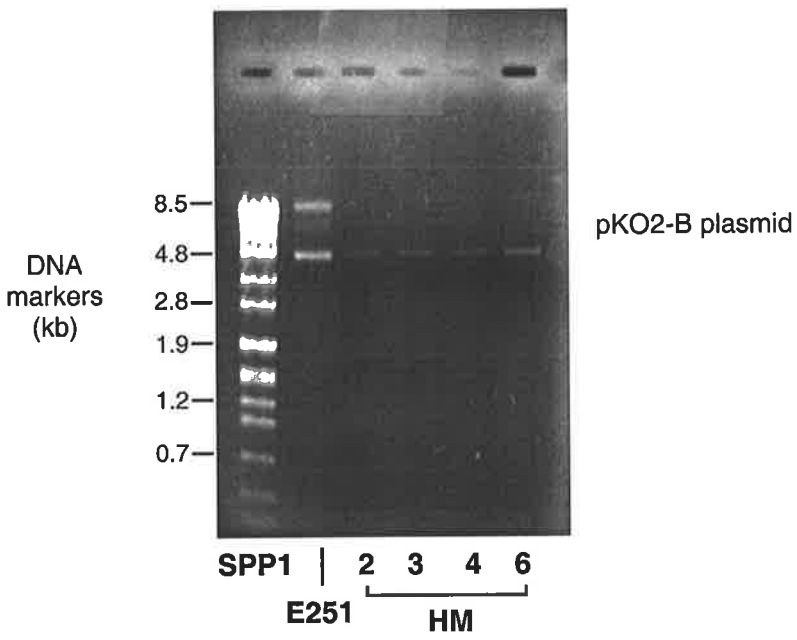
A.

host	λ*pL.lacZ* LacZ units	λ*pL.lacZ* **% E251 units**	pTL61T-*pL.lacZ* LacZ units	pTL61T-*pL.lacZ* **% E251 units**
E251 *lacZ*	52 ± 9	**100** ± 17	592 ± 81	**100** ± 14
HM2 *lacZ*	59 ± 5	**112** ± 9	58 ± 9	**10** ± 2
HM3 *lacZ*	58 ± 4	**112** ± 8	55 ± 6	**9** ± 1
HM4 *lacZ*	57 ± 4	**109** ± 7	52 ± 7	**9** ± 1
HM6 *lacZ*	56 ± 3	**108** ± 5	109 ± 7	**18** ± 1

B.

	λpV.*lacZ* **LacZ units**		*pV* **activity**	
host	+ pKO2-Bam	**+ pKO2-B**	LacZ units	**% E251**
E251 *lacZ*	3.0 ± 0.7	**139** ± 39	136	**100**
HM2 *lacZ*	1.7 ± 0.4	**11** ± 5	9	**7**
HM3 *lacZ*	2.0 ± 0.3	**7** ± 3	5	**4**
HM4 *lacZ*	1.7 ± 1.1	**14** ± 3	12	**9**
HM6 *lacZ*	1.2 ± 0.7	**36** ± 5	35	**25**

C.



2.B.3.3. The copy number of ColE1 plasmids is reduced in the Host Mutants.

The pTL61T plasmid is based on the pBR322 plasmid and is therefore replicated from a ColE1 origin (Linn and St Pierre, 1990). If the Host Mutants carried a mutation that reduced replication from this plasmid origin, the mutation would be expected to also affect other ColE1-based plasmids, such as pKO2-B, which was used in the original selection system. To determine if this was the case, activation of a chromosomal *pV.lacZ* reporter by B protein expressed from pKO2-B was assayed in the Host Mutants, and plasmid minipreps were also performed to directly compare the levels of plasmid DNA present.

Lysogens of the λ*pV.lacZ* reporter were made in E251 *lacZ*::Km^R or the Host Mutant *lacZ*::Km^R strains, and were transformed with the pKO2-B (or pKO2-Bam control) plasmid. The results of LacZ assays are shown in Figure 2.5B. As expected, *pV* activity (above background) in the presence of the pKO2-B plasmid was significantly reduced in the Host Mutant strains compared to the wildtype E251 host. The *pV.lacZ* reporter in HM2, HM3 & HM4-derived strains gave between 4 and 9% of wildtype activity, while in HM6 cells the level of *pV* activation was ~25% of that seen in E251.

pKO2-B plasmid DNA was then isolated from equal volumes of overnight cultures of each of these strains, which had been diluted to approximately the same cell density. An agarose gel of samples of each of these minipreps (Figure 2.5C) clearly shows that the level of pKO2-B plasmid DNA in the Host Mutant strains was significantly reduced compared to the wildtype host. A slightly higher level of plasmid DNA was present in the HM6 culture compared to the other Host Mutants, but this was still much less than in E251.

Activation of a chromosomal *pV.lacZ* reporter by B protein expressed from the pZA-B plasmid was also decreased in HM2 *lacZ*::Km^R cells (data not shown) suggesting that the copy number of plasmids with p15A origins was also reduced in the Host Mutants. Plasmids with other origins were not tested.

Thus, the Host Mutants appeared to have a mutation which reduced the copy number of plasmids with ColE1 (or p15A) origins to ~10-20% of their normal level. Such a mutation would have affected the copy number of the plasmids used in the original selection system – pKill and pKO2-B – and in subsequent plasmid-based reporter assays of B protein activity. Therefore, B activation of the 186 late promoters was reduced in the Host Mutants,

not by removal of a host function vital for B protein activity, but by a host mutation reducing the level of B and late protein expression from the plasmids.

2.B.3.4. The Host Mutants carry truncating mutations in the pcnB *gene.*

Replication of plasmids with ColE1 and p15A origins is similar and is dependent on bacterial replication proteins whose mutation would be expected to have a general deleterious effect on the bacterial cell. However, several researchers have found that mutation of the host gene, *pcnB*, gives a reduction in the copy number of plasmids with ColE1-like origins, without having a dramatic effect on the cell (Lopilato *et al.*, 1986; Liu and Parkinson, 1989; March *et al.*, 1989). Therefore, it seemed possible that the Host Mutant strains had a mutation in the *pcnB* gene, or another mutation with a similar effect. I decided to sequence the *pcnB* gene of the Host Mutants for my own satisfaction, even though the results would not actually improve our understanding of the control of 186 late gene expression by the B protein!

ColE1 plasmid replication (reviewed by Helinski *et al.*, 1996; del Solar *et al.*, 1998) is initiated by an RNA molecule, RNAII, which is transcribed from the plasmid origin and anneals to the origin DNA. Subsequent cleavage of this DNA-RNA hybrid by RNaseH allows leading strand synthesis to begin from RNAII, and replication proceeds using DNA polymerase I and other host replication machinery. The frequency of plasmid replication (and hence plasmid copy number) is controlled at the level of initiation. A smaller RNA molecule (RNAI) is also made from the plasmid origin in the opposite orientation and overlapping the 5' end of the RNAII sequence. Annealing of the complementary RNAI-RNAII molecules is stabilized by a plasmid-encoded protein Rop, and hybrid formation prevents RNAII from acting as a primer for plasmid replication.

Normally in the cell, RNAI is present in ~100-fold excess over RNAII (He *et al.*, 1993), and presumably limits plasmid replication to maintain a stable number of copies of the plasmid per cell. The RNAI molecule has a very short half-life of ~1-2 minutes (He *et al.*, 1993; Xu *et al.*, 1993) to allow a rapid response in RNAI concentration to fluctuations in plasmid copy number (He *et al.*, 1993). Rapid RNAI degradation is thought to be facilitated by the addition of a poly(A) tail by the Poly(A) polymerase I (PAP I) encoded by the *pcnB* gene (Cao and Sarkar, 1992; He *et al.*, 1993; Masters *et al.*, 1993; Xu *et al.*, 1993). In the absence of PAP I, the half-life of RNAI increases more than 10-fold (He *et al.*, 1993; Xu *et al.*, 1993), and plasmids with ColE1 origins show a reduction in copy number to ~5-25% of the wildtype level – although they are still maintained by antibiotic selection (Lopilato *et*

al., 1986; March *et al.*, 1989; Masters *et al.*, 1993). Polyadenylation also appears to be involved in the decay of some bacterial mRNAs; however, other mRNA degradation pathways have also been described which may be used in the absence of the PAP I protein (reviewed by Kushner, 1996; Regnier and Arraiano, 2000). The copy number of plasmids not dependent on an RNA molecule for regulation of replication (including pSC101 and F-based plasmids), would not be expected to be reduced by the *pcnB* mutation (Masters *et al.*, 1993; Helinski *et al.*, 1996).

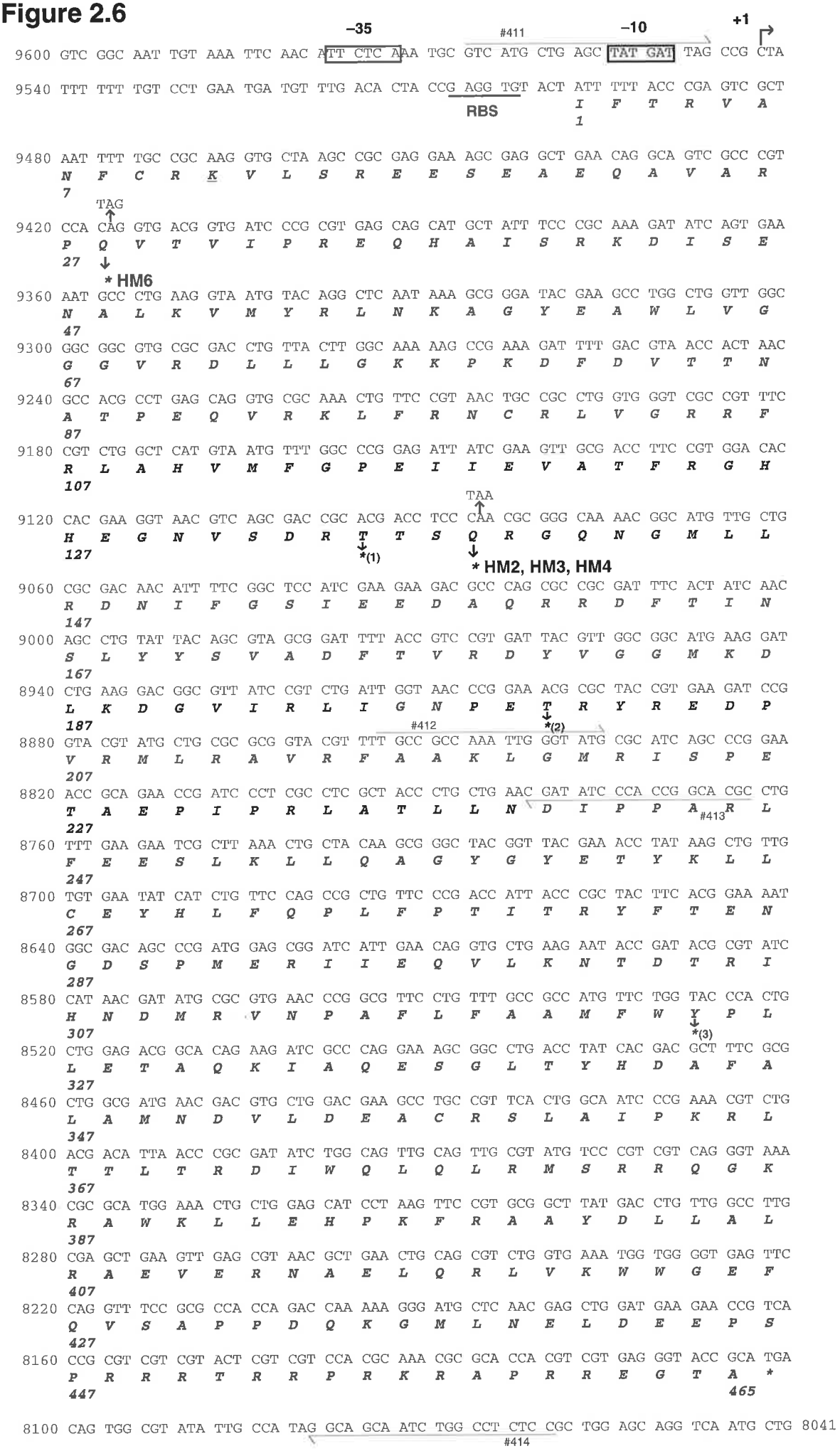
The *pcnB* gene was originally sequenced by Liu and Parkinson (1989) and Cao and Sarker (1992). The sequence of the complete E. coli K-12 MG1655 genome by Blattner *et al.* (1997) shows one change to this region (deletion of one basepair at position 8153 of AE000123), which results in some alteration to the originally proposed C-terminal amino acid sequence. The wildtype *pcnB* sequence from the E251 strain was determined here and proved to be identical to that of Blattner *et al.* (1997) – see Figure 2.6. Characterization of this gene by Binns and Masters (2002) showed that PAP I protein translation begins at the rare initiation codon AUU at position 9498, implying that the wildtype protein is 465 residues in length.

Sequencing of the coding region of the *pcnB* gene of the Host Mutant strains (in at least one direction) showed that each carries a nonsense mutation in the *pcnB* gene which would result in truncation of the Poly(A) polymerase protein made (see Figure 2.6). HM2, HM3 and HM4 carried an identical mutation at position 9078, resulting in truncation of the PAP I protein to 140 residues; while in HM6 cells, a mutation at position 9417 would result in expression of a protein only 27 residues in length. Other researchers have shown that PAP I protein activity is abolished by truncation to 200 residues or less (Cao and Sarkar, 1992; Raynal and Carpousis, 1999), implying that the nonsense mutations found in the Host Mutants would prevent PAP I function. The PAP I protein produced in HM6 should be shorter than that made in the other Host Mutants, however the copy number of ColE1-based plasmids appeared to be less drastically reduced in this strain compared to the others (see Figure 2.5). Presumably this effect was due to a higher level of read-through of the TAG truncating mutation found in HM6, compared to the TAA mutation in other strains, resulting in a higher level of active PAP I protein being expressed.

Figure 2.6. Mutations of the *pcnB* gene found in the Host Mutant strains.

The sequence of the *pcnB* gene, which encodes the Poly(A) polymerase I (PAP I), is shown as determined for *E.coli* K-12 MG1655 by Blattner *et al.* (1997 – Genbank accession number AE000123) and identical to that seen here for the E251 strain. (Note that this sequence has a single basepair deletion at position 8153 compared to the original sequence of Liu and Parkinson (1989 – position 1410).) The –10 and –35 promoter sequences, ribosome binding site (RBS) and transcription start point (+1) of the gene are as identified by Binns and Masters (2002). The positions of primers #411-#414 which were used to sequence the coding region of the *pcnB* gene are also given, and truncating mutations found in the Host Mutant strains HM2, HM3, HM4, and HM6 are shown. (1), (2), and (3) indicate truncating mutations of PAP I which have previously been found to eliminate polymerase activity (Raynal and Carpousis, 1999; Cao and Sarker, 1992).

Figure 2.6



Thus, the Host Mutants each carry a mutation in the *pcnB* gene which reduces the copy number of ColE1-based plasmids, and resulted in lower activity of the 186 late activator protein in the original plasmid-based selection system and reporter assays.

2.B.4. B PROTEIN DOES INTERACT WITH THE RNA POLYMERASE α-SUBUNIT – RE-EXAMINATION OF THE *RPOA*109 STRAIN.

It was initially reported that B activity during a phage infection was not affected by the *rpoA*109 mutation (Sauer, 1979), which results in a leucine-to-histidine substitution at residue 290 in the C-terminal domain of the RNA polymerase α-subunit. The subsequent, unpublished results of Tina Rathjen supported this observation, as shown in Figure 2.1A (Section 2.A.2.1.). She found that E251 cells transduced with the *rpoA*109 mutation showed no reduction in 186 plating and that a pMRR9-*pV.lacZ* reporter plasmid was still activated to ~30% of wildtype levels in these cells by B protein expressed from pTMC10-B. However, I decided to repeat these experiments on the *rpoA*109 mutant strain using my modified single-copy *lacZ* reporter assay.

Therefore, E251 *rpoA*109 cells were transduced with the *lacZ*::Km^R insertion mutation, then lysogenized with the λ*pV.lacZ* reporter and transformed with the pKO2-B (or pKO2-Bam control) plasmid. LacZ assays were performed on the resultant strains and wildtype E251 *lacZ*::Km^R cells as a control. As shown in Figure 2.7, activation of the *pV* reporter by B protein measured here was reduced to ~2% of wildtype by the *rpoA*109 mutation – from 136 LacZ units in E251 to 3 LacZ units in the E251 *rpoA*109 strain. Therefore, this assay showed a dramatic reduction in B function caused by the *rpoA* mutation.

The efficiency of 186 plating was also re-assayed on the E251 *rpoA*109 strain in comparison to the wildtype E251 host. As shown in Figure 2.7, in this experiment 186^+ efficiency of plating was reduced to 0.39 by the *rpoA* mutation, and plaques were smaller and less distinct than on a wildtype E251 host. It was not clear why such a difference in the efficiency of 186 phage plating on the *rpoA*109 strain was seen in this assay compared to that of Tina Rathjen (0.39 compared to 0.98).

Therefore, I found that B activation of a 186 late promoter and 186 lytic development were both significantly reduced in the E251 *rpoA*109 strain. These results imply that activation of the late promoters by B protein is mediated by an interaction with the RNA polymerase α-subunit and that residue 290 of alpha is involved in this contact.

Figure 2.7. 186 plating and B activity in the *rpoA*109 strain.

The average efficiency of plating (eop) of 186^+ phage on E251 *lacZ*::Km^R *rpoA*109 relative to the wildtype E251 *lacZ*::Km^R host is shown with standard deviation errors from 4 assays. Microtitre plate LacZ assays were performed on the same strains lysogenized with the λ*pV.lacZ* reporter phage and transformed with the pKO2-B or pKO2-Bam plasmids. Average LacZ units ± 95% confidence limits are given for assays on at least 13 colonies of each strain. *pV* activity (above background) is the difference between the average LacZ units measured with the pKO2-B and pKO2-Bam plasmids in each strain and is also shown as a percentage of the *pV* activity (above background) measured in E251.

Figure 2.7.

		λ*pV.lacZ*			
		LacZ units		***pV* activity**	
host	**186 eop**	+ pKO2-Bam	**+ pKO2-B**	LacZ units	**% E251**
E251 ***lacZ***	**1.0**	3.0 ± 0.7	**139** ± 39	136	**100**
E251 ***lacZ rpoA*****109**	**0.39** ± 0.10	3.7 ± 0.3	**6.7** ± 1.5	3	**2**

2.C. DISCUSSION.

At the start of this project it was envisioned that novel bacterial functions required for B protein activation of the 186 late promoters could be selected by mutagenesis of *E. coli* cells carrying a multiple copy late gene expression plasmid which was lethal upon transformation of a compatible, multi-copy B-expressing plasmid. Resistance to 186 phage infection by a small fraction of the survivors was expected to reflect the severity of the mutation on B protein activity, and plasmid-based reporters in these strains appeared to confirm that B activation of the late promoter, *pV*, was indeed abolished.

In each the four survivors of the selection process which were characterized, the mutations conferring resistance to 186 infection were mapped by Tina Rathjen to a region of the chromosome between ~81.5' and 82' (Figure 2.1B). I showed here that these mutations lay within an operon encoding the *waa* genes required for biosynthesis of the cell surface lipopolysaccharide structure (Figure 2.2). In isolation, the *waa* mutation from one of these mutant strains did not affect 186 lytic development in general, or B-stimulated transcription from a 186 late promoter reporter (Figure 2.3B). Instead, it seemed likely that resistance to 186 infection was conferred by removal of the molecules required for adsorption to the cell, though this was not tested directly. Further experiments suggested that the receptor necessary in 186 phage binding contains the second and third glucose and sidechain galactose sugars of the LPS outer core (Figure 2.4). Reduced B activation of the late promoters in the original screen and in *lacZ* reporter assays on the Host Mutants was therefore due to additional mutations in these cells. However, these mutations also did not directly affect B protein function, but in fact reduced the copy number of the plasmids involved (Figure 2.5). These cells were subsequently found to carry nonsense mutations in the *pcnB* gene (Figure 2.6), whose wildtype product normally reduces inhibition of plasmid replication at ColE1-like origins, caused by the RNAI molecule. I also found that the ~10-fold variation in *pV* reporter activation seen previously for different length promoter fragments (Dibbens and Egan, 1992, Tina Rathjen, unpublished) was not due to downstream binding of a co-activator protein, but to alteration of the *lacZ* leader sequence, which probably affected the stability or translation of the *lacZ* message (Figure 2.3A).

These results have highlighted a number of flaws in the original system used to select bacterial mutants with apparently reduced 186 late activator protein function. Firstly, the plasmid-based nature of this system led to the isolation of *pcnB* mutants, which survived simply by reducing the copy number of the plasmids involved in the selection. Secondly,

the nitrosoguanidine treatment resulted in a proportion of cells carrying (at least) two mutations, such that resistance to 186 infection in some survivors was not due to the mutation required for survival, but a second (presumably random) mutation affecting phage adsorption. Finally, three of the four Host Mutants studied had the same basepair mutation in the *pcnB* gene and possibly also the same mutation in the *waa* locus (which showed similar transduction linkage and the same efficiency of 186 phage plating). Therefore, it seems likely that overnight culturing of the mutagenized cells before selection resulted in several survivor colonies being derived from the same original cell. These problems would need to be addressed if the selection process were to be repeated.

However, it was obviously not worthwhile repeating this selection process for the following reasons. It was initially thought that the B protein may have made a novel interaction with the RNA polymerase complex, because, unlike the homologous Ogr protein of the closely related phage P2, B protein activity and 186 infection did not appear to be adversely affected by the *rpoA*109 mutation. However, since the time this project was started, the interactions of other activator proteins with other RNA polymerase subunits – β, β' and σ have been well-documented (Severinov, 2000). In addition, it seems that all activators, although they may make different contacts with the RNA polymerase complex, act in a similar way to increase polymerase binding, open complex formation and/or release from the promoter (Hochschild and Dove, 1998). Therefore, even if the 186 B protein was found to interact with a different part of RNAP, this may not give additional information about possible mechanisms of transcription activation.

In addition, re-examination of the effect of the *rpoA*109 mutation of the RNA polymerase α-subunit on 186 plating and B function demonstrated that interaction between the B protein and residue 290 of the α-subunit is important for activation of 186 late promoters (Figure 2.7). This mutation reduced the efficiency of 186 plating to ~40% of wildtype and plaques that were formed were much smaller than on E251. The effect on 186 plating was most likely due to a reduction in B-stimulated transcription of the 186 late promoters, as only ~2% of wildtype activation of a *pV.lacZ* reporter was seen in the *rpoA*109 strain. Since the 186 B and P2 Ogr proteins are 63% identical (Kalionis *et al.*, 1986a) and activate phage late promoters by binding to very similar DNA sequences (Van Bokkelen *et al.*, 1991 – see Section 1.C.4.2.; Julien and Calendar, 1995; Pountney *et al.*, 1997), it is likely that they make very similar contacts with the RNA polymerase α-subunit. These contacts have been well-characterized for the Ogr protein (Wood *et al.*, 1997), by use of a library of *rpoA*

mutant strains. Similar experiments with 186 B protein could be used to confirm the residues of alpha (and B) required for this interaction, but were not pursued here.

CHAPTER 3:

THE ROLE OF REPLICATION IN THE PROVISION OF LATE FUNCTIONS DURING 186 LYTIC DEVELOPMENT

3.A. INTRODUCTION.

The structural and lysis genes of bacteriophage 186 are expressed from three promoters, *pV*, *p12* and *pJ* (Dibbens and Egan, 1992; Xue and Egan, 1995b), and possibly also a fourth promoter '*p32*' (see Section 1.C.3.2.). Transcripts of these genes can be detected from 20-25 minutes after temperature induction of a 186 *cI*ts lysogen (Finnegan and Egan, 1981; Richardson, 1993), following the expression of early lytic functions from the phage and replication of the phage DNA (Hocking and Egan, 1982d; Orsborn, 1986). The 186 late activator protein, B, is required to enhance transcription from the 186 late promoters, as shown by *galK* reporter assays (Dibbens and Egan, 1992). Increased levels of late transcripts during phage development also require the phage replicase protein, A (Finnegan and Egan, 1981), probably to increase the late gene dosage (see Section 1.D.3.1.). However, mRNA of the late activator protein can be detected as early as 2.5 minutes after removal of lysogenic repression of the *B* promoter by the transfer of a 186 *cI*ts lysogen to high temperature (Richardson, 1993). Thus, the late activator gene is transcribed early during 186 lytic development, but only functions to enhance the 186 B-dependent promoters much later in the cycle. The main aim of this chapter was to explain this apparent inconsistency and determine how 186 late gene expression is delayed after phage infection.

Dibbens and Egan (1992) proposed that the increased *B* gene dosage provided by phage replication could be necessary for B-dependent activation of the late promoters during 186 lytic development. This would provide an explanation for the delay in late gene transcription and the requirement for replication to enhance the level of late transcripts during 186 development. Therefore, I decided to test this proposal directly by expressing the B protein from an increasing number of copies of its native gene, and assaying the ability of the protein produced to activate the 186 late promoters on the phage (by measuring complementation of a 186 B^- mutant), or as transcriptional fusions to the *lacZ* reporter gene. Previous experiments had also suggested that replication of the late genes themselves may be important for phage production, suggesting that enhanced late promoter activity early in the phage infection may not actually be detrimental to the phage burst. This idea was also investigated here by assaying phage production in a cell expressing high levels of B protein from the start of a 186 infection. However, I initially wished to confirm the difference in timing of transcription of the *B* gene and the promoters it activates during 186 lytic development, by assaying the expression of *lacZ* reporters of these promoters.

3.B. RESULTS.

3.B.1. CONFIRMATION OF THE TIMING OF *B* AND LATE PROMOTER ACTIVITY DURING 186 LYTIC DEVELOPMENT.

3.B.1.1. pB *and* pV *reporter activity during temperature induction of a 186* cI*ts lysogen.*

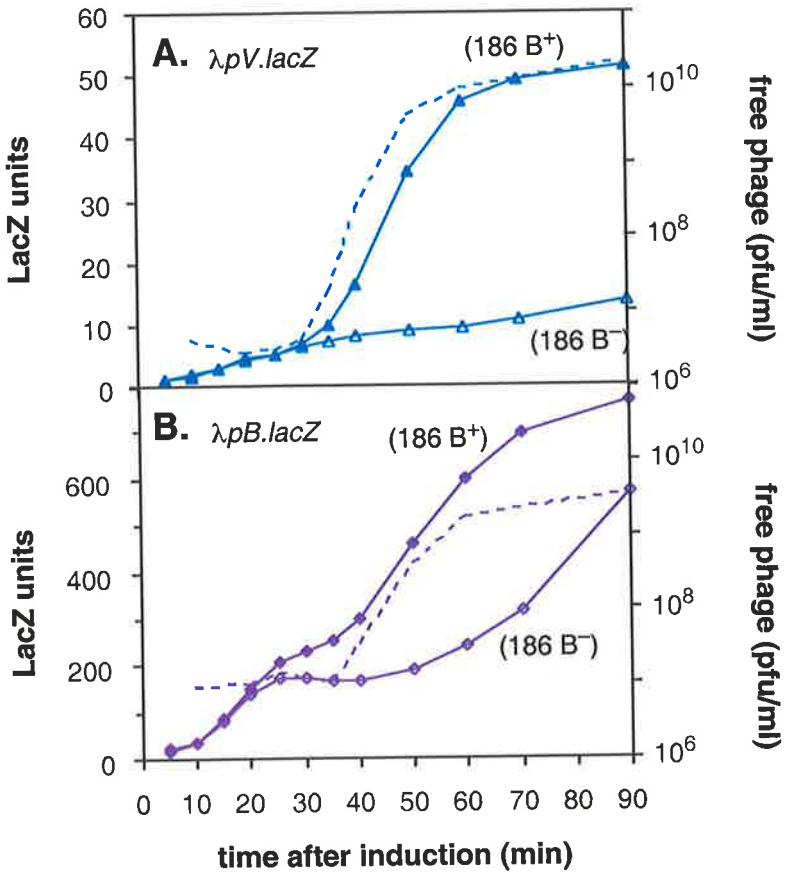
A single-copy, chromosomal *lacZ* reporter system was used to measure the level of transcription from the *B* promoter, *pB*, and 186 late promoter, *pV*, during 186 lytic development. Transcriptional fusions of these promoters to the *lacZ* reporter gene were made in pTL61T, transferred to the λRS45Δ*YA* phage and mono-lysogens of the resultant phage were made of E251 *lacZ*::Km^R cells carrying a 186 *cI*ts prophage. Single-copy reporters were also made in E251 *lacZ*::Km^R (186 *cI*ts *B*am17) cells as a control. (The pTL61T plasmid was used as a *lacZ* reporter vector in this assay as, unlike other reporter plasmids, such as pMRR9R, it lacks the *trpt* terminator sequence upstream of the *lacZ* gene. The activity of this terminator varies considerably at different temperatures (Liang *et al.*, 1998), and its presence could affect transcription of the *lacZ* gene from the promoter fusion during the temperature induction assay.) The 186 *cI*ts prophages of the reporter strains were induced by transfer of the cells to 39°C and aliquots were taken at different times to follow the phage burst and LacZ activity of the culture (see Sections 6.B.2.7 and 6.B.4.2 for details). Due to the nature of the assay, LacZ units were calculated using the optical density of the cells (at 600nm) immediately prior to the temperature transfer, so changes in the culture density due to cell growth and phage development were not taken into account. However, these effects were expected to occur equally for both reporters, and therefore should not affect comparison of the *B* and late promoter activities during phage development. Results of these experiments are shown in Figure 3.1.

The presence of the lambda reporter prophage did not significantly affect the kinetics of phage production by the temperature-induced 186 *cI*ts B^+ lysogen in comparison with previous experiments (Finnegan and Egan, 1981). The levels of free phages in the cultures (indicated by dashed lines in Figures 3.1A & B) increased from 30-35 minutes after transfer to high temperature, and reached a level of 10^9-10^{10} pfu per ml by 60 minutes after induction. There was no change in the number of free phages found in a culture of 186 *cI*ts *B*am17 lysogens up to after 90 minutes after induction, as expected in the absence of wildtype B protein expression (data not shown).

Figure 3.1. The timing of *pB* and *pV* reporter expression during 186 development.

A. and **B.** show LacZ units measured from chromosomal *lacZ* reporters of the 186 *pV* (λ*pV.lacZ*) and *pB* (λpB_{64}.*lacZ*) promoters respectively, during temperature induction of 186 *cI*ts B^+ or 186 *cI*ts *B*am17 lysogens of E251 *lacZ*::Km^R. Assays were performed as described in the text and Sections 6.B.2.7. and 6.B.4.2., where LacZ units were calculated using the starting OD_{600} of the cultures. The increase in free phage levels during temperature induction of the 186 *cI*ts lysogens (also carrying the respective reporter prophage) are shown as dotted lines, with y-axis values on the right-hand side of the graphs.

Figure 3.1


A. λpV.lacZ
(186 B+)
(186 B−)
LacZ units
free phage (pfu/ml)
B. λpB.lacZ
(186 B+)
(186 B−)
LacZ units
free phage (pfu/ml)
time after induction (min)

Transcription from the chromosomal *pV* reporter, as shown in Figure 3.1A, was initially very low, giving only 1.3 LacZ units at 5 minutes after 186 *c*Its B^+ induction, due to the lack of B protein expression in a 186 lysogen. However, when a wildtype *B* gene was present on the 186 prophage, *pV* reporter activity increased dramatically from 35 minutes after induction, before leveling off between 60 and 90 minutes presumably due to cell lysis. In the absence of B protein expression (186 *c*Its *B*am17 prophage induction), there was only a slight increase in LacZ expression from the *pV* reporter up to 90 minutes after induction.

As expected, the *pB* reporter initially showed a low level of transcription following temperature induction of a 186 *c*Its B^+ prophage (~20 LacZ units after 5 minutes), due to lysogenic repression of the *B* promoter. LacZ activity of cells carrying the *pB* reporter began to increase 5-10 minutes after the temperature transfer, to be 10-fold higher after 20 minutes. After a slight plateau at 25 minutes, LacZ activity continued to increase until cell lysis occurred between 70 and 90 minutes after induction.

These results confirm the large difference between the time of transcription of the B protein (from ~5-10 minutes after induction) and its function in enhancing transcription of the 186 late promoter, *pV* (from ~30-35 minutes after induction), during 186 lytic development. LacZ expression from each promoter in this assay, increased somewhat later than the level of transcript measured previously by primer extension experiments – where *pB* mRNA was detected 2.5 minutes after infection and *pV* transcripts after ~20 minutes (Richardson, 1993). This delay between promoter transcription and LacZ reporter activity was presumably a result of the time required for transcription and translation of the ~110kD LacZ protein, and its assembly into tetramers.

3.B.1.2. Investigating autoregulation of the B *promoter.*

The *B* gene is expressed from a promoter of moderate intrinsic strength for which 186 CI repression is the only known regulation. Therefore, it was expected that activity of the *pB.lacZ* reporter during 186 lytic development would increase after removal of CI repression until the maximum activity was reached. Since the *B* promoter is not known to be autoregulated, it was also expected that the reporter would show similar activity whether B protein was expressed during phage development, or not.

As anticipated, similar LacZ expression from *pB* was seen for the first 30 minutes after temperature induction of a 186 *c*Its B^+ lysogen or 186 *c*Its *B*am17 lysogen – LacZ units

increased before plateauing after 25 minutes. However, after this time *pB* reporter expression increased further during 186 B^+ lytic development but not 186 B^- development, except for a late rise in LacZ activity. These differences may have been due to the different effects of phage development in the presence or absence of B protein function. Thus, increased LacZ activity from 35 minutes after 186 *c*Its B^+ induction may have been due to cell lysis providing enhanced access of the LacZ enzyme to its substrate, compared to the polymixin B-induced permeabilization used in the assay. Indeed, a *lacZ* reporter of a constitutive, 186-independent promoter also showed higher LacZ activity from ~30 minutes after induction of a 186 *c*Its B^+ lysogen compared to a 186 *c*Its *B*am17 lysogen (data not shown). Similarly, the increase in reporter activity late after 186 *c*Its *B*am17 prophage induction could have been due to cells dying after an excessive amount of phage replication (in the absence of *B* and late gene expression).

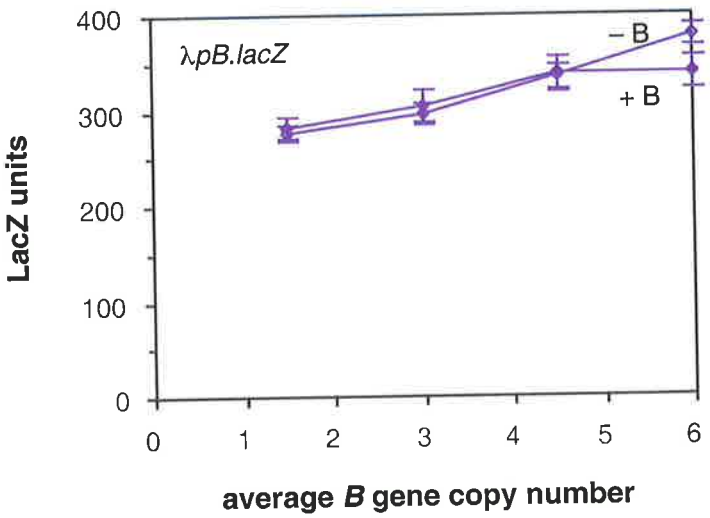
Alternatively, the 3-fold increase in *pB* expression seen during 186 B^+ compared to 186 B^- prophage induction could, in theory, have been due to stimulation of promoter transcription by the wildtype B protein or a 186 late protein. Previous investigation of *pB* autoregulation determined promoter activity with a single level of B protein (Dibbens, 1990); therefore to examine this more thoroughly I decided to assay the response of the *B* promoter to a range of B concentrations.

Figure 3.2 shows activity of a chromosomal *lacZ* reporter of *pB* transcription in the E251 *lacZ*::Km^R host, in the presence of increasing levels B protein expressed from one to four copies of the *B* gene cloned into the single copy number plasmid, pZC320. These plasmids, pZC-B1, pZC-B2, pZC-B3 and pZC-B4, express sufficient B protein to complement a 186 B^- phage burst and activate a *lacZ* reporter of the 186 late promoter, *pV*, to ~54 LacZ units (see Figures 3.8 and 3.9). Control plasmids carry the same number of copies of the mutant *B*am17 gene. No significant difference in *B* promoter transcription was seen in the presence of multiple copies of the *B* gene, verifying that the B protein does not directly autoregulate its own promoter. (Similar results were seen with higher levels of B protein induced from the *plac* promoter on a multiple copy plasmid – data not shown.) A small increase in LacZ expression from *pB* was seen in cells carrying an increasing number of copies of either the wildtype or mutant *B* gene on the pZC320 plasmid. This effect may have been due to the plasmids having a slight, non-specific effect on cell growth or LacZ protein production.

Figure 3.2. The effect of B protein on transcription from the *B* promoter.

LacZ expression from a single lysogen of the 186 *pB* reporter phage, λ*pB.lacZ*, in E251 *lacZ*::Km^R was assayed in the presence of B protein expressed from the pZC-B1, pZC-B2, pZC-B3 and pZC-B4 plasmids. These plasmids carry one to four copies of the wildtype native *B* gene cloned into pZC320, which is maintained at 1-2 copies per cell (Shi and Biek, 1995 – and see Figure 3.7). The promoter was also assayed in the presence of control plasmids carrying the mutated *B* gene, *B*am17. Average LacZ activity is plotted against the average number of copies of the *B* (or *B*am17) gene expected to be present in the cell, and error bars show 95% confidence limits. At least 11 colonies were examined for each strain using microtitre plate LacZ assays.

Figure 3.2


400
300
200
100
0
λpB.lacZ
– B
+ B
LacZ units
0
1
2
3
4
5
6
average B gene copy number

Therefore, B does not autoregulate its own promoter over a range of protein concentrations. The difference in *pB* reporter activity during temperature induction of 186 *c*Its B^+ or 186 *c*Its *B*am17 lysogens can instead be explained by a differential effect of productive or non-productive phage development on LacZ activity.

3.B.2. MULTIPLE COPIES OF THE *B* GENE ARE REQUIRED FOR 186 LYTIC DEVELOPMENT.

3.B.2.1. Expressing B protein from multiple copies of the native B *gene.*

The delay in transcription of the 186 late genes compared to that of *B* itself could be explained if an increased number of *B* genes, provided by phage replication, were required to produce an active level of protein. In order to test this and reproduce the effect of phage replication on B protein expression, the native *B* gene was cloned into plasmids of increasing copy number.

The region of 186 containing the wildtype *pB* promoter, the *B* gene and transcriptional terminator, *tB*, was cloned as a module (*pB-B-tB*), with flanking *rrnB T1T2* terminator sequences to isolate B expression from plasmid transcription. (The complete *T1T2* terminator sequence from the *E. coli rrnB* gene would be expected to give ~100% termination of transcription in either the native or reverse orientation (Orosz *et al.*, 1991).) This cassette was initially inserted into a high copy number, spectinomycin-resistant plasmid, pZE41. The resultant plasmid, pZE-B, carried the ColE1 origin sequence, and was expected to be maintained at about 50-70 copies per cell (Lutz and Bujard, 1997). (Note that the plasmid copy numbers referred to here were usually determined relative to the number of bacterial chromosomes in the cell.) The modular nature of this plasmid allowed simple replacement of the origin sequence with that of lower copy number origins: p15A, pSC101 and pSC101* (Lutz and Bujard, 1997). The resulting plasmids pZA-B, pZS-B and pZS*-B were expected to be maintained at about 20-30, 10-12 and 3-4 copies per cell respectively (Lutz and Bujard, 1997). The origin of pZE-B was also replaced with the mini-F origin of pZC320, which is maintained at 1-2 copies per cell (Shi and Biek, 1995), to give pZF-B. The corresponding series of control plasmids expressing the *B*am17 gene were made in the same way. The wildtype or mutant *B* gene sequence was confirmed in the original cloning, and in pZF-B and pZF-Bam plasmids; and replacement of the plasmid origin was confirmed by PCR with specific primers and/or by restriction enzyme digestion. Diagrams of the pZF-B and pZS*-B plasmids are shown in Figure 3.3A, and construction of these plasmids is described in detail in Section 6.A.3 and Figure 6.3.

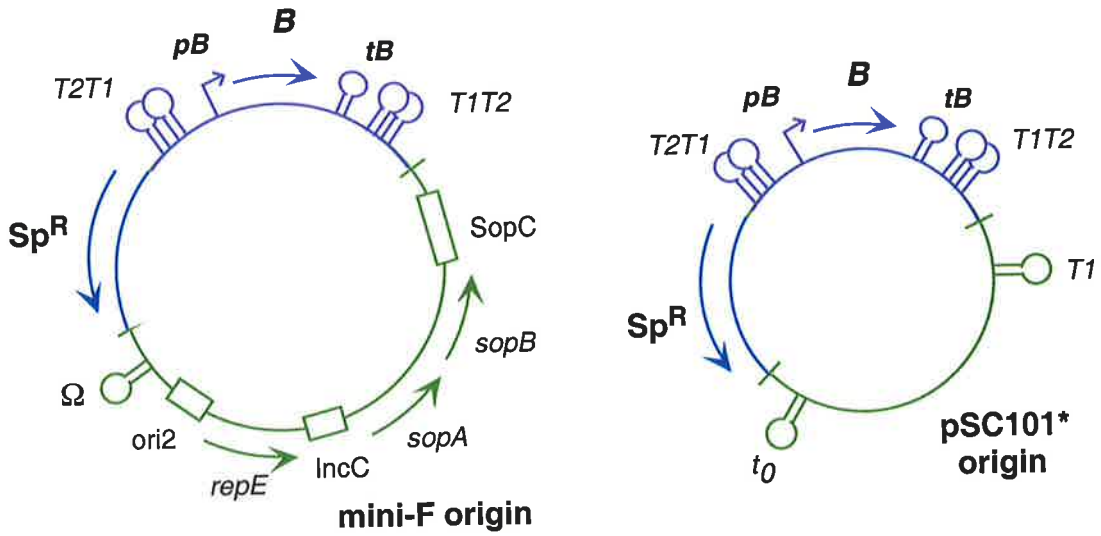
Figure 3.3. Complementation of 186 B⁻ phage plating by native *B* expression plasmids of increasing copy number.

A. *Low copy number native* B *gene plasmids, pZF-B and pZS*-B.*
The pZF-B and pZS*-B plasmids carry a native *B* gene cassette, which consists of the wildtype 186 *B* promoter, gene and terminator, flanked by the transcriptional terminators from *rrnB*, *T1T2*. Each also has the Spectinomycin-resistance gene (Sp^R). pZS*-B has the pSC101* origin (Lutz and Bujard, 1997), flanked by single terminator sequences, t_0 and *T1*. The pZF-B plasmid instead carries the mini-F origin derived from pZC320 (Shi and Biek, 1995), which contains the *repE*, *sopA* and *sopB* genes, the protein-binding sequences IncC and SopC, and the origin of replication, ori2. A transcriptional terminator (Ω) from pZC320 separates the ori2 and Sp^R gene sequences in pZF-B. Details of the plasmid cloning procedures are given in Figure 6.3.

B. *186 B⁻ phage plating on native B plasmids of increasing copy number.*
The plating efficiency of 186 *c*Its *B*am17 phages was determined at 37°C on an E251 (non-suppressor) host carrying a B-expressing plasmid, relative to the phage titre determined on a C600 (*supE*) host. The average efficiency of plating (eop) is shown with standard deviation errors, for two assays with the pZS-B and pZE-B plasmids, five assays with pZS*-B and seven assays with pZF-B. The expected *B* gene copy number for the respective plasmid origins (per chromosome in the cell), is as described by Shi and Biek (1995) for the mini-F origin, or Lutz and Bujard (1997) for the other origins.

Figure 3.3

A.



B.

B plasmid	plasmid origin	*B* gene copy no.	186 B^- eop
pZF-B	mini-F	**1-2**	**1.0** ± 0.2
pZS*-B	pSC101*	**3-4**	**1.9** ± 0.5
pZS-B	pSC101	**10-12**	**1.7** ± 0.0
pZE-B	ColE1	**50-70**	**1.3** ± 0.1

3.B.2.2. Complementation of 186 B⁻ phage plating by multiple copies of the B *gene.*

The plasmids expressing B from an increasing number of copies of the native *B* gene were then assayed for their ability to complement a 186 B⁻ phage burst in plating and temperature induction experiments.

The non-suppressor host, E251, was transformed with pZF-B, pZS*-B, pZS-B and pZE-B plasmids or their *B*am17 equivalents, and the efficiency of 186 *c*Its *B*am17 phage plating was determined for the resultant strains relative to the *supE* indicator, C600. Results are shown in Figure 3.3B. 186 B⁻ phages plated with an efficiency ≥1 on each of the plasmids expressing wildtype B protein, but gave larger and more clearly defined plaques at a higher efficiency on plasmids maintained at more than 2 copies per cell. No plaques were seen on E251 cells carrying any of the *B*am17 control plasmids.

These results suggest that even 1-2 copies of the *B* gene give sufficient B and late protein expression for progeny phages to be released from a 186 B⁻ infection. Plaques were more numerous and larger when more copies of the *B* gene were present, but this was not a very sensitive measure of complementation by the B plasmids. The formation of more 186 *c*Its *B*am17 plaques on the high copy number B plasmids compared to C600 also demonstrates that the amber mutation is incompletely repressed in this strain.

3.B.2.3. Multiple copies of the B *gene are required for a normal 186 B⁻ phage burst.*

To more sensitively measure complementation of a 186 B⁻ phage burst by different copy number B plasmids, 186 *c*Its *B*am17 lysogenic cells carrying the B-expressing plasmids were temperature induced and the timing and size of the phage burst was measured. Thus, 186 *c*Its or 186 *c*Its *B*am17 lysogens of the non-suppressor strain E251 were transformed with the pZF-B or pZF-Bam, and pZS*-B or pZS*-Bam plasmids respectively. Cells at log-phase were transferred to 39°C, and the numbers of free phages and infectious centres in the culture were determined at various times after induction. The burst size was calculated as the total number of phages released (the average number of free phages present after lysis) relative to the number of lysogenic cells induced (the average number of infectious centres measured prior to the burst). The timing of the phage burst was quantitated by determining the time taken to reach the mid-point of the rise in free phage levels (expressed on a logarithmic scale) – called here the 'mid-rise time' (see Section 6.B.2.7. for details). (Classic measurements of the timing of the phage burst such as the latent period and eclipse period could not be calculated accurately in these assays as timepoints were not taken very frequently.)

Figure 3.4A shows the levels of free phages measured during a representative temperature induction assay performed on 186 *cI*ts lysogens carrying the pZF-Bam plasmid, and186 *cI*ts *B*am17 lysogens carrying the pZF-B plasmid or pZF-Bam control plasmid. The average burst sizes and mid-rise times given in Figure 3.4C were calculated from three assays on each strain. Temperature-induced 186 *cI*ts B^+ lysogens carrying the pZF-Bam plasmid gave an average burst of 92 ± 6 phages per cell, and an average mid-rise time of 33 minutes – with all phages released by ~60 minutes after induction. In contrast, the number of free phages present after temperature induction of a 186 *cI*ts *B*am17 lysogen carrying the pZF-B plasmid required up to 120 minutes for all phages to be released, with mid-rise times varying between 63 and 96 minutes in three assays, with an average time of 74 minutes. After cell lysis, the number of free phages present was similar to the number of lysogenic cells induced – resulting in an average 'burst size' of only 0.9 ± 0.4 phages per cell.

As a control, a 186 *cI*ts *B*am17 lysogen was also induced in the presence of the pZF-Bam plasmid. Despite the lack of wildtype B protein expression, the culture initially had a low level of free phages, which increased about 10-fold by four hours after transfer to high temperature. This result suggested that there was some 'leakiness' of the *B* amber mutation in the non-suppressor host E251, which allowed a very small amount of active B protein, and therefore progeny phages, to be produced. However, this level of phage production – in the order of one phage per 100 cells – was not enough to give 186 *cI*ts *B*am17 plaques on an E251 bacterial lawn.

Thus, a single copy of the native *B* gene on the pZF-B plasmid gave insufficient B protein to efficiently complement temperature induction of a 186 *cI*ts *B*am17 prophage. The resulting phage burst was reduced ~100-fold and delayed by about 40 minutes compared to that of a 186 *cI*ts B^+ prophage.

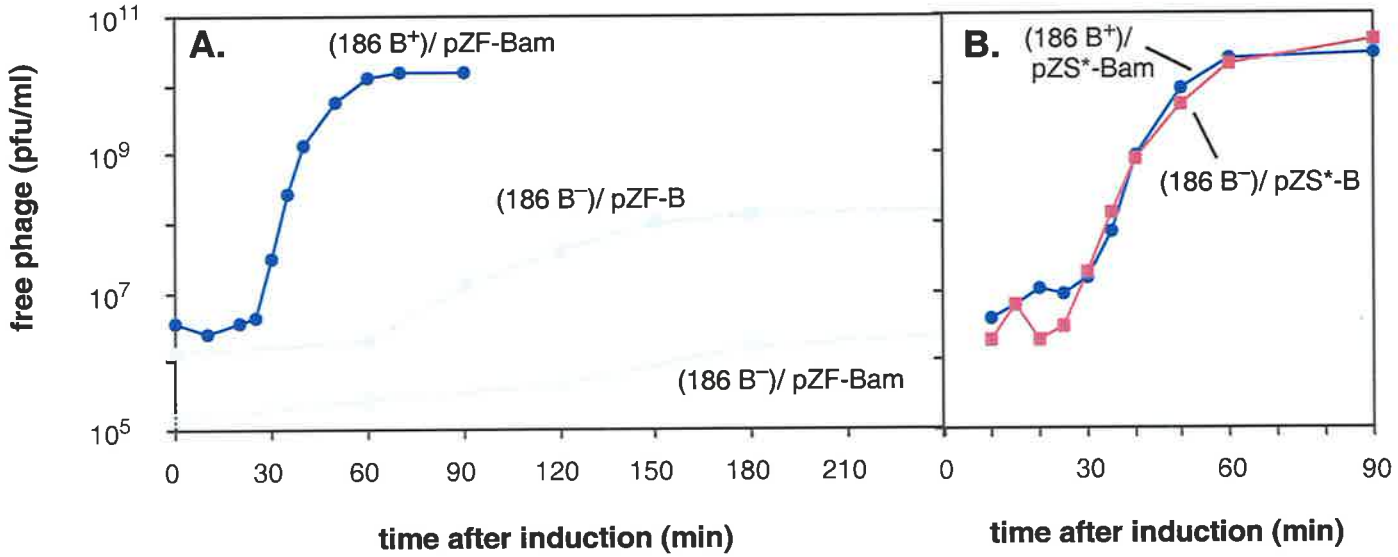
These temperature induction experiments were then repeated with 186 lysogens carrying the pZS*-B or pZS*-Bam plasmids, which were expected to be maintained at about 3-4 copies per cell. Representative burst curves are given in Figure 3.4B, and the average burst sizes and mid-rise times determined from at least three assays on each strain, are shown in Figure 3.4C.

Figure 3.4. The 186 B^- phage burst in the presence of the pZF-B and pZS*-B plasmids.

A. and **B.** show the increase in free phage levels measured during representative temperature induction assays on 186 c*I*ts Bam17 lysogens of E251 carrying the pZF-B or pZS*-B plasmids respectively. The phage bursts of 186 c*I*ts B^+ lysogens in the presence of the respective control plasmids, pZF-Bam or pZS*-Bam, induced on the same day, are also shown. The level of free phages measured after transfer of a 186 c*I*ts *B*am17 lysogen carrying the pZF-Bam control plasmid to high temperature, is also shown in **A. C.** shows the average burst sizes and mid-rise times (with standard deviation errors) determined from at least three assays on each strain (as described in the text and Section 6.B.2.7.). For each plasmid, the burst size of the induced 186 c*I*ts *B*am17 prophage in the presence of the native *B* expression plasmid was also determined as a percentage of the 186 c*I*ts B^+ burst size on the same day, and the average (± standard deviation) is also shown in **C.**

For 186 c*I*ts B^+ lysogens, average free phage and infectious centre levels measured prior to 30 minutes (pre-burst) or later than 50 minutes (post-burst) after induction were used to calculate the burst sizes and mid-rise times. The same timepoints were used for induced 186 c*I*ts *B*am17 lysogens carrying the pZS*-B plasmid. For 186 c*I*ts *B*am17 lysogens carrying the pZF-B plasmid, the burst sizes and mid-rise times were calculated using values prior to 50 minutes (pre-burst) and later than 90 minutes (post-burst) on one day, or prior to 70 minutes and later than 120 minutes on another day, depending on the burst curve seen.

Figure 3.4



C.

186 *cI*ts lysogen	B plasmid	mid-rise time (min)	burst size (phage/cell)	% (186 B+)
B+	pZF-Bam	**33** ± 3	**92** ± 6	
B−	**pZF-B**	**74** ± 19	**0.9** ± 0.4	**1.0** ± 0.5
B+	pZS*-Bam	**38** ± 1	**134** ± 24	
B−	**pZS*-B**	**38** ± 3	**220** ± 48	**170** ± 20

Temperature-induced 186 *c*Its B$^+$ lysogens showed similar phage production whether the cells carried the pZS*-Bam or pZF-Bam plasmid. In the presence of pZS*-Bam, 186 *c*Its B$^+$ gave an average burst size of 134 ± 24 phages per cell and a mid-rise time of 38 minutes on average. Surprisingly, however, the level of B protein expressed from 3-4 copies of the native *B* gene allowed a 186 B$^-$ phage burst that was not largely different from the wildtype burst. The time taken to reach the mid-point of the rise in free phages was similar for induced 186 *c*Its *B*am17 lysogens carrying the pZS*-B plasmid and 186 *c*Its B$^+$ control lysogens induced on the same day. These cells also produced a full phage burst with an average of 220 ± 48 phages released per cell. This larger burst size may indicate that B protein expression from the plasmid is slightly limiting compared to a normal infection, thus allowing additional 186 B$^-$ phage replication and assembly before lysis of the cells. However, overall the temperature induction of a 186 B$^-$ lysogen was essentially completely complemented in cells carrying the pZS*-B plasmid, or 3-4 copies of the native *B* gene.

In summary, the level of B protein expressed from one to two copies of the native *B* gene gave insufficient late functions for a normal burst of 186 B$^-$ phages, with less than two progeny released per cell. At least 3 copies of the native *B* gene were needed to produce sufficient B protein to allow normal production of 186 B$^-$ phage particles. These results demonstrated that multiple copies of the *B* gene, which would be supplied by replication of the phage DNA during 186 lytic development, are needed for normal 186 late gene expression and phage production.

3.B.3. MULTIPLE COPIES OF THE *B* GENE ARE REQUIRED FOR ACTIVATION OF THE 186 LATE PROMOTERS.

3.B.3.1. The 186 late promoters respond equally to increasing copies of the B *gene.*

The level of 186 B$^-$ phage complementation seen with different copy number B plasmids was presumably due to the ability of the B protein produced to activate 186 late gene transcription on the phage. In order to test this directly, chromosomal *lacZ* reporters of each of the 186 late promoters were assayed in the presence of the different B-expressing plasmids. The sequence of the putative *p32* promoter was also included in this assay, since a preliminary primer extension showed transcription from this region after temperature induction of a 186 *c*Its lysogen (see Section 1.C.3.2.).

When sequencing clones of the 186 *pJ* and putative *p32* promoters, several changes were identified in comparison with the complete 186 sequence found in Genbank (accession number NC_001317). At *pJ*, a T-A basepair was deleted at position 14075 or –84 of the promoter, and a G-C basepair was inserted at 14063 or –97 of the promoter, which would result in a longer stem-loop structure of the *t45* terminator found immediately upstream of *pJ*. In addition, a G-C to C-G change was found at 14278 or +120 of the promoter, which would result in a cysteine instead of a tryptophan amino acid at residue 32 of the J protein. At the '*p32*' promoter, deletion of a T-A basepair was found at position 9723 of the phage, 5 basepairs upstream of the putative +1 site at 9728. This change, which results in five basepairs separating the putative +1 site and –10 sequence is shown in Figure 1.8.

Transcriptional fusions of the 186 late promoters to the *lacZ* reporter gene were made in pMRR9R, transferred to λRS45 and mono-lysogens of the resultant phage were made of the Lac$^-$ host strain, NK7049. (This reporter system gives very low background LacZ expression, and was therefore expected to allow accurate measurement of low promoter activity.) Reporter strains were then transformed with each of the native B expression plasmids of increasing copy number, pZF-B, pZS*-B, pZS-B, pZA-B and pZE-B, and LacZ assays performed on the resultant strains. Background promoter activity was determined in the presence of the respective plasmids carrying the *B*am17 gene. Results of these assays are given in Figure 3.5.

The reporters showed little activity in the absence of the B protein, giving between –0.2 and 1.2 LacZ units in the presence of the *B*am17 control plasmids, as shown in Figure 3.5A. Enhanced LacZ expression was seen for each of the promoter sequences, including *p32*, when wildtype B protein was produced from an increasing number of copies of its native gene. Strikingly, none of the 186 late promoters was activated above background by the 1-2 copy plasmid, pZF-B, but reporter activity was significantly increased in the presence of pZS*-B. Further stimulation of the late promoters was apparent with higher levels of B, although saturation of promoter activity or functional B protein expression appeared to occur with the highest copy number plasmid, pZE-B.

As shown in Figure 3.5B, there was a large difference in the strengths of the different 186 late promoters at each B protein concentration. The *pJ* reporter showed the highest activity, giving ~340 LacZ units with the pZE-B plasmid. The *pV* and *p32* promoters were also quite strong, ~75% and 50% as active as *pJ*, respectively; while the *p12* promoter was only

Figure 3.5. Activation of the 186 late promoters by native B expression plasmids of increasing copy number.

Activation of transcription from each of the known 186 late promoters by different B expression plasmids, pZF-B, pZS*-B, pZS-B, pZA-B or pZE-B was determined using single lysogens of the reporter phages, λ*pV.lacZYA*, λ*p12.lacZYA*, λ*pJ.lacZYA* and λ*p32.lacZYA* in NK7049. Microtitre plate LacZ assays were performed on 4-8 colonies of each strain.

A. shows average LacZ activity ± 95% confidence limits from reporters of each promoter as indicated, with plasmids carrying a wildtype *B* gene or *B*am17 gene. The level of activity (above background) of each promoter, calculated as the difference in LacZ units with the respective B^+ and *B*am17 plasmid, is also given. The number of copies of the *B* gene expected per cell for each plasmid is given as described in Figures 3.3. Promoter activity (above background) with B expression plasmids also used in temperature induction assays, pZF-B and pZS*-B, are shown in bold.

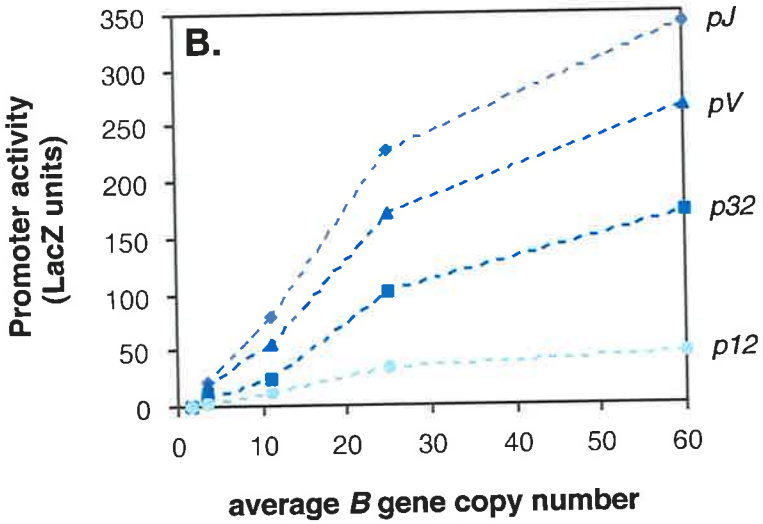
B. shows the average level of activity (above background) of each of the late promoters with each B expression plasmid, plotted against the average number of copies of the *B* gene expected for that plasmid.

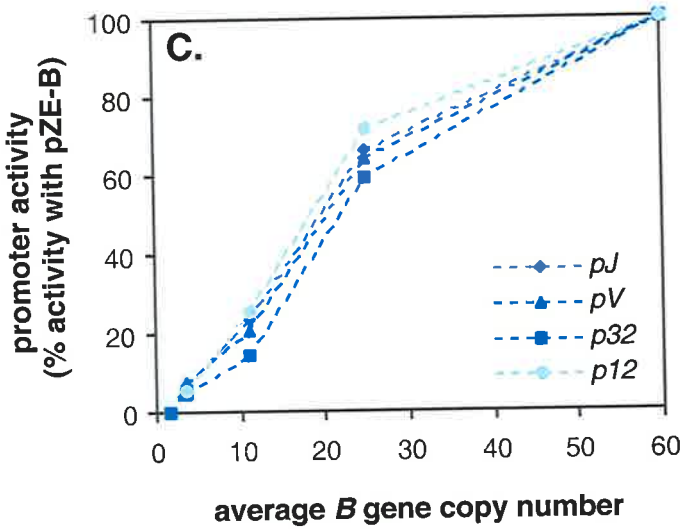
In **C.** promoter activity (above background) of the 186 late promoters with each B expression plasmid is shown as a percentage of the maximal promoter activity (above background) measured with the pZE-B plasmid, and is graphed relative to the average number of copies of the *B* gene expected to be present.

Figure 3.5

A.

plasmid	*B* gene copy no.	λ*pV.lacZYA* LacZ units – B	λ*pV.lacZYA* LacZ units + B	λ*pV.lacZYA* activity units	λ*p12.lacZYA* LacZ units – B	λ*p12.lacZYA* LacZ units + B	λ*p12.lacZYA* activity units	λ*pJ.lacZYA* LacZ units – B	λ*pJ.lacZYA* LacZ units + B	λ*pJ.lacZYA* activity units	λ*p32.lacZYA* LacZ units – B	λ*p32.lacZYA* LacZ units + B	λ*p32.lacZYA* activity units
pZF-B	**1-2**	–0.2 ±0.1	–0.3 ±0.1	**–0.1**	–0.1 ±0.5	–0.3 ±0.2	**–0.2**	–0.1 ±0.2	–0.1 ±0.3	**0.0**	–0.1 ±0.4	–0.3 ±0.2	**–0.2**
pZS*-B	**3-4**	0.1 ±0.0	20 ±3	**20**	0.0 ±0.2	2.6 ±0.9	**2.6**	0.3 ±0.2	23 ±6	**22**	0.3 ±0.1	8.5 ±4.4	**8.2**
pZS-B	10-12	0.4 ±0.1	55 ±3	54	0.2 ±0.2	12 ±1	12	1.0 ±0.1	81 ±4	80	0.5 ±0.1	25 ±2	25
pZA-B	20-30	1.1 ±0.2	171 ±21	170	0.1 ±0.1	34 ±3	34	1.2 ±0.3	227 ±28	226	0.8 ±0.1	102 ±11	101
pZE-B	50-70	1.1 ±0.1	267 ±13	266	0.0 ±0.1	47 ±3	47	0.6 ±0.1	342 ±22	341	0.5 ±0.1	171 ±10	171





activated to ~15% of the level of *pJ* at the highest B concentration. (A similar disparity between *pV* and *p12* promoter expression was also seen by Dibbens and Egan (1992).) However, when promoter activation by each B plasmid was expressed relative to the maximal activity of that promoter seen in this assay (with pZE-B) – see Figure 3.5C, each 186 late promoter showed a similar response to the increasing levels of B.

Thus, the response of *lacZ* reporters of the 186 late promoters to native B plasmids of increasing copy number showed very little activation with the pZF-B plasmid, but substantially more activity with pZS*-B. These results explain why at least 3 copies of the *B* gene were required to give a normal burst of 186 B^- progeny phages – activation of the 186 late promoters required the expression of B protein from more than 1-2 copies of the native *B* gene. The putative late promoter sequence, *p32*, showed no LacZ expression without B but considerable LacZ activity in the presence of the high copy number B plasmids, demonstrating that this sequence does contain a B-activated promoter. Comparison of the four 186 late promoters showed variation in the level of promoter transcription at a particular B concentration, with *pJ>pV>p32>p12* in order of strength, but similar sensitivity to increasing levels of B protein.

3.B.3.2. Is B expression from pZS-B comparable to that from a 186 phage during lytic development?*

The activity of the late promoters in response to the pZS*-B plasmid, while much higher than with pZF-B, was less than a tenth of the maximal level of promoter activity seen in the assay (see Figure 3.5). This suggested that although the level of B made from pZS*-B was sufficient to complement a 186 B^- phage burst, a much higher level of B may be made by the phage during a wildtype 186 infection.

A *pV.lacZ* reporter assayed during temperature induction of a 186 *c*Its B^+ lysogen reached ~52 LacZ units by the end of the phage burst (see Figure 3.1A), while in the presence of the pZS*-B plasmid a *pV* reporter gave ~20 units of LacZ activity (Figure 3.5A). However, these assays are not directly comparable. The experiments were performed in different host strains, and lytic development of the 186 phage appeared to affect the measurement of LacZ activity (see Section 3.B.1.2.). In addition, LacZ expression from the *pV* promoter probably did not reach an equilibrium or steady state of expression during temperature induction of the 186 *c*Its B^+ lysogen, but did in the presence of the pZS*-B plasmid. Therefore, to directly compare B expression from the phage or plasmid in the same system, activity of a chromosomal *pV* reporter was assayed during temperature induction of a 186

*cI*ts *B*am17 lysogen carrying the pZS*-B plasmid, and compared to that seen during induction of a 186 *cI*ts B^+ prophage.

Therefore, E251 *lacZ*::Km^R cells lysogenized with the λ*pV.lacZ* reporter, and carrying a 186 *cI*ts or 186 *cI*ts *B*am17 prophage, were transformed with the pZS*-Bam (control) or pZS*-B plasmids respectively. The resultant strains were temperature-induced and the levels of free phages and LacZ activity in the culture at various times after induction were determined as previously described (see Section 3.B.1.1.). Results are shown in Figure 3.6.

As seen previously, (see Figure 3.4B) the 186 *cI*ts *B*am17 phage burst was complemented by the pZS*-B plasmid, giving a mid-rise time and level of free phages after cell lysis which were comparable to those seen after induction of a 186 *cI*ts B^+ prophage (see Figure 3.6A).

The pattern of LacZ expression from the *pV* reporter was also similar during 186 lytic development whether B protein was expressed from a wildtype phage or 3-4 copies of the native *B* gene, from pZS*-B. *pV* promoter activity was initially low (due to B expression from the wildtype *B* promoter on the phage or plasmid being repressed by CI in the 186 lysogen), increased rapidly between 20-70 minutes after induction, and leveled off after 70 minutes due to complete cell lysis. LacZ expression from *pV* rose earlier when B was supplied by pZS*-B, presumably because more B protein was produced by the 3-4 copy number plasmid compared to the phage, prior to 186 DNA replication. However, the final level of LacZ activity (71 cf 57 LacZ units respectively), and the rate at which LacZ expression increased from the *pV* reporter, was not dramatically different whether B protein was expressed from the plasmid or phage.

Therefore, these results suggested that a similar level of B protein was expressed from the pZS*-B plasmid, as from a wildtype *B* gene on the phage during temperature induction of a 186 *cI*ts lysogen.

3.B.4. Non-linear activation of the 186 *pV* promoter by B protein.

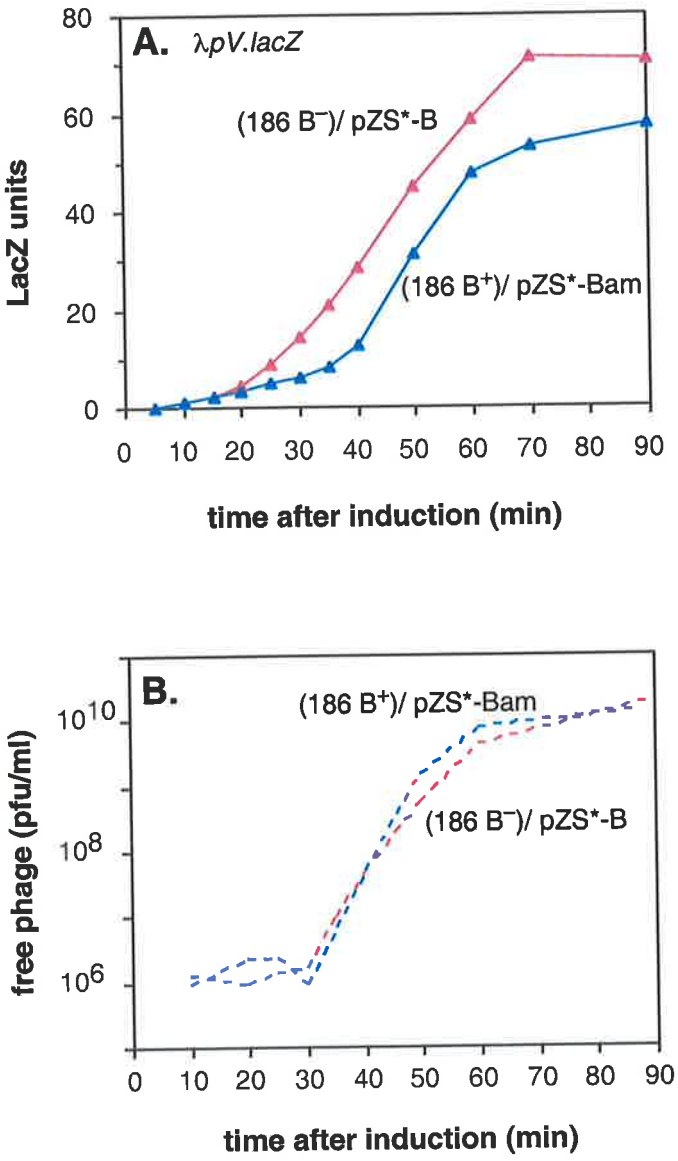
3.B.4.1. Cloning multiple copies of the native B gene onto a single-copy plasmid.

LacZ reporters showed that multiple copies of the *B* gene, expressed from the wildtype *B* promoter, were required to produce sufficient activator protein to significantly enhance transcription from the 186 late promoters. In fact, the response of the late promoters to B

Figure 3.6. *pV* activation during 186 development with B expressed from the phage or pZS*-B plasmid.

The activity of the λ*pV.lacZ* chromosomal reporter was determined after temperature induction of a 186 c*I*ts *B*am17 lysogen of E251 *lacZ*::Km^R carrying the pZS*-B plasmid; or during induction of a 186 c*I*ts B^+ lysogen carrying the pZS*-Bam control plasmid as described in Sections 6.B.2.7. and 6.B.4.2. **A.** shows LacZ activity from the *pV* reporter and **B.** shows the increase in free phage levels, measured for each strain during the temperature induction assays.

Figure 3.6


A. λpV.lacZ
(186 B⁻)/ pZS*-B
(186 B⁺)/ pZS*-Bam
LacZ units
time after induction (min)
B.
(186 B⁺)/ pZS*-Bam
(186 B⁻)/ pZS*-B
free phage (pfu/ml)
time after induction (min)

produced from four copies of the native gene per cell or less was strikingly non-linear. No increase in promoter transcription above background was seen when B was expressed from 1-2 copies of the native *B* gene (with pZF-B), but more than four-fold higher reporter activity was seen in the presence of 3-4 copies of the *B* gene (with pZS*-B). This result could be due to cooperativity in B activation of late promoter transcription. However, this conclusion depends on the assumptions that plasmids were maintained at the expected copy number. Therefore, I wished to confirm this result and further characterize the apparent non-linearity in the response of the 186 late promoters to low levels of late activator protein.

Normally, to investigate potential cooperativity in the activation of a promoter, the promoter response to a range of known activator concentrations is determined. This can be done *in vitro*, with purified activator protein, or *in vivo*, where protein concentration can be quantitated, for example by Western blot analysis. However, the B protein is very insoluble and is therefore difficult to purify in an active form in sufficient quantities for *in vitro* experiments (Pountney *et al.*, 1997), and antiserum previously raised against the B protein was not of sufficient quality for Western blot assays (Keith Shearwin, personal communication). Therefore, to investigate non-linear activation of the 186 late promoters, I decided to express levels of B protein which increased by a known amount over the concentration range produced by the pZF-B and pZS*-B plasmids, and determine the late promoter response *in vivo*.

Stepwise increments in B protein levels could be achieved by expressing B individually from 1, 2, 3 or 4 copies of the native gene in the cell. Ideally, the *B* gene(s) would be encoded by the bacterial chromosome, however, attempts to transfer multiple copies of the *B* gene from the pTL61T plasmid onto a lambda reporter phage in order to lysogenize a host strain, were unsuccessful. Instead, I decided to clone an increasing number of copies of the native *B* gene into the polylinker of the mini-F plasmid, pZC320. This would give a set of plasmids that were identical except for the number of copies of the *B* gene present and would therefore be expected to express levels of B protein that increased in a linear fashion. (The pZF-B plasmid was not used in this experiment since the procedure used to create this plasmid (see Section 6.A.3 and Figure 6.3) removed most of the polylinker sequences of both parent plasmids, pZE-B and pZC320, and left few convenient restriction enzyme sites for cloning.)

Therefore, one to four copies of a native *B* gene cassette – which contained the wildtype *B* promoter, gene and terminator sequences, preceded by the *rrnB T1T2* terminator – were initially sub-cloned into the high copy number plasmid, pTL61T. (Two copies of the *B* cassette also carrying downstream *rrnB T1T2* sequences – as used in pZF-B – could not be cloned in tandem, presumably due to instability of the adjacent terminator sequences.) DNA fragments containing one, two, three or four copies of the *B* cassette were then inserted into the MCS of the pZC320 plasmid (in which the *plac* sequence had been deleted), to give pZC-B1, pZC-B2, pZC-B3 and pZC-B4 respectively. The *B*am17 gene was cloned in the same way to give the control plasmids: pZC-Bam1 to pZC-Bam4. A diagram of the resultant plasmids is shown in Figure 3.7A; and details of the cloning procedure can be found in Section 6.A.3 and Figure 6.4. The number of copies of the *B* or *B*am17 cassette present was confirmed by restriction enzyme digestion.

3.B.4.2. Complementation of a 186 B⁻ phage burst by B protein expressed from the pZC-B plasmids.

As an initial characterization of the mini-F plasmids carrying one to four copies of the native *B* gene, complementation of a 186 B⁻ phage burst by the pZC-B plasmids was assayed in plating and temperature induction experiments.

The efficiency of 186 *c*Its *B*am17 phage plating on E251 cells carrying any of the pZC-B plasmids (or Bam controls) was determined relative to the *supE* strain, C600 (as shown in Figure 3.7B). 186 B⁻ phages plated on the E251 pZC-B1 host with an efficiency of 1.7 ± 0.4, but gave plaques which were smaller and less distinct than those formed on C600. When more copies of the *B* gene were present on pZC320 in the host cell, 186 B⁻ phages formed larger and more distinct plaques, and at an efficiency of plating almost two times higher than that on C600.

186 *c*Its *B*am17 lysogens of E251 were then temperature-induced in the presence of the pZC-B plasmids carrying one, two or three copies of the *B* gene, and the timing and size of the subsequent phage bursts were determined. 186 *c*Its lysogens carrying the pZC-Bam control plasmids were temperature-induced on the same day for comparison. Representative burst curves of lysogens induced in the presence of the pZC-B or pZC-Bam plasmids carrying one, two and three copies of the *B* gene are shown in Figure 3.8A, B & C respectively. Average burst sizes and mid-rise times determined for these strains are given in Figure 3.8D. The bursts of 186 *c*Its B⁺ phages in the presence of the pZC-Bam plasmids were similar to those seen previously (see Figure 3.4A & B) although burst sizes were

Figure 3.7. Complementation of 186 B⁻ phage plating by single-copy plasmids carrying an increasing number of copies of the native *B* gene.

A. *Single-copy plasmids carrying one to four copies of the native B gene: pZC-B1 to pZC-B4.*

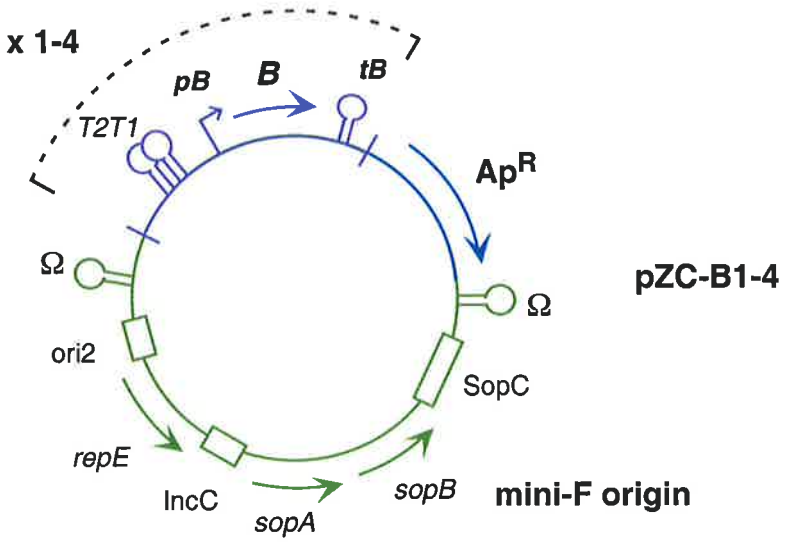
The pZC-B plasmids contain 1 to 4 copies of a *B* gene cassette, consisting of the native *B* gene (*pB-B-tB*) and upstream transcriptional terminators (*T1T2*), inserted into the polylinker of pZC320, with *pB* transcription towards the Ampicillin-resistance gene (Ap^R). Single transcriptional terminators (Ω) flank the mini-F origin sequence, which contains of the *repE*, *sopA* and *sopB* genes, the protein-binding sequences IncC and SopC, and the origin of replication, ori2. Details of the plasmid cloning procedures are given in Figure 6.4 and Section 6.A.3.

B. *186 B⁻ phage plating on the pZC-B1 to pZC-B4 plasmids.*

Plating efficiency of the 186 c*I*ts *B*am17 phage was determined at 37°C on an E251 (non-suppressor) host carrying a B expression plasmid, relative to the phage titre determined on a C600 (*supE*) host. The average efficiency of plating (eop) is shown with standard deviation errors for two to four assays on each strain. The *B* gene copy number is the number of copies of the *B* gene present on the plasmid, multiplied by the plasmid copy number expected for a mini-F origin, 1-2 copies per chromosome in the cell (Shi and Biek, 1995).

Figure 3.7

A.



B.

B plasmid	plasmid origin	*B* gene copy no.	186 B^- eop
pZC-B1	mini-F	**1-2**	**1.7** ± 0.4
pZC-B2	mini-F	**2-4**	**1.9** ± 0.2
pZC-B3	mini-F	**3-6**	**2.1** ± 0.8
pZC-B4	mini-F	**4-8**	**2.1** ± 0.5

somewhat smaller – with 58 to 67 phages released per cell). Induction of a 186 *cI*ts *B*am17 lysogen in the presence of the pZC-Bam1 plasmid gave no significant increase in free phages or infectious centres after 90 minutes, as seen previously (data not shown).

Surprisingly, in the presence of the pZC-B1 plasmid the induced 186 *cI*ts *B*am17 prophage gave a large burst – in three assays, the burst size averaged 202 ± 48 phages per cell, about three-fold larger than that of the 186 *cI*ts B^+ lysogen on the same day. However, the timing of the phage burst was considerably delayed compared to that of an induced prophage with a wildtype *B* gene – giving a mid-rise time ~17 minutes longer. In E251 cells carrying the pZC-B2 plasmid, the 186 B^- phage also gave a phage burst which was larger and slightly later compared to the 186 *cI*ts B^+ lysogen control. Approximately 2.5-fold more phages were released per cell, with a mid-rise time delayed by ~7 minutes. Release of a delayed but larger burst size probably indicates that the level of B protein, and late promoter activation, was sub-optimal in these cells, and that additional phage replication and assembly of progeny was able to occur before cell lysis.

However, in the presence of the pZC-B3 plasmid, the induced 186 *cI*ts *B*am17 phage gave a burst that was essentially the same as that of the 186 *cI*ts B^+ control – the mid-rise time was identical (~33 minutes) and the phage burst was only slightly larger (~1.3-fold). Thus, the level of B protein expressed from 3 copies of the native *B* gene on a single-copy plasmid was sufficient to give a normal burst of 186 B^- phages.

These results confirmed that at least three copies of the *B* gene are required for a wildtype 186 phage burst. As expected, a similar level of B protein appeared to be expressed from three copies of the *B* gene on a single copy number plasmid (pZC-B3) as from one *B* gene present on a 3-4 copy number plasmid (pZS*-B) in the cell. In addition, the level of B protein expressed from a single copy of the *B* gene on a single copy number plasmid was unable to fully complement lytic development of a 186 B^- phage.

3.B.4.3. Non-linear activation of the 186 pV *promoter by B protein expressed from the pZC-B plasmids*

To characterize the late promoter response to stepwise increments of B protein, the activity of a *lacZ* reporter of the *pV* promoter was assayed in the presence of one to four copies of the native *B* gene on the pZC320 plasmid.

Therefore, E251 *lacZ*::Km^R cells lysogenized with the λ*pV.lacZ* reporter phage, were

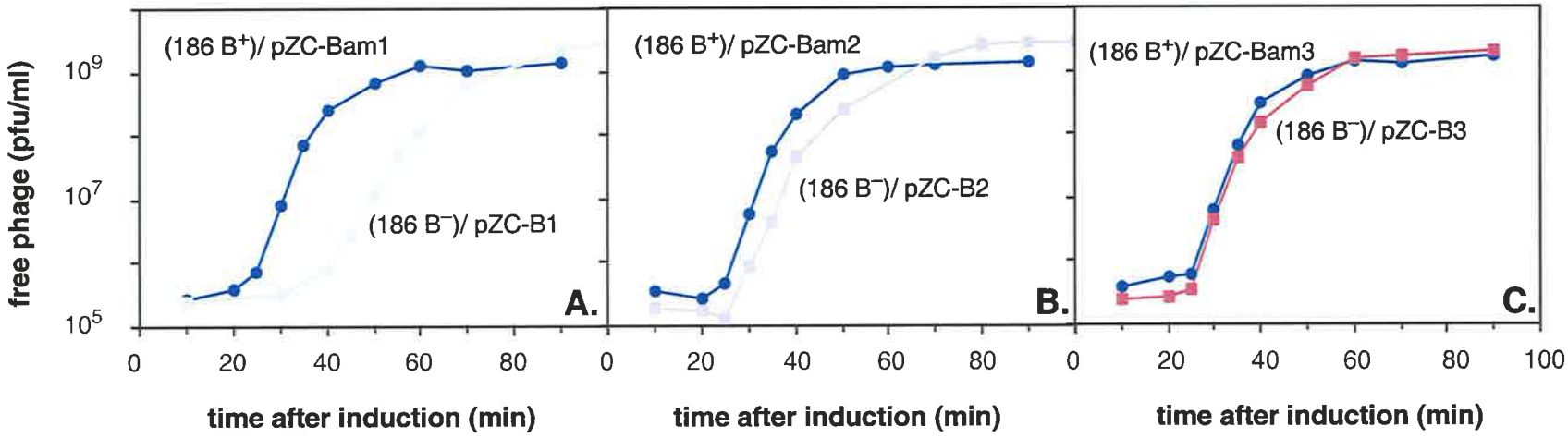
Figure 3.8. The 186 B^- phage burst in the presence of the pZC-B1, pZC-B2 and pZC-B3 plasmids.

A., **B.** and **C.** show the increase in free phage levels measured during representative temperature induction assays on 186 c*I*ts Bam17 lysogens of E251 carrying the pZC-B1, pZC-B2 or pZC-B3 plasmids respectively. The phage bursts of 186 c*I*ts B^+ lysogens in the presence of the respective control plasmids, pZC-Bam1, pZC-Bam2 or pZC-Bam3, induced on the same day, are also shown.

Mid-rise times and burst sizes were determined as described in Section 6.B.2.7. The burst size of the induced 186 c*I*ts *B*am17 prophage in the presence of the native *B* gene plasmid was also calculated as a percentage of the 186 c*I*ts B^+ control burst on the same day. Average values with standard deviation errors, determined from one or two assays on each strain are shown in **D.**

For 186 c*I*ts B^+ lysogens, free phage and infectious centre levels prior to 30 minutes (pre-burst) or later than 50 minutes (post-burst) after induction were used to calculate the burst sizes and mid-rise times. The same timepoints were used for induced 186 c*I*ts *B*am17 lysogens carrying the pZC-B3 plasmid. For 186 c*I*ts *B*am17 lysogens carrying the pZC-B1 plasmid, the burst sizes and mid-rise times were calculated using values prior to 30 minutes (pre-burst) and later than 70 minutes (post-burst); while in the presence of the pZC-B2 plasmid, values prior to 30 minutes (pre-burst) and later than 60 minutes (post-burst) were used.

Figure 3.8



D.

186 *c*I*ts* lysogen	B plasmid	mid-rise time (min)	burst size (phage/cell)	% (186 B+)
B+	pZC-Bam1	**33**	**62** ± 3	
B−	**pZC-B1**	**50** ± 4	**202** ± 48	**320** ± 70
B+	pZC-Bam2	**36** ± 4	**58** ± 8	
B−	**pZC-B2**	**43** ± 7	**149** ± 71	**250** ± 90
B+	pZC-Bam3	**33**	**67**	
B−	**pZC-B3**	**33**	**87**	**130**

transformed with the pZC-B1-4 plasmids (or *B*am17 controls) and LacZ assays performed. Figure 3.9A shows the activation of the *pV* reporter by the different B expression plasmids, and promoter activity is plotted against the average number of copies of the wildtype *B* gene present in Figure 3.9B. Activity of the *pV* reporter was also assayed in cells carrying the pZF-B to pZE-B set of plasmids for comparison, as previous assays with these plasmids had been performed using a different reporter system and host. Overall, the response of the *pV* promoter to the pZF-B to pZE-B plasmids was similar using this reporter system (see Figure 3.9A) although LacZ activity was generally slightly higher (compare with Figure 3.5A).

Stimulation of the *pV* reporter by B protein expressed from one to four copies of the native *B* gene on the pZC-B plasmids was dramatically non-linear. In the presence of the pZC-B1 plasmid, LacZ expression from *pV* was only slightly (0.8 units) above background levels. However, when two copies of the *B* gene were present on the pZC320 plasmid, *pV* activation was increased ~8-fold. When the level of B protein was expected to be four times higher, in the presence of the pZC-B4 plasmid, LacZ transcription from *pV* was ~68-fold higher.

Similar activation of the *pV* reporter was seen when B protein was expressed from a similar number of copies of the *B* gene, with pZC-B3 (~25 units) or pZS*-B (~30 units), in agreement with the complementation results. In addition, *pV* activation by the pZC-B1 plasmid, although only 0.8 LacZ units, was more than that seen with the pZF-B plasmid (0.0 units above background).

Thus, activation of the *pV* promoter was strikingly non-linear with the concentrations of B protein expressed from one to four copies of the native *B* gene *in vivo*. This presumably reflects cooperativity in the activation of this promoter by the B protein. It is expected that the *pV* promoter is representative of the other 186 late promoters, since these promoters were equally responsive to a range of B proteins *in vivo* (see Figure 3.5C) and during 186 lytic development (Finnegan and Egan, 1981; Richardson, 1993).

Thus, multiple copies of the *B* gene were required to express sufficient B protein to activate the 186 late promoters, and give a normal 186 phage burst. This suggests that late promoter activation during 186 lytic development is delayed due to the requirement for replication to increase the *B* gene dosage. The final section of this chapter explores the expectation that

Figure 3.9. Activation of the *pV* promoter by single-copy plasmids carrying an increasing number of copies of the native *B* gene.

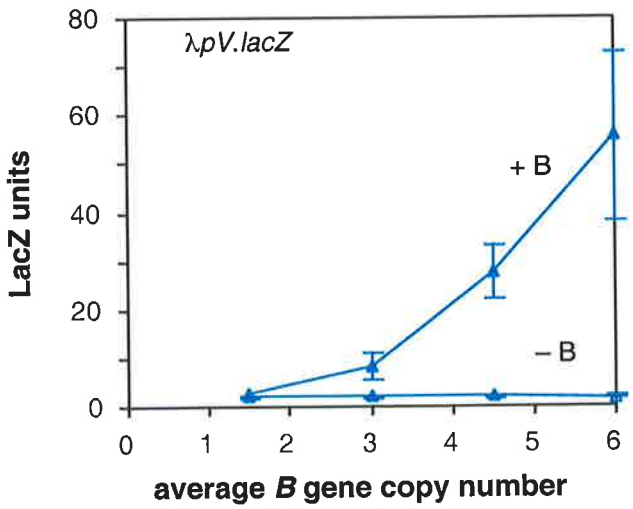
Activation of transcription from the *pV* promoter by different B expression plasmids was determined using a single lysogen of the *lacZ* reporter, λ*pV.lacZ* in the E251 *lacZ*::Km^R host, and microtitre plate LacZ assays. Average LacZ activity, with 95% confidence limits, from *pV* in the presence of plasmids expressing the wildtype *B* or *B*am17 gene, is given in **A.** LacZ activity was determined for least 13 colonies of each strain, except for cells carrying the pZA-B plasmid, where 6 colonies were assayed. *pV* activity (above background), the difference in average LacZ units with the respective B^+ and *B*am17 plasmids, is also shown. For each plasmid, the number of copies of the *B* gene expected per cell is given as described in Figures 3.3 and 3.7. Results are shown in bold for plasmids used in temperature induction assays to measure complementation of a 186 B^- phage burst (Figures 3.4 and 3.8). In **B.** LacZ units from the *pV* reporter in the presence of the pZC-B plasmids, from **A.**, are plotted against the average number of copies of the *B* gene expected to be present in the cell. Error bars are 95% confidence limits.

Figure 3.9

A.

B plasmid	*B* gene copy no.	λ*pV.lacZ* LacZ units – B	λ*pV.lacZ* LacZ units + B	*pV* activity LacZ units
pZC-B1	**1-2**	1.9 ± 0.3	2.7 ± 0.4	**0.8**
pZC-B2	**2-4**	2.0 ± 0.4	8.2 ± 2.7	**6.2**
pZC-B3	**3-6**	2.0 ± 0.4	27 ± 5	**25**
pZC-B4	4-8	1.8 ± 0.3	56 ± 17	54
pZF-B	**1-2**	1.1 ± 0.3	1.1 ± 0.3	**0.0**
pZS*-B	**3-4**	2.2 ± 0.3	32 ± 4	**30**
pZS-B	10-12	2.9 ± 0.3	98 ± 10	95
pZA-B	20-30	4.1 ± 0.5	220 ± 35	216
pZE-B	50-70	4.1 ± 0.2	337 ± 21	333

B.



replication of the 186 late genes is also required for necessary expression of the late functions.

3.B.5. Replication of the 186 late genes is also required for a normal phage burst.

Premature expression of the late functions, particularly the lysis proteins, can be detrimental to a phage burst, leading to early release of a smaller number of progeny (Johnson-Boaz *et al.*, 1994; Markov *et al.*, 2004). However, the experiments of Hocking and Egan (1982a, b, c, d) suggest that replication of the 186 late genes is also required to increase the gene dosage and express sufficient levels of the 186 late proteins for a normal phage burst. This implies that early transcription from the 186 late promoters may not actually affect the timing of substantial late gene expression or the production of 186 phages. I decided to test this idea directly by expressing a high level of B protein from the start of a 186 infection and following the size and timing of the phage burst.

3.B.5.1. Early B expression does not dramatically affect a 186 phage burst.

To determine the effect of early B expression, and therefore premature late promoter activation, on 186 lytic development, a cell expressing high levels of B protein was infected by wildtype 186 phages and the phage burst assayed. The pZS*-B plasmid was used to express B protein in this assay, since it has previously been shown to produce sufficient late activator protein to complement a 186 B^- phage, and to substantially activate the 186 late promoters (see Sections 3.B.2. and 3.B.3.). (Note that in the previous experiments which involved temperature induction of 186 prophages, B expression from the pZS*-B plasmid was initially repressed by CI, whereas in this case B expression from pZS*-B is at equilibrium prior to the infection.)

Therefore, E251 cells carrying the pZS*-B (or pZS*-Bam plasmid, as a control) were infected with 186^+ phages and the numbers of free phages and infectious centres present with time were assayed. Figure 3.10A shows the levels of free phages present during representative 186^+ infections, while Figure 3.10B shows the average mid-rise times and burst sizes determined from three separate assays.

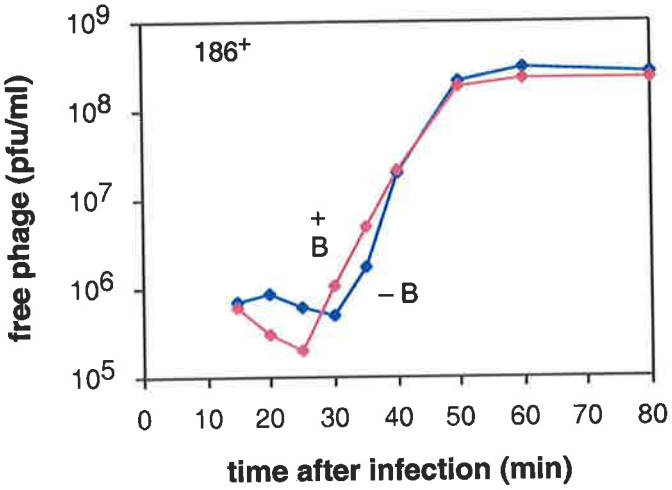
Overall, the 186^+ phage burst was only slightly earlier and smaller when high levels of B protein were present from the start of phage infection. On average, the mid-rise time of the 186^+ infection was ~3 minutes shorter when B was expressed from pZS*-B – 37 ± 2 minutes compared to 40 ± 0 minutes in the presence of the Bam plasmid. The burst size of

Figure 3.10. The effect of B protein expression from the start of a 186^+ infection.

The increase in free phage levels for representative assays of 186^+ infection of E251 cells carrying the B expression plasmid, pZS*-B or control plasmid, pZS*-Bam are shown in **A.** Mid-rise times and burst sizes were calculated as described in Section 6.B.2.7. using average free phage and infectious centre levels measured prior to 30 minutes (pre-burst) and following 50 minutes (post-burst) after the start of the phage infection. The burst size of the 186^+ phage in the presence of the B plasmid was also calculated as a percentage of the 186^+ burst measured in cells carrying the *B*am17 plasmid on the same day. The average mid-rise times and burst sizes (with standard deviations) determined from three assays on these strains, are given in **B.**

Figure 3.10

A.



B.

phage	B plasmid	mid-rise time (min)	burst size (phage/cell)	% pZS*-Bam
186^+	pZS*-Bam	40 ± 0	55 ± 24	
186^+	pZS*-B	37 ± 2	37 ± 17	68 ± 4

186^+ in the presence of early, high levels of B was about 70% of that in the absence of early B expression (37 ± 17 phages per cell compared to 55 ± 24 phages per cell); however, standard deviation errors (as shown) overlap.

Therefore, early B expression, and presumably early late promoter activation, did not have a dramatic effect on the size or timing of the 186 phage burst. This result is compatible with a requirement for replication of the 186 late genes to increase late gene dosage and provide the necessary levels of structural and lysis functions for normal assembly and release of progeny phages.

3.C. DISCUSSION.

The main aim of this chapter was to investigate how late gene expression is delayed when transcripts of the late activator appear to be expressed shortly after the start of 186 lytic development (Richardson, 1993).

Firstly, transcriptional *lacZ* reporters of *pB* and the 186 late promoter, *pV*, were assayed after temperature induction of a 186 *cI*ts lysogen, to confirm the differential timing of transcription from these promoters. Activity of the *pB* reporter increased soon after induction, while the late promoter, *pV*, showed no increase in transcription until about 25 minutes later. These results, and previous assays with a *galK* reporter of *pV* (Dibbens and Egan, 1992), suggested that the initial level of B protein made during 186 lytic development is inadequate to stimulate the late promoters, and that B activity must be enhanced later to facilitate activation of the late promoters and expression of the morphogenetic functions.

B activity was not increased late during lytic development by the B protein activating transcription from its own promoter. Little positive (or negative) autoregulation of a *pB.lacZ* reporter was apparent when increasing levels of B protein were present (Figure 3.2). Instead, simply expressing B protein from an increasing number of copies of the *B* gene on a plasmid, equivalent to the effect of phage replication during wildtype development, enhanced B function to allow stimulation of the late promoters (Figures 3.5 and 3.9) and provide sufficient amounts of late products to complement a 186 B^- phage burst (Figures 3.4 and 3.8). When B was expressed from its own promoter in single copy in the cell, as expected at the start of 186 infection, *lacZ* reporters of the 186 late promoters gave <1 unit of activity, and the 186 B^- phage burst was considerably delayed. Multiple

copies of the *B* gene, however, gave sufficient activator protein to substantially activate the late promoters and restore the timing and number of progeny phages produced by temperature induction of a 186 *c*Its *B*am17 lysogenic cell.

B protein expressed from only three to four copies of the native *B* gene gave essentially full complementation of a 186 B^- burst, although reporters of the 186 late promoters were activated to only a fraction of their potential. The activity of a *pV.lacZ* reporter suggested that this level of B protein (produced from the pZS*-B plasmid) was similar to that expressed from a wildtype *B* gene on the phage during temperature induction of a 186 *c*Its lysogen (Figure 3.6). Thus, it appears that a relatively low level of B protein is produced during 186 lytic development, and that the B protein is not fully expressed from all copies of the 186 genome after phage DNA replication. This is not unreasonable, since replication would be expected to occur concurrently with B activation of the late promoters, expression of structural proteins and formation of progeny phages. Therefore, it could be imagined that soon after being replicated, some phage genomes would become sequestered by packaging and unavailable for transcription. However, the comparison of B activity produced by the phage or expression plasmid was probably not exact as lysis of the cells after prophage induction may have prevented full expression of the *lacZ* reporter in this assay. The concentrations of B protein expressed in this assay could be quantitated more accurately by Western blot analysis, if antibodies specific for the B protein could be generated.

Fusion of the 186 sequence immediately preceding the *orf32* gene to a *lacZ* reporter demonstrated that this region contains a promoter, *p32*, which is intrinsically inactive but can be stimulated to moderately high levels of transcription by the 186 B protein (Figure 3.5). This promoter showed equal sensitivity to increasing B protein concentrations, compared to the other 186 late promoters – although each promoter showed a different maximal level of activity. The *pJ* and *pV* promoters were the strongest, *p32* showed moderate activity and *p12* was quite weak. These differences in late promoter strength could be due to the difference in *lacZ* transcript sequences affecting stability of the message, but these variations should be reduced by the presence of an RNaseIII site between the promoter and reporter sequence. Instead it seems more likely that they reflect the intrinsic ability of the promoter sequences to allow contact between the B protein and RNA polymerase, or to facilitate polymerase binding, open complex formation or initiation of transcription more generally. The similar response of these promoters to increasing levels of B suggests that the affinity of B protein for these binding sites is comparable,

despite their differences in sequence. This was somewhat surprising, especially for the *p32* promoter, since this region contains only a poor match to a weight matrix of predicted B binding sites from 186 and P2 late promoters (Portelli *et al.*, 1998). Confirmation of *p32* as being a B-activated promoter suggests that this matrix may not correctly identify basepairs important for B protein binding. Further implications of the *p32* promoter in terms of characterizing B recognition sequences and its importance in the 186 phage are discussed in more detail later (Section 5.B.3.).

The response of the 186 late promoters to B protein expressed from an increasing number of copies of the native *B* gene was also non-linear. A single copy of the *B* gene on pZF-B gave ≤0 LacZ units above background from any of the 186 late promoter reporters, while up to 20 units were seen with 3-4 copies of the *B* gene. This effect was confirmed with a second system expressing the native *B* gene, where at least 8-fold more LacZ expression from the *pV* reporter was seen when the number of *B* genes present on the pZC320 plasmid was increased from one to two. It is possible that this effect was due to non-linear B protein expression from the plasmids, perhaps due to a titratable inhibitor or degradation system which reduced B production, but this seems unlikely. Instead, it seems probable that stimulation of the late promoters by binding of an oligomeric activator complex is highly cooperative at these B protein concentrations. Ideally, this cooperative effect could be characterized *in vitro* using purified B protein, if soluble, active protein could be obtained (Pountney *et al.*, 1997).

Two plasmids were used to express B from a single copy of the native *B* gene: pZF-B and pZC-B1. Both carried a single copy of the native *B* gene and a mini-F origin of replication (expected to maintain the plasmid at one to two copies per cell) but differed in construction and the antibiotic-resistance gene present (see Figures 6.3 and 6.4). A small difference in the activation of a chromosomal *pV.lacZ* reporter (0.0 compared to 0.8 LacZ units with pZF-B or pZC-B1, respectively – see Figures 3.5 and 3.9), suggests that the levels of B produced by these plasmids differ slightly, presumably due to some plasmid transcription affecting B expression. This small effect was probably amplified by the sensitivity of the *pV* promoter to low B protein concentrations. Complementation of a 186 B^- phage burst also differed between these plasmids (compare Figures 3.4 and 3.8), but showed that at least some increase in late promoter expression must occur with each, despite activation of the promoters by the pZF-B plasmid being below the detection level of the LacZ assay. In the presence of the pZF-B plasmid, an average of 1-2 186 *c*Its *B*am17 phages were released

per cell, ~40 minutes later than normal, and small plaques were formed with an efficiency of ~1.0 compared to a suppressor host. Although quite small this burst of 186 B^- phages was larger than that seen in the absence of B expression. The 186 *cI*ts *B*am17 phage alone in a non-suppressor host gave only a very small increase in free phage levels, and this was probably due to some leakiness of the amber mutation in the *B* gene. In contrast, when B was produced by pZC-B1, 186 *cI*ts *B*am17 gave a burst of ~200 phages per cell, ~15 minutes later than wildtype; and plated with an efficiency of ~1.7 compared to a suppressor host. The high efficiency of plating of 186 B^- phages on strains carrying either plasmid suggests that each infection produced phages, even if it was only one or two per cell. However, the small difference in late promoter activity with these plasmids appears to be magnified in the level of complementation of 186 B^- phages seen. This difference is unlikely to be due to disproportionately higher activation of the other 186 late promoters by these plasmids, given that each of the late promoters responded similarly to higher copy number B plasmids. Therefore, it was probably due to the amplifying effect of phage replication on late gene dosage and therefore late function expression. Presumably, when late promoters are partly activated, reduced expression of the lysis genes permits further rounds of phage replication and assembly of progeny phages to occur before cell lysis. Thus, it seems that the timing and size of the 186 phage burst is very sensitive to the level of B protein present.

Late gene dosage is also important for the production of the necessary levels of late functions for a normal phage burst. This was shown by the experiments of Hocking and Egan (1982a, b, c), who found that when the late genes were expressed from a phage which was unable to replicate (or a prophage), no complementation was seen of a co-infecting phage mutated in an essential late gene. Instead, progeny were only released by recombination between the two phages. Consistent with this requirement, I found here that high B protein expression from the start of a 186 infection, and presumably premature late promoter activation, didn't significantly affect the phage burst produced (Figure 3.10).

Results with the pZF-B plasmid were also comparable to those seen previously when B protein was expressed from one copy of the *B* gene, by Hocking and Egan (1982a, b, c). In phage burst experiments, co-infection of a 186 B^- phage with a replication-defective 186 phage expressing wildtype B protein gave a burst ~4% of that of a wildtype phage, or ~1.7 phages per cell (Hocking and Egan, 1982c). This was similar to the 186 B^- phage burst seen here in the presence of the pZF-B plasmid (Figure 3.4C). Hocking and Egan (1982a, b) also

found that 186 *vir B*am17 phages could plate on a non-suppressor host carrying a 186 B^+ prophage with an efficiency of plating ~20% of that on a suppressor host strain. This showed that B expression from a single copy of the genome could partly complement a 186 B^- phage in plating experiments, as found here with pZF-B (Figure 3.3B).

Thus, overall, despite *pB* being transcribed soon after the start of 186 lytic development, the B protein made (if any) is insufficient to activate the late promoters. The increase in the number of copies of the *B* gene provided by replication increases B protein concentration to a level able to activate late promoters and therefore start production of the morphogenetic functions. Sensitivity of 186 late promoters to low levels of B protein, and probably cooperative promoter activation, means that little B protein is needed in infection to enhance transcription of the late genes. However, replication of late genes is also necessary to provide sufficient amounts of the late products for the production of large numbers of progeny in the cell. Therefore, this reliance on increased gene dosage both of *B* and of the late genes ensures that substantial late gene expression is delayed until after phage replication has occurred.

CHAPTER 4:

THE ROLE OF CI REPRESSION OF THE 186 *B* PROMOTER

4.A. INTRODUCTION.

Previous results have shown that high levels of B protein are lethal to a 186 lysogen (Dibbens and Egan, 1992), probably due to the expression of a lethal 186 late product (Ravi Tiwari, unpublished). However, the amount of B protein produced by a single copy of its native gene gives very little activation of the 186 late promoters and is insufficient to fully complement a 186 B^- phage burst (see Chapter 3). A similar level of B also does not appear to be lethal to a 186 prophage (Richardson and Egan, 1989 – see Section 1.D.3.2.). Therefore, the role of 186 CI repression of B transcription in a 186 lysogen is not immediately obvious.

It is possible that unrepressed B expression from the prophage, though not lethal to the lysogen, may still be harmful to the cell due to low levels of late gene expression; or may affect establishment or stability of the lysogen in an as yet uncharacterized way. Alternatively, or in addition, CI bound at *pB* may form a DNA loop to bind cooperatively to other sites and influence CI regulation of the developmental switch region. The 186 CI CTD has been found to form a 14-mer by X-ray crystallography (Pinkett *et al.*, 2005), and a multimer bound over the *pR* promoter can interact with flanking recognition sites ~350bp away (*FL* or *FR*) to enhance *pR* repression (Dodd and Egan, 2002). A hybrid repressor consisting of the 186 CI CTD and lambda CI N-terminal DNA-binding domain can also simultaneously bind to lambda CI operators separated by 3.8kb (Ian Dodd, personal communication). Therefore, it seems possible that a CI multimer could cooperatively bind to *pR* and *pB* operators separated by ~2.5kb in the circular phage template, to enhance *pR* repression (for example, see Figure 1.4C(4)). Interaction of a *pR*-bound CI multimer with the *pB* operators could perhaps occur at lower CI concentrations than interactions with *FL* or *FR*, since CI has a higher affinity for the *B* promoter sequence compared to the flanking sites (Dodd and Egan, 1996). Such an interaction may be prevented in a 186 lysogen since integration at the attachment site, which lies between the *pR* and *pB* promoters (see Figure 1.2), results in these sequences lying at opposite ends of the phage genome, separated by ~30kb.

In order to address these possibilities, the effect of unrepressed B protein expression on different stages of lysogenic development was examined. Repression of the B promoter by lysogenic levels of CI protein was eliminated by mutation of the CI operator sequence on the phage, and the effect on the growth rate, stability, establishment and induction of the mutant lysogen was assayed. Reporter studies were also used to determine the effect of the

pB operator sequences on CI regulation of the *pR* promoter. As an initial assay, however, the growth rates of strains lysogenic for 186 were measured in the presence of a range of B concentrations expressed from an inducible plasmid.

4.B. RESULTS.

4.B.1. THE EFFECT OF B EXPRESSION FROM A PLASMID ON GROWTH OF A 186 LYSOGEN.

4.B.1.1. Low levels of B protein from a plasmid do not affect the growth rate of a 186 lysogen.

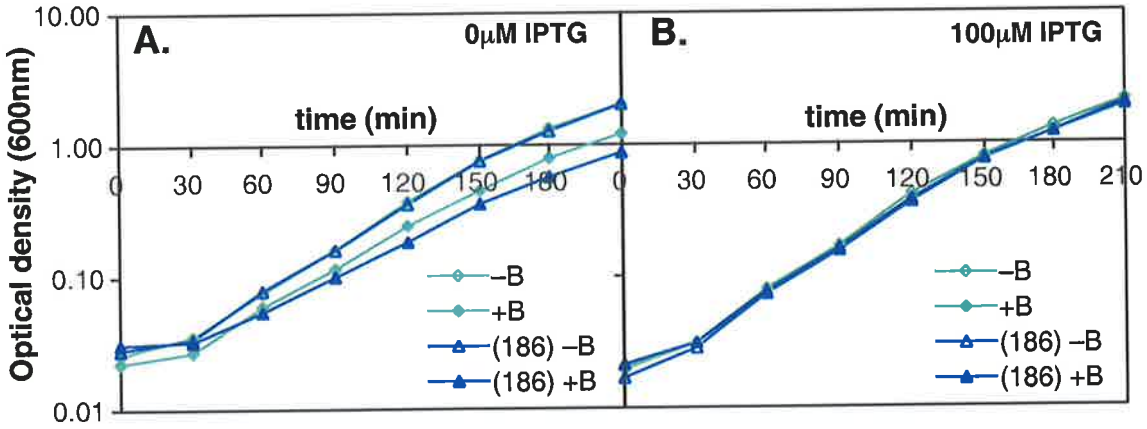
To determine if B protein expression has a detrimental effect on the growth of a 186 lysogen, the growth rate of lysogenic and non-lysogenic bacterial cultures was examined in the presence of several different levels of B protein expressed from a plasmid. The B protein was supplied from the multi-copy plasmid, pZE15-B which expresses the *B* gene from the wildtype *lac* promoter. A compatible plasmid, pDM1.1, was used to express high levels of the *lac* repressor, LacI, allowing IPTG control of B transcription. This expression system was used to induce a range of B concentrations as shown by activation of a chromosomal *pV.lacZ* reporter (see Figure 4.1C). The parental plasmid pZE15 was also used as a control for the effect of IPTG on the growth of lysogenic or non-lysogenic cultures.

Therefore, NK7049 or NK7049 (186^+) cultures were transformed with the pZE15-B (or pZE15) plasmid and LacI plasmid pDM1.1, and the generation (or doubling) time of the cultures was determined. Overnight cultures grown at 37°C in selective medium containing 0, 10, 30 or 100μM IPTG, were subcultured into the same medium in a microtitre plate and the optical density at 620nm (OD_{620}) was followed with time using a microtitre plate reader. OD_{620} measurements were converted to equivalent optical density at 600nm (OD_{600}) using the empirical relationship determined by Dodd *et al.* (2001), and the generation time was calculated for OD_{600} values between 0.05 and 1.0 (for which the slope of the $\log_{10}(OD_{600})$ plotted against time was approximately linear – see Figure 4.1A & B). Figures 4.1A&B show semi-log plots of OD_{600} with time for a single colony of the lysogenic or non-lysogenic strains carrying the pZE15-B plasmid or pZE15 control, in the presence of 0 (A) or 100μM (B) IPTG. Figure 4.1C shows average generation times of the cultures with different IPTG concentrations, and the *pV.lacZ* activation measured with these plasmids.

Figure 4.1. Growth rates of lysogenic and non-lysogenic cultures in the presence of different levels of B protein.

Generation times were determined for cultures of non-lysogens or 186^+ lysogens of NK7049 carrying pDM1.1 and the B expression plasmid, pZE15-B or parental control, pZE15, grown at 37°C in selective medium including IPTG as indicated (see Section 6.B.1.4.). **A.** and **B.** show representative growth curves of optical density at 600nm (with LB OD_{600} subtracted) against time on a semi-log scale, for strains grown in the presence of 0μM and 100μM IPTG respectively. Average generation times (± standard deviation) calculated for 2-4 colonies of each strain grown in the presence of 0, 10, 30 or 100μM IPTG are given in **C.** NK7049 cells lysogenic for the λ*pV.lacZ* reporter were also transformed with these plasmids and microtitre LacZ assays were performed on 3-5 colonies of each strain, cultured in the same IPTG concentrations (as described in Section 6.B.4.2.). *pV* activity (above background), or the difference in average LacZ units measured with the pZE15-B and pZE15 plasmids, is also shown in **C.**

Figure 4.1



C.

IPTG (μM)	λp*V.lacZ* activity	generation time (min) NK7049 + pZE15	NK7049 + pZE15-B	NK7049 (186⁺) + pZE15	NK7049 (186⁺) + pZE15-B
0	**1.7**	**26.6** ± 0.3	**27.1** ± 0.6	**27.0** ± 0.6	**26.3** ± 0.6
10	5.7	26.1 ± 0.8	27.1 ± 0.7	26.8 ± 0.0	26.7 ± 0.3
30	22	26.5 ± 0.3	29.3 ± 1.0	27.2 ± 0.3	29.4 ± 2.4
100	**102**	**26.8** ± 0.2	**30.6** ± 0.2	**27.4** ± 0.2	**36.1** ± 0.8

As expected, cultures showed an initial growth lag in the first 30 minutes after subculture, then the $\log_{10}$ of OD_{600} values increased approximately linearly with time until an OD_{600} of ~2 was reached. In the absence of B protein, lysogenic and non-lysogenic strains had similar log-phase doubling times of between ~26 and 27 minutes, and IPTG concentrations up to 100μM had little effect on cell growth. Low levels of B protein which gave up to 10 LacZ units from the *pV* reporter, expressed from pZE15-B using 10μM IPTG, had little effect on the generation time of 186-lysogenic or non-lysogenic cultures. However, the doubling times of both cultures were increased by 2-3 minutes when a level of B was expressed which gave ~22 units from *pV.lacZ* (30μM IPTG). With B concentrations giving ~95 LacZ units from the *pV* reporter (100μM IPTG), generation times were increased further, by ~4 minutes for the non-lysogen and ~10 minutes for the 186 lysogen. (Overnight cultures of the same strains showed a similar reduction in OD_{600} values with B protein induced from this system using 30 and 100μM IPTG (data not shown).)

These results confirmed that high concentrations of B protein were harmful to a 186 lysogenic cell, as seen by Dibbens & Egan (1992), and showed that high expression of B alone could be detrimental to growth of a non-lysogen. However, low levels of B protein which gave <10 units from a *pV.lacZ* reporter, had no effect on the generation times of the lysogen, or non-lysogen. Given that one copy of the native *B* gene gave ≤0.8 LacZ units from a chromosomal *pV.lacZ* reporter (see Figures 3.5 and 3.9), this suggests that unrepressed B expression from a 186 prophage should not be detrimental to the growth of the lysogenic cell.

4.B.2. Initial characterization of 186 phages carrying a CI operator mutation at *pB*

4.B.2.1. The OB1 *mutation at* pB *removes repression by lysogenic levels of CI.*

To confirm that low levels of B do not affect growth of a lysogen and to look for other effects of unrepressed B expression on 186 lysogenic development, I next mutated the CI operators at *pB* in an attempt to knock-out lysogenic repression of the promoter, but maintain intrinsic *pB* activity (and hence B protein expression).

The 186 CI repressor binds to two A-type inverted repeat sequences centered at –28 and +5 on the *B* promoter (Dodd and Egan, 1996). *In vitro*, a single A-type site shows very little affinity for purified CI protein (Ian Dodd, unpublished), suggesting that knocking out CI

binding to one inverted repeat at *pB* would similarly reduce repressor binding to the whole region. Previous mutation of the A-type binding site at *FR* showed that changes to at least 4/6 bases in a CI halfsite were necessary to remove CI binding to the inverted repeat sequence in this operator (Keith Shearwin, unpublished).

Therefore, in order to prevent CI repression of the *B* promoter, I initially mutated all bases of the CI halfsite situated between the –10 and –35 sequences of *pB* to their complementary bases: GTGAAT to CACTTA (as shown in Figure 4.2A). This mutation, called *OB1*, was chosen to have as little effect on *B* transcription and translation as possible by maintaining the recognition sequences for RNAP binding, the AT content of the promoter (which could affect the kinetics of open complex formation) and the sequence of the B mRNA. Thus, Quickchange mutagenesis was performed on a –213 to +178 fragment of the *B* promoter cloned into pBluescript to give the pBS-pB OB1 plasmid, and the mutation was confirmed by sequencing.

To determine the effect of this mutation on intrinsic *pB* activity and lysogenic CI repression, transcriptional fusions of the wildtype promoter sequence or *OB1* mutant from the respective pBluescript plasmids were made to the *lacZ* reporter gene in pTL61T, and transferred to λRS45Δ*YA*. Mono-lysogens were then formed of the E251 *lacZ*::Km^R or E251 *lacZ*::Km^R (186^+) host strains, and LacZ assays performed. In this system, the wildtype *pB* promoter gave ~230 LacZ units and was repressed about 12-fold in the 186 lysogen (see Figure 4.2B). The *OB1* mutation caused a 1.5-fold increase in intrinsic *pB* activity (to ~360 LacZ units), but abolished repression by lysogenic levels of CI protein (see Figure 4.2B). The increase in intrinsic activity of *pB* caused by the *OB1* mutation would give slightly higher levels of B protein than from the wildtype *B* promoter on the phage, but I decided to proceed with phage experiments and keep this in mind.

4.B.2.2. 186 phages carrying the OB1 *mutation give turbid plaques and normal lytic development.*

The *OB1* mutation was then transferred onto 186 by plating 186 *cI*ts *B*am57 phages onto suppressor cells carrying the pBS-pB OB1 plasmid and selecting recombinant phages in which the amber mutation in *B* had been replaced by wildtype sequence (~1 in 10^4-10^5 phage). Sequencing of two purified recombinant phages showed that each had a wildtype *B* gene and one also carried the *OB1* mutation, giving the 186 *cI*ts *OB1* phage.

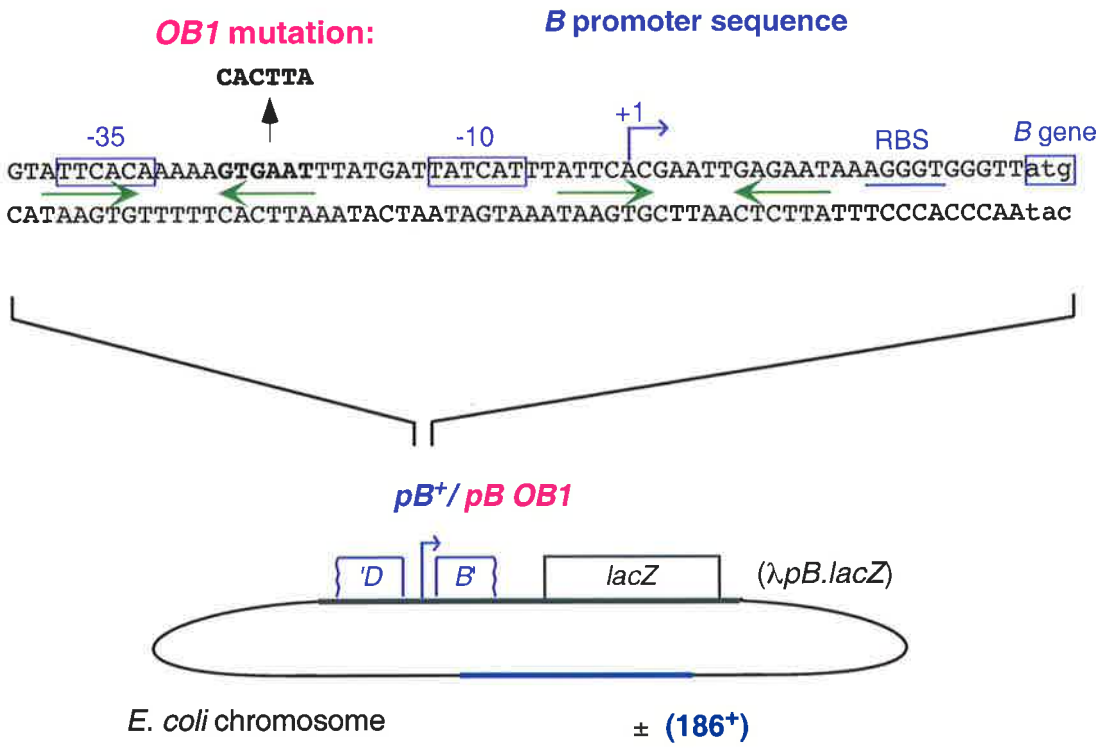
Figure 4.2. The effect of the *OB1* operator mutation on *pB* activity.

A. *The* OB1 *mutation and the reporter used to measure its effect on* pB *promoter activity.* The nucleotide sequence from –37 to +28 of the *B* promoter (20 547 to 20 611 in the 186 genome) is shown. –10 and –35 RNAP σ^{70} recognition sequences and the first codon of the *B* gene are boxed, the initiation site (+1) indicated by an arrow, and the ribosome binding site (RBS) is underlined. Inverted-repeat sequences bound by the 186 CI protein are indicated by converging arrows (in green), and the sequence of the operator mutant, *OB1*, which changes one halfsite of one CI operator to the complementary sequence, is shown above the top strand.

B promoter activity was measured using a transcriptional fusion of a larger region of the wildtype or *OB1* mutant promoter sequence to the *lacZ* reporter gene in a λ reporter phage (made as described in Section 6.A.2.). The resultant phages, λ*pB.lacZ* or λ*pB OB1.lacZ*, were lysogenized in the E251 *lacZ*::Km^R host and LacZ activity measured in the presence (+CI) or absence (–CI) of a wildtype 186 prophage. The average LacZ units ± 95% confidence limits measured in microtitre plate assays on 33 to 44 colonies of each strain are shown in **B.** The average fold repression of *pB* activity by CI expressed from the 186 prophage is also given.

Figure 4.2

A.



B.

λ*pB.lacZ* reporter	LacZ units – CI	LacZ units + CI	fold repression
***pB*⁺**	234 ± 7	20 ±1	**12**
***pB* OB1**	357 ±23	373 ±16	**1.0**

When the 186 CIts protein was inactivated by plating at 37°C, 186 c*I*ts *OB1* gave large, clear plaques that were identical to those of 186 c*I*ts. At 30°C, when the CIts protein was active, 186 c*I*ts *OB1* phages gave turbid plaques, indicating that the *OB1* mutation did not prevent formation of lysogens. By eye, the turbid centres of the mutant plaques were slightly smaller than those of 186 c*I*ts, suggesting that the *OB1* mutation had a slight negative effect on lysogenic development of the phage. However, 186 *cI*ts *OB1* lysogens of the E251 host could be isolated, demonstrating that the mutation did not prevent establishment or maintenance of the prophage.

As an initial characterization, temperature induction of E251(186 *cI*ts *OB1*) and E251(186 *cI*ts) lysogenic cultures were then compared to determine any effects of the mutation on lytic growth of the phage. 186 c*I*ts and 186 c*I*ts *OB1* phages gave almost identical production and release of progeny in two separate temperature induction assays (see Figure 4.3A for an example), and had very similar burst sizes and mid-rise times (Figure 4.3B). (A similar number of progeny were released by both phages on the same day, but some variation in the size of the burst measured on different days led to the large errors given in Figure 4.3B.) Thus, the *OB1* mutation had little effect on lytic development of the phage.

4.B.2.3. The OB1 *mutation has little effect on growth of a lysogenic cell or stability of a 186 prophage.*

When B was expressed from its wildtype promoter in single copy in the cell, the level of protein produced was insufficient to substantially activate reporters of the late promoters, so it was expected that, even when *pB* was fully active on a prophage, little increase in late promoter expression would be seen. To test this directly, lysogens of 186 *cI*ts or 186 *cI*ts *OB1* were isolated of E251 *lacZ*::Km^R cells carrying a chromosomal *pV.lacZ* reporter and the LacZ activity was assayed in cultures grown at 30°C (to ensure maintenance of the 186 prophage). In the 186 *cI*ts lysogen, when *B* transcription was repressed by CI, the *pV* reporter gave 1.6 ± 0.3 LacZ units, with 95% confidence limits from assays on 35 colonies. Similarly, in the presence of the 186 *cI*ts *OB1* prophage, transcription from *pV* gave 1.3 ± 0.2 LacZ units, with 95% confidence limits from assays on 30 colonies. Thus, no significant increase in activity of a *pV* transcriptional reporter was seen when B expression from the prophage was unrepressed (or even slightly increased) due to the *OB1* mutation.

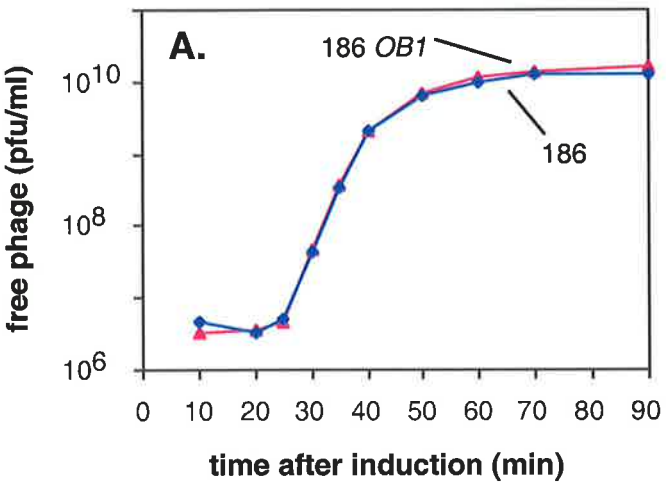
To confirm that this low level of *B* and late gene expression was not detrimental to the cell, the growth of lysogens carrying a wildtype *B* promoter or the *OB1* mutation was compared. Therefore, the generation times of E251(186 c*I*ts) or E251(186 c*I*ts *OB1*) cultures were

Figure 4.3. Effect of the *OB1* mutation on temperature induction, growth rate and stability of the 186 *c*I*ts* lysogen.

Cultures of 186 *c*I*ts* and 186 *c*I*ts OB1* lysogens of E251 were temperature-induced at 39°C, and the burst sizes and mid-rise times were calculated using free phage and infectious centre measurements taken prior to 30 minutes (pre-burst) or later than 60 minutes (post-burst) after induction (as described in Section 6.B.2.7.). **A.** shows free phage levels after induction for representative assays on each strain, and **B.** shows average burst sizes and mid-rise times (± standard deviations) calculated for assays performed on two colonies of each.

Generation times of E251(186 *c*I*ts*) and E251(186 *c*I*ts OB1*) cultures grown in LB at 30°C were determined using OD_{600} values between 0.05 and 1.0 (as described in Section 6.B.1.4.). Average values ± 95% confidence limits from assays on 15-16 colonies of each strain are shown in **B.** The levels of free phages in the supernatants of E251(186 *c*I*ts*) and E251(186 *c*I*ts OB1*) lysogenic cultures grown at 30°C to OD_{600} ~0.55-0.73, were also determined (as described in the text and Section 6.B.2.6.). **B.** shows average free phage levels (pfu per 100 cells) ± standard deviations calculated from assays on 7-9 colonies of each strain.

Figure 4.3



B.

186 lysogen	Phage burst		Growth rate	Stability
	burst size (pfu/cell)	mid-rise time (min)	gen time (min)	free phage (pfu/100 cells)
*c*Its	51 ± 23	35 ± 1	35.5 ± 0.5	3.2 ± 0.8
*c*Its *OB1*	58 ± 37	35 ± 1	35.8 ± 0.6	2.6 ± 0.6

determined as described above (Section 4.B.1.1.), except that cultures were grown at 30°C in LB alone. 186 c*I*ts and 186 c*I*ts *OB1* lysogens were each found to have an average doubling time of ~36 minutes (see Figure 4.3B); indicating that the *OB1* mutation had no significant effect on the growth rate of the lysogenic cell.

186 lysogens revert spontaneously to lytic development at a low frequency, resulting in a low level of progeny phage in the supernatant of a lysogenic culture. The level of free phages in a culture depends on how often induction has occurred (i.e. the stability of the lysogen) and the size of the phage burst, and the rates of readsorption and other loss of phage particles. It seemed unlikely that low B protein expression from the prophage could affect stability of the lysogen, but to be certain the levels of free phages in E251(186 c*I*ts) or E251(186 c*I*ts *OB1*) log-phase cultures grown at 30°C, were compared. As shown in Figure 4.3B, cultures of each lysogen had a similar level of free phages in the supernatant of ~3 phages per 100 cells. (Similar results were seen with overnight cultures of these strains – data not shown.) Since the *OB1* mutation did not disrupt lytic development of the phage (see Section 4.B.2.2.) and should not affect phage adsorption, these results imply that it also had no effect on stability of the prophage.

Thus, the levels of B protein produced from an unrepressed *B* promoter on the 186 prophage did not significantly activate a reporter of the late promoter, *pV*, or affect growth or stability of the lysogen. I next considered whether the CI bound to *pB* could be interacting with other CI binding sites to assist lysogenic development of the phage.

4.B.3. Do CI multimers bind cooperatively to operators at *pB* and *pR*?

4.B.3.1. Repression of pR *by 186 CI protein is unaffected by* pB *operators 3kb away.*

It is possible that CI multimer binding to the *pB* sequence allows a cooperative interaction with CI operators at *pR*/*pL* in order to enhance repression of the early lytic promoter, *pR*, during establishment of a 186 lysogen. To examine this possibility, I wished to assay the response of the *pR* promoter to increasing levels of CI protein, in the presence of the wildtype *pB* operators positioned an appropriate distance away. I therefore adapted the method of Dodd *et al.* (2001) to create a λ*pR.lacZ* reporter with the *pB* operator sequence downstream of the *lacZ* gene and at a distance of ~3.8kb from *pR*. The steps involved are described briefly here and explained in detail in Figure 6.1.

The plasmid and lambda phage reporter vectors of Simons *et al.* (1987), or modified versions of these, were used in this process, and the presence of wildtype *lacZ* sequences were screened by plating on X-gal for either blue colonies (for reporter plasmids) or blue plaques (for reporter phages) in a Lac^- host. Briefly, the CI operator sequence from *pB*, *OB*, (from –57 to +50 of the promoter) was cloned within the *lacYA* genes of the pRS308 reporter vector of Simons *et al.* (1987), which carries a deleted *lacZ* gene (*'lacZ*). A λRS45 phage carrying a transcriptional fusion of a strong promoter, *ptet*, to the *lacZYA* operon was plated on cells carrying the pRS308-Δ*YA.OB* plasmid, and recombinant phages were screened for the presence of the *'lacZ* gene and Δ*YA.OB* sequences. The resultant phage, λRS45Δ*YA.OB* was recombined with a reporter plasmid carrying a transcriptional fusion of the 186 *pR* promoter and *lacZ* gene, to give the λ*pR.lacZ.OB* phage. The *pR* promoter fragment used includes all 186 CI operators over the switch region, as shown in Figure 4.4A, to replicate the wildtype situation, and carries *cI*ts and *apl*am mutations. This process was performed using wildtype *pB* operators, OB^+, or *OB1* mutant sequence, and the *B* terminator sequence was included upstream of the operators to reduce *lacZ* transcription over the CI binding sites.

Lysogens of the resultant reporter phage – λ*pR.lacZ.*OB^+/*OB1* (see Figure 4.4A) – in the NK7049 host were transformed with the p*lac-cI* expression plasmid pZC320-cI and the compatible LacI plasmid pUHA1, to replicate the system used in previous assays of *pR* repression (Dodd and Egan, 2002). Activity of the *pR* reporter was then assayed in the presence of increasing levels of CI induced with IPTG. Assays were also performed on cells carrying the pZC320 parental vector to control for effects of IPTG on LacZ production. A λ*pR.lacZ* reporter, which lacks the *pB* operator sequence (supplied by Ian Dodd), was also assayed for CI protein repression at the same time to compare the results with those of previous experiments (Dodd and Egan, 2002).

In the absence of the *OB* sequence, the 186 *pR* reporter gave ~1000 LacZ units without IPTG, repressed down to ~3 units with the highest concentration of IPTG (and therefore CI protein) used – see Figure 4.4B. Some variation in *pR* activity was seen with each ITPG concentration, as shown by 95% confidence limits, but this is not unusual when measuring high promoter activity with this assay system. For cells carrying the pZC320 control plasmid, activity of each of the *pR* reporters was not significantly changed by the addition of IPTG (data not shown).

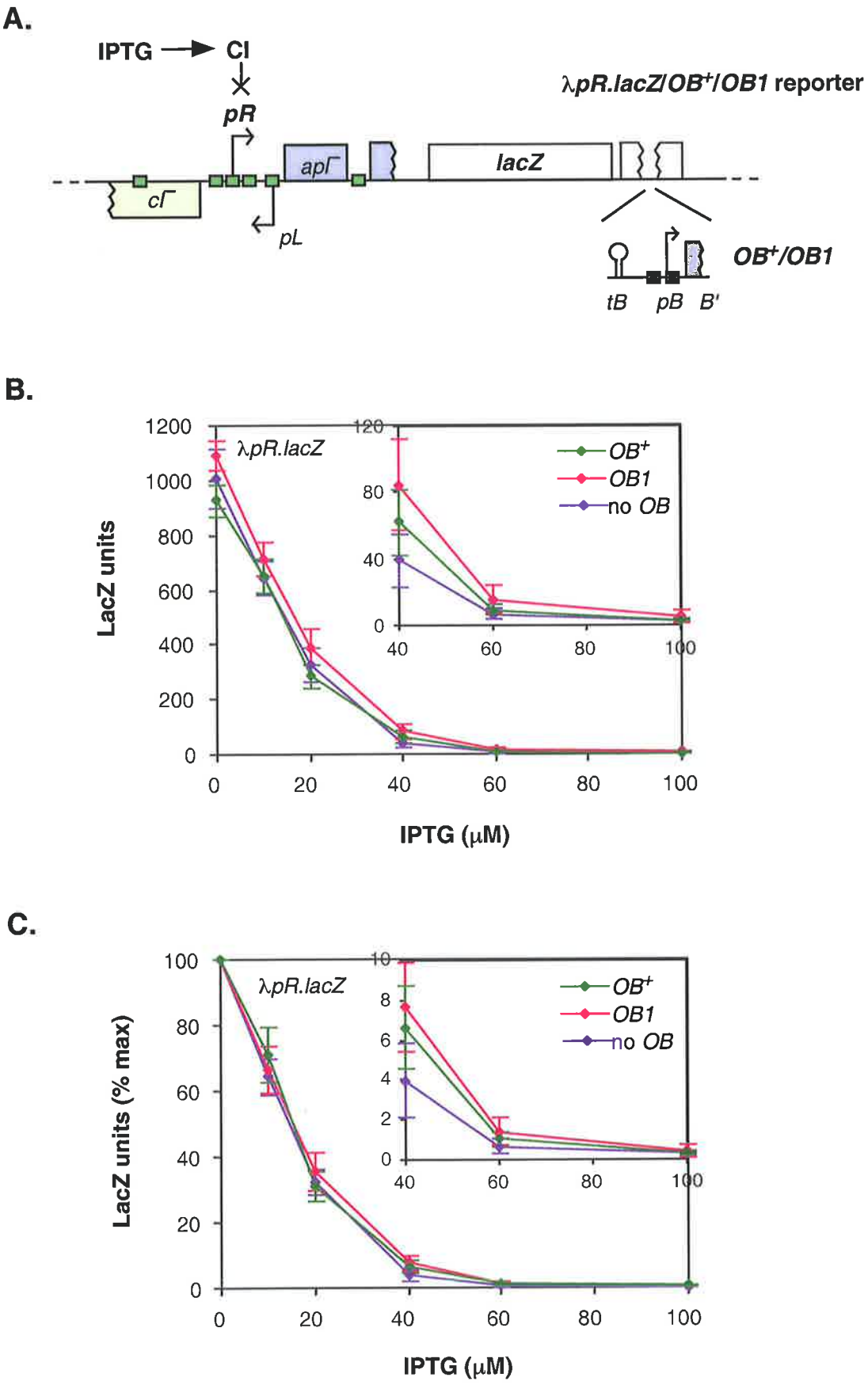
Figure 4.4. CI regulation of the *pR* promoter in the presence of the *pB* operators.

The reporter system used to measure *pR* activity in the presence of the *pB* operators is given in **A.** A 186 fragment which included the *pR* and *pL* promoters (shown by arrows), CI operators (green boxes) and *cI*ts and *apl*am mutations, was fused to the *lacZ* gene on a reporter phage carrying or lacking the *pB* operator sequence (*OB*), as described in detail in Figure 6.1. When present, the wildtype or *OB1* mutant *B* promoter sequence, preceded by the *B* terminator (*tB*), was inserted into the *lacYA* genes downstream of *lacZ*. CI protein expression was induced with IPTG from *plac* on the pZC320-cI plasmid, in the presence of LacI produced by pUHA1.

B. shows the results of microtitre plate LacZ assays on single lysogens of the λ*pR.lacZ*, λ*pR.lacZ.OB*$^+$ or λ*pR.lacZ.OB1* reporter phages in NK7049, transformed with the pZC320-cI and pUHA1 plasmids and grown in IPTG at the concentration indicated. Average LacZ units from *pR*, with 95% confidence limits, are shown plotted against IPTG concentration, for assays performed on 18 colonies for reporters carrying the wildtype or mutant *OB* sequence, or 6 colonies for the reporter lacking *OB*. The inset shows a magnification of *pR* activity with IPTG concentrations above 40μM.

LacZ activity of each *pR* reporter at each IPTG concentration, as measured in **B.,** was expressed as a percentage of the maximum activity seen for that colony, with 0μM IPTG. Averages for each reporter are shown against IPTG concentration in **C.**, with 95% confidence limits as error bars. The inset shows a magnification of the graph for 40μM or more IPTG.

Figure 4.4


A.
IPTG
CI
pR
λpR.lacZ/OB+/OB1 reporter
apr
lacZ
cI
pL
OB+/OB1
tB
pB
B'
B.
λpR.lacZ
OB+
OB1
no OB
LacZ units
IPTG (μM)
C.
λpR.lacZ
OB+
OB1
no OB
LacZ units (% max)
IPTG (μM)

LacZ expression from *pR* was slightly lower in the presence of the wildtype *OB* operator sequence compared to the *OB1* sequence, in the absence of IPTG and with increasing levels of CI protein (Figure 4.4B). However, when the activity of each reporter was expressed as a percentage of the maximal promoter expression, with 0μM IPTG, no clear effect of the *pB* operator sequence was seen (Figure 4.4C). Therefore, no significant enhancement of *pR* repression by the 186 CI protein was seen in the presence of the operators from *pB* situated ~3.8kb from the promoter. This implies that if cooperative binding of a CI multimer to the switch region and *pB* sequence occurs in the phage, it does not affect regulation of *pR* transcription by the immunity repressor.

4.B.4. The effect of B protein expression on the establishment of 186 lysogens.

4.B.4.1. Measuring the frequency of lysogeny of the 186 cIts *phage.*

Removal of CI repression of *pB* appeared to have no effect on a 186 lysogen once it was established, and the CI operators at *pB* did not assist *pR* repression by CI (which is required for establishment of lysogeny), yet the *OB1* mutation did appear to affect the turbidity of phage plaques. I therefore wondered if CI repression of B production could be important in the establishment of 186 lysogens.

Establishment of lysogeny can be measured by determining the 'yield of lysogens' – the number of lysogens formed as a percentage of the total number of phages added – or the 'frequency of lysogeny' – the percentage of the total number of infections which give rise to lysogens. The wildtype 186 phage gives a yield of lysogens of around 7% on a C600 host (Dodd *et al.*, 1993) in assays performed at 37°C – at which temperature 186^{+} phages infect ~25% of cells (Ian Dodd, personal communication). The 186 *c*Its *OB1* phage is only able to form lysogens at permissive temperatures such as 30°C, when the CIts protein is active, therefore, the method of Dodd *et al.* (1993) was adapted to measure the establishment of lysogeny at this temperature (see Section 6.B.2.9.).

In three assays at 30°C on the E251 strain (used for phage assays in this thesis), 186 *c*Its phages infected ~15% of cells, and gave an average yield of lysogens of 2.3% and frequency of lysogeny of 14.5% (see Figure 4.5). Similar adsorption and yield of lysogens results were seen with 186 *c*Its phages on C600 cells at 30°C (data not shown), suggesting that the differences in these results compared to those of Dodd *et al.* (1993) were due to the lower temperature used. 186 *c*Its also gave a 2-3-fold higher frequency of lysogeny on a

C600 host compared to that on the E251 host (at 30°C), presumably due to some differences between these strains.

4.B.4.2. Unrepressed B protein expression does affect the establishment of lysogeny in 186.

The yield of lysogens and frequency of lysogeny of the 186 c*I*ts *OB1* phage on E251 at 30°C were then assayed in the same way as for 186 *cI*ts. Surprisingly, the *OB1* mutation did have a significant effect on the formation of lysogens of the phage. Overall, from three independent assays, the yield of lysogens of 186 c*I*ts *OB1* was reduced by an average of 2.6-fold, and the frequency of lysogeny reduced by 3.3-fold compared to 186 c*I*ts (see Figure 4.5). The number of 186 c*I*ts *OB1* lysogens formed was also reduced ~5-fold compared to 186 *cI*ts in the host strain C600, indicating that this was not a strain-specific effect (data not shown.)

To determine if the effect of the *OB1* mutation on the formation of 186 lysogens was specifically due to unrepressed expression of the late activator, the frequency of lysogeny of 186 *cI*ts phages was determined on E251 cells carrying a B-expression plasmid. B protein was supplied from the pZC-B1 or pZS*-B plasmids, which express B from the wildtype *pB* promoter and give 0.8 or 30 LacZ units above background, respectively, expressed from a chromosomal reporter of the 186 late promoter, *pV* (see Section 3.B.4.3.). As a control, experiments were also performed on cells carrying equivalent plasmids encoding the *B*am17 gene, which fails to express active B protein in the non-suppressor host, E251. The presence of either plasmid carrying a wildtype or mutant B gene gave a slight (up to two-fold) increase in the level of adsorption of the 186 *cI*ts phage. Low levels of B, from pZC-B1, had a small effect on the formation of 186 *cI*ts lysogens, reducing the frequency of lysogeny and yield of lysogens by 1.7-or 1.8-fold, respectively, compared to the *B*am17 control. (Similar results were seen with the pZF-B plasmid, which also expresses a low level of B protein from a single copy of the native *B* gene – data not shown.) Higher levels of B protein, which were able to fully complement a 186 B^- phage burst in previous assays (see Section 3.B.2.3.), had a larger effect on the number of 186 *cI*ts lysogens established. The frequency of lysogeny and yield of lysogens of 186 *cI*ts were, respectively, 4.1- or 3.5-fold lower in the presence of the pZS*-B plasmid, compared to cells carrying pZS*-Bam.

Thus, unrepressed B protein expression from the 186 phage reduced the establishment of 186 lysogens by about 3-fold – providing a purpose for the repression of B transcription by

Figure 4.5. The frequency of lysogeny of 186 *cI*ts in the presence of B protein expressed from the phage or a plasmid.

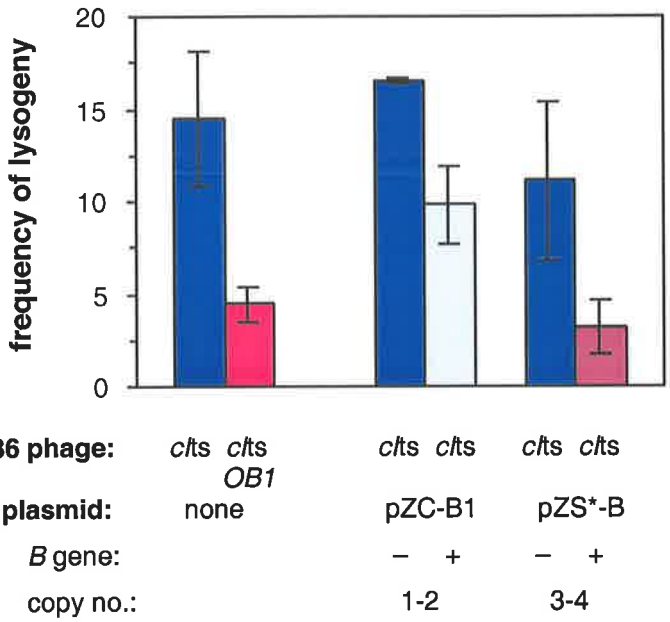
The frequency of lysogeny and yield of lysogens of 186 *cI*ts or 186 *cI*ts *OB1* phages were determined at 30°C on E251 cells carrying or lacking the indicated B plasmid (as described in Section 2.B.2.9.). Average values (± standard deviations) are shown in **A.** and displayed graphically in **B.**, for assays performed on 3 different days on wildtype E251, or on 2 or 3 days for E251 cells carrying a B or *B*am17 plasmid. The expected copy numbers of the plasmids are also given (as described in Figures 3.3 and 3.7). The fold reduction in the frequency of lysogeny or yield of lysogens of 186 *cI*ts compared to 186 *cI*ts *OB1*, and of 186 *cI*ts in the presence of a plasmid expressing wildtype B compared to the *B*am17 mutant, was also determined for assays on the same day. Average fold reduction values with standard deviations are given in **A.**

Figure 4.5

A.

186	B plasmid plasmid	copy no.	frequency of lysogeny	fold reduction	yield of lysogens	fold reduction
*c*Its	none		14.5 ± 3.7		2.3 ± 1.8	
c*Its *OB1	none		4.4 ± 1.0	**3.3** ± 0.2	1.1 ± 0.7	**2.6** ± 1.5
*c*Its	pZC-Bam1	1-2	16.5 ± 0.1		4.0 ± 0.5	
***c*Its**	**pZC-B1**	1-2	9.8 ± 2.2	**1.7** ± 0.4	2.3 ± 0.1	**1.8** ± 0.2
*c*Its	pZS*-Bam	3-4	11.1 ± 4.2		3.5 ± 1.7	
***c*Its**	**pZS*-B**	3-4	3.2 ± 1.5	**4.1** ± 1.5	1.2 ± 0.7	**3.5** ± 1.6

B.



the 186 lysogenic repressor protein, CI. B protein alone when expressed from a plasmid showed a similar, and concentration-dependent, negative effect on the formation of 186 lysogens.

4.C. DISCUSSION.

In this chapter, I determined a function for binding and repression of the *B* promoter by the 186 lysogenic repressor, by investigating the effects of unrepressed B protein expression from the phage or a plasmid, on different stages of lysogenic development.

When B protein was expressed from a plasmid, relatively high concentrations of activator protein (giving between 20 and 100 LacZ units from a chromosomal *pV* reporter) increased the generation time of cells lysogenic for 186 (Figure 4.1), suggesting that these levels of B stimulated harmful late gene expression from the prophage. However, when these B protein levels were expressed in non-lysogens, the growth rate of the cells was also reduced, though to a lesser extent. This effect may have been due to non-specific DNA binding by the late activator protein or perhaps the formation of some insoluble aggregates, which accumulate when B protein is over-expressed (Pountney *et al.*, 1997). However, lower levels of B, which gave <10 units from a *pV.lacZ* reporter, and were similar to those expected if B was expressed from the prophage, had no effect on the doubling time of lysogenic (or non-lysogenic) cells.

To determine the effect of unrepressed B expression from the phage on lysogenic development, a CI operator mutation was made and transferred to the phage. The *OB1* mutation, caused by changing all basepairs in one halfsite of a single CI operator at *pB* to the complementary sequence, increased basal activity of *pB* slightly but abolished repression of the promoter by lysogenic levels of CI protein (Figure 4.2).

186 *cI*ts phages carrying the *OB1* mutation showed normal lytic phage development (Figure 4.3) and gave turbid plaques at a permissive temperature, suggesting that the mutation did not prevent lysogens forming. Lysogens of the mutant phage also showed no reduction in growth or stability, and the activity of a chromosomal *pV.lacZ* reporter in a mutant lysogen showed no increase in late promoter expression from the prophage compared to a wildtype lysogenic cell (Section 4.B.2.3.). However, assays of the frequency of 186 *cI*ts or 186 *cI*ts *OB1* lysogen formation showed that the number of lysogens established after infection was reduced ~3-fold by the mutation (Figure 4.5). This reduction

in lysogen formation was apparently due to unrepressed B protein expression from the *OB1* phage, since B protein alone produced by a plasmid had a similar effect on an infecting 186 phage.

Interestingly, B expressed from the *OB1* phage had an approximately 2-fold larger effect on lysogen formation, than B protein produced from a single copy of the native gene, suggesting that more B protein was made from the *OB1* phage. This could have been due to the ~1.5-fold increase in basal activity of *pB* caused by the *OB1* mutation. Alternatively, the B protein may have been expressed from more than one copy of the *OB1* phage genome soon after infection, due to some phage replication occurring prior to complete establishment of the lysogenic state.

It seemed possible that cooperative interaction of a CI multimer with operators at *pR* and *pB* could improve *pR* inhibition, and that removal of this interaction could cause reduced establishment of 186 lysogens. However, the presence of the CI operators from *pB* downstream of the *lacZ* gene on a chromosomal reporter of *pR* activity, had little effect on the response of the *pR* promoter to increasing levels of CI protein (Figure 4.4). In this experiment the *pB* operator sites were placed downstream of *pR*, in tandem and ~3.8kb away, however in the phage prior to integration ~2.5kb separates the CI binding sites at *pR* and *pB* and the *pB* promoter is upstream of *pR* in tandem. Conceivably, CI protein could interact with both operator sites in the phage, but be prevented from doing so in the reporter system by the larger distance and different orientation of the operators, but this seems unlikely.

It is also possible that the *pB* operators specifically affect CI autoregulation of *pL*, which was not assayed here, but this seems unlikely for the following reasons. CI positively regulates its own promoter, *pL*, by repressing interfering transcription from *pR* (Dodd & Egan, 2002); and this repression was unaltered in the presence of the *OB* sequence. Negative autoregulation of *pL* by CI is expected to be most important to tightly maintain the concentration of CI protein in a 186 lysogen (Dodd and Egan, 2002). It seems unlikely that CI bound at *pB* could affect negative regulation of *pL* by CI in a lysogen, since the *pB* and *pR*/*pL* operators are at opposite ends of the prophage, ~30kb apart. In addition, when CI binding to *pB* was removed in the lysogen, by the *OB1* mutation, the stability of the prophage was unchanged, implying that a normal level of CI protein was present. To

confirm this reasoning however, the response of a *pL.lacZ* reporter to CI protein could also be determined in the presence of the *OB* (or *OB1*) sequence downstream of *lacZ*.

Thus, it seems that the 186 CI protein represses the *B* promoter primarily to prevent the expression of B protein which disrupts the normal formation of 186 lysogens.

It is not known at this stage exactly how B protein reduces the number of cells forming lysogens after 186 infection. This effect could be due to the death of cells which would otherwise enter lysogeny, due to B activation of a late function whose expression is lethal to the cell. If this is the case, even low levels of this protein, from one or perhaps a few copies of the phage genome, must ultimately result in death of the cell. Obvious candidates for this function are the lysis proteins of the phage. The lethal effect of high levels of B protein expressed from a plasmid in a 186 lysogenic cell can also be prevented by amber (and polar) mutations in the *V* operon (Ravi Tiwari, unpublished). Therefore, to test whether the lysis genes or other genes of the *V* operon are responsible for the B-dependent reduction in the establishment of 186 lysogens, mutants could be assayed to see whether their lysogenization is insensitive to excess B.

Alternatively the B protein could be having an unexpected effect on the lytic/lysogenic decision – perhaps by activating an additional promoter in the switch or early lytic region which results in a higher proportion of cells going lytic. The finding of *p32* as a B-activated promoter in 186, despite having only a poor match to a weight matrix of B recognition sequences (Section 3.C.), suggests that there could be other B binding sites in the 186 genome (perhaps upstream of other B-activated promoters) that do not score well using this technique. These promoters would be difficult to identify from the sequence, as they would also be expected to have poor matches to the RNAP sigma70 consensus –10 and –35 recognition sequences. To test for B-activated promoters, different sections of the switch region or early lytic region could be fused to a *lacZ* reporter and activation of transcription determined in the presence of B protein. If purified B protein could be obtained, *in vitro* transcription of phage DNA could also be assayed in the presence of B to look for B-responsive promoters, and activator binding could be confirmed by gel-shift assays on these sequences. In addition, the effect of B protein on the regulation of the 186 lytic/lysogenic switch region could be determined. The activity of the *pR* and *pL* promoters and their response to CI protein could be assayed in the presence of B, and the influence of B on CII regulation of the switch could also be determined.

CHAPTER 5:

FINAL DISCUSSION

The aims of this work were to investigate several unexplained aspects of regulation of morphogenetic gene transcription during infection by the coliphage 186. Three main projects were explored in the process, as described in Chapters 2, 3 and 4. These were, respectively: characterization of four bacterial mutants showing reduced B-dependent activation of 186 late genes; investigation of the role of replication in controlling the timing of late gene expression during phage lytic development; and examination of the role of the 186 lysogenic repressor protein, CI, in binding to and inhibiting transcription from the *B* promoter. The findings of these investigations are summarized here and discussed in terms of late gene regulation in the phage as a whole. Comparison is also made with late gene regulation in two other double-stranded DNA coliphages, the homologous phage, P2 and the unrelated phage, lambda.

5.A. HOST REQUIREMENTS FOR B ACTIVATION OF THE 186 LATE PROMOTERS.

5.A.1. CHARACTERIZATION OF FOUR BACTERIAL MUTANTS WITH REDUCED B ACTIVITY.

My initial aim was to characterize four bacterial mutants previously isolated in this laboratory by Ravi Tiwari (unpublished) which showed a reduction in B-activated expression of lethal 186 late genes in a plasmid-based selection system, and were unable to support a 186 phage infection. The presumption was that these two phenotypes were due to the same mutation.

Here, I found that resistance to 186 infection in these strains was conferred by mutations mapping in the *waa* genes required for biosynthesis of the cell membrane lipopolysaccharide (LPS) structure (Figure 2.2). These mutations did not in fact reduce B activity (Figure 2.3B), but instead appeared to prevent phage infection by removing the cell surface receptor for phage adsorption. Plating of 186 and P2 phages on several known *waa* gene mutants indicated that the second and third glucose residues of the LPS outer core are important for adsorption of both phages, and that 186 also requires the side-chain galactose of the outer core (Figure 2.4).

I also demonstrated that B activation of the plasmid-encoded 186 late genes in the original mutant strains, was prevented by a second chromosomal mutation. Further experiments showed that the apparent reduction in B activity in these cells could be explained by a decrease in the copy number of the plasmids involved (Figure 2.5), due to a mutation in the *pcnB* gene of each strain (Figure 2.6). Thus, no novel host interactions required for B activity were found by this selection system.

5.A.2. B PROTEIN INTERACTS WITH THE RNAP ALPHA-SUBUNIT TO ACTIVATE TRANSCRIPTION.

It was initially suggested that the 186 B protein differed from the homologous late activator protein of P2, Ogr, by not requiring an interaction with the RNAP α-subunit for activation of transcription. This idea was proposed because the 186 Hy5 phage, which carries the 186 late activator and immunity region of 186 and late genes of P2, was able to plate on the *rpoA*109 mutation, which prevented Ogr activity and P2 infection (Sauer, 1979). However, preliminary assays using 186 late promoter reporters showed some reduction in B activity in this strain (Tina Rathjen, unpublished), and the *rpoA*109 mutant was therefore reexamined here in detail. B-dependent transcription from a *lacZ* reporter of the 186 late promoter, *pV*, was reduced to only 2% of wildtype activity by the *rpoA*109 mutation; and 186^+ phages plated with a lower efficiency (~0.4) and gave smaller plaques on the *rpoA* mutant compared to a wildtype strain (Figure 2.7). To determine if the B protein contacts different residues of alpha compared to P2 Ogr, a library of *rpoA* mutants could be assayed for their effect on B activation of a 186 late promoter reporter, or 186 phage plating. Compensatory substitutions in the B protein could then be isolated to determine which amino acids of the activator protein are involved.

Therefore, the 186 late activator protein, like several of its homologues, requires an interaction with the RNAP α-subunit to increase initiation of transcription at target promoters.

5.B. REGULATION OF THE TIMING OF LATE GENE EXPRESSION DURING 186 LYTIC DEVELOPMENT.

5.B.1. PHAGE REPLICATION INCREASES B ACTIVITY DURING 186 LYTIC DEVELOPMENT.

The second aim of my thesis was to determine how 186 late promoter activation is delayed despite the late activator protein being transcribed very early during the phage infection; and what the role of 186 replication is in this regulation.

Primer extension experiments have shown that, although transcripts of the *B* gene can be readily detected as early as 2.5 minutes after the start of 186 lytic development, late promoters on the phage are only activated 15-20 minutes later (Richardson, 1993). This startling finding was confirmed here using chromosomal *lacZ* reporters of the *B* promoter and the 186 late promoter, *pV*, during temperature induction of a 186 *cI*ts prophage (Figure 3.1). Prior experiments also suggested that phage replication is important for increased transcription of the late genes (Finnegan and Egan, 1981; Hocking and Egan, 1982c, d; Richardson, 1993) and that this is not due to a requirement for the replication proteins or a replicating template as such (Dibbens and Egan, 1992).

Here I found that simply expressing the 186 late activator from an increasing number of copies of its native gene gave levels of protein which could activate *lacZ* reporters of the 186 late promoters (Figures 3.5 and 3.9) and could give sufficient late gene expression to complement a temperature-induced 186 *cI*ts B^- prophage (Figures 3.4 and 3.8). When the *B* gene was present on a mini-F origin plasmid, expected to be maintained at 1-2 copies per cell, <1 LacZ unit was seen from the late promoter reporters, and the 186 B^- phage burst was considerably delayed. However, if at least three copies of the *B* gene were present in the cell, the 186 late promoters were substantially activated and the induced 186 B^- prophage gave a normal burst. In addition, no autoregulation of the *B* promoter was seen using a chromosomal *lacZ* reporter of *pB* with a range of B protein concentrations (Figure 3.2), and it seems unlikely that the *B* promoter is activated by another phage protein during infection (see Section 1.D.3.1.). These results imply that activation of the 186 late promoters during lytic development requires the enhanced level of B protein that is produced when phage replication increases the number of copies of the *B* gene. This provides a simple way to delay late gene expression until after multiple copies of the phage

genome are present, and also provides an explanation for why replication of the phage is required for increased transcription of the 186 late genes (Finnegan and Egan, 1981).

This dependence of 186 late promoter transcription on B concentration is due to a markedly non-linear response of the late promoters to low levels of B protein (see Figures 3.5 and 3.9). With B expressed from 1-2 copies of the *B* gene chromosomal *lacZ* reporters of the 186 late promoters each gave <1 LacZ unit, while a three-fold increase in the number of *B* genes present resulted in a >3-fold increase in LacZ activity. These results suggest that B activation of the promoters is highly cooperative over this activator concentration range, presumably due to binding of a multimeric protein complex in order to stimulate initiation of transcription (see Section 1.C.4.3.). This cooperativity could occur if the B protein multimerizes in solution to give an active, DNA-binding species; or if binding of B monomers to the DNA triggers the formation of an active, multimeric complex on the promoter. If purified, soluble B protein could be obtained, these models could be distinguished by following multimer formation *in vitro* in the presence or absence of DNA using, for example sedimentation equilibrium experiments.

Late promoters of other phages also show non-linear stimulation of transcription by increasing concentrations of the positive regulator protein. These include late promoter activation by the B-related Ogr and Delta proteins of P2 and P4, respectively (Lee and Christie, 1990; Julien and Calendar, 1995), and antitermination of late transcription by the Q protein of lambda (Yang *et al.*, 1987; Kobiler *et al.*, 2005). This may therefore represent a universal mechanism to make late gene expression very sensitive to activator concentration, thus allowing a switch from low to high late gene expression with a small change in activator protein concentration.

5.B.2. THE 186 PHAGE BURST ALSO REQUIRES INCREASED GENE DOSAGE OF THE LATE GENES.

In the lambda phage, early activity of the holin protein results in a severely reduced burst released only a short time after infection (Johnson-Boaz *et al.*, 1994), presumably due to lysis occurring before significant phage replication and assembly of progeny has occurred. A similar result would be expected for the early expression of lysis and structural functions in other phages such as 186. Therefore, the result found here, that premature activation of the 186 late promoters during lytic development is not actually very disruptive to virion production and release is quite surprising. When a level of B protein able to substantially

activate the 186 late promoters (and complement a 186 B^- phage burst) was present from the start of a 186 phage infection, progeny were produced only 5 minutes earlier and at ~70% of the wildtype level (see Figure 3.10). This suggests that even if the 186 late proteins (including lysis functions) are expressed from early during the phage infection their activity is still delayed until an approximately normal time. These results can be explained by the findings of Hocking and Egan (1982a, b, c, d) that the level of an essential late product produced from a single copy of the 186 DNA (present as a non-replicating genome or uninduced prophage) is insufficient to complement infection by a 186 phage defective in that gene. Therefore, an increase in late gene dosage, provided by phage replication during normal 186 lytic development, also seems be required in order to supply high levels of late functions and give a normal 186 phage burst.

Thus, the expression of morphogenetic proteins of 186 appears to be linked to phage replication in two ways to ensure that no premature activity of these proteins occurs. Replication is required to increase the gene dosage of the late activator and supply levels of protein able to stimulate the late promoters; and also to increase the late gene dosage in order to produce sufficient amounts of late functions for normal phage production.

The size of the phage burst appears to be dependent both on the level of late promoter activation, and the timing of cell lysis (which determines the amount of replication of the phage genome). With the pZF-B plasmid, chromosomal *lacZ* reporters of the 186 late promoters showed ~0 LacZ units above a background level of expression (Figure 3.5), and this gave a substantially delayed burst of 186 B^- phage (~40 minutes later than wildtype) of only 1-2 virions per cell (Figure 3.4). Note, however, that late promoter transcription in the presence of pZF-B must actually be increased, since in the absence of this plasmid each cell produces, on average, only ~0.01 186 B^- progeny (Figure 3.4A). Presumably, the level of promoter stimulation with pZF-B is below the detection threshold level of the LacZ assay. When 186 late promoter activity is increased slightly, to ~0.8 LacZ units above background (Figure 3.9), with the pZC-B1 plasmid, the phage burst is still delayed by ~15-20 minutes, but the number of phage produced per cell is actually larger than during induction of a 186 *cI*ts B^+ lysogen (Figure 3.8). This suggests that production of morphogenetic and lysis proteins occurs later than normal – B expression is still sub-optimal – but that high levels of these proteins can be produced, even though promoter activity is very low. The delay in the timing of cell lysis appears to allow additional phage replication, which results in ~3-fold more progeny than during wildtype phage development. With higher levels of B protein

expression, for example, with the pZS*-B and pZC-B3 plasmids, late promoters are activated to a higher level (Figure 3.9), the delay in the timing of cell lysis is reduced, and the number of phage produced decreases to levels similar to wildtype (Figures 3.4 and 3.8), presumably as less phage replication occurs. The level of B protein produced from only 3-4 copies of the native *B* gene actually gives sufficient late promoter activation for a wildtype burst of 186 B^- phages, despite the fact that higher levels of activator can give as much as 10-fold higher late promoter transcription (Figure 3.5). In addition, the level of B protein produced during wildtype 186 lytic development appears to be similar to that made from 3-4 copies of the native *B* gene in the cell, as assayed using a chromosomal *pV.lacZ* reporter (Figure 3.6). This suggests that production of the B protein is restricted during phage development, perhaps due to B and late protein expression occurring concurrently with phage replication, and phage templates being sequestered by packaging into progeny particles.

Plating experiments also show that a large burst of 186 particles is not required to produce plaques on a bacterial lawn overnight. Even when only 1-2 phages were released per cell (as for 186 B^- phage infection of a cell carrying the pZF-B plasmid) plaques were reduced in size but formed with the same efficiency compared to a wildtype infection (Figure 3.3B). A similar result was seen plating 186^+ phages on an *rpoA*109 host (Figure 2.7). This mutation reduced B activity to ~2% of wildtype but still gave a high efficiency of 186 plating albeit with a reduced plaque size. (For comparison, if a chromosomal *pV.lacZ* reporter gives ~30 LacZ units during a wildtype phage infection, 2% of B activity should give ~0.6 units from the *pV* reporter, similar to that seen with the pZF-B or pZC-B1 plasmids.)

The results of complementation experiments performed with the single-copy native *B* expression plasmid, pZF-B, were similar to those seen in previous experiments by Hocking and Egan (1982a, b, c) with a single copy of the *B* gene. When a 186 B^- phage co-infected a cell with a replication-defective 186 phage expressing B, the 186 B^- phage burst produced was ~4% of wildtype, or ~1.7 phages per cell. In addition, a virulent 186 B^- phage gave ~20% of the wildtype level of plaques when plated on an uninducible 186 prophage able to express wildtype B protein – although in this case it is hard to know how much the *pB* promoter on the prophage was derepression.

5.B.3. COMPARISON OF THE FOUR B-ACTIVATED 186 LATE PROMOTERS.

LacZ reporter assays performed here (Figure 3.5) confirmed a preliminary primer extension experiment which suggested that 186 contains a fourth B-activated late promoter, *p32*. This promoter is upstream of the *orf32* gene and is expected to express a number of proteins required for tail formation (Figure 1.2). Upstream of the transcription start site (at 9727 or 9728) are –10 and –35-like sequences that partly match the consensus sequences for RNAP σ^{70} recognition and binding (Figure 1.7). Sequences centred at –56 on the promoter show some similarity in one halfsite to a consensus inverted-repeat sequence for recognition by B-homologous proteins (see Figure 1.8), but were not identified in a weight matrix of B-activated promoter sequences (Portelli *et al.*, 1998).

Interestingly, despite the poor match to the B binding site consensus, *p32* responded to increasing levels of B protein in a comparable way to the other 186 late promoters, *pV*, *p12* and *pJ* (Figure 3.5C). This suggests that each promoter has an equivalent level of occupation at a particular B protein concentration, and therefore, has a similar affinity for B binding, despite considerable variation in the upstream promoter sequences. It also implies that the promoters will be activated in parallel during a 186 phage infection. Although DNaseI footprinting has not been used to confirm the sequences of 186 *pJ* and *p32* which are bound by B protein, it seems likely that the B recognition sites will also be centred at –56 of these promoters, as these regions show the most identity with other sequences bound by B or functionally interchangeable B-homologues. A consensus for recognition by B-like proteins which includes the 186 *pJ* and *p32* promoter sequences does not differ much from one without these promoters (Figure 1.8, discussed in Section 1.C.4.2.).

Despite the equivalent response of the 186 late promoter reporters to increasing levels of B protein, large variation was seen in the absolute level of activity for distinct late promoters at the same activator concentration (Figure 3.5A & B). At the highest B protein concentration used the *pJ* promoter showed the greatest reporter expression, *pV* gave ~80% of this level, *p32* ~50% and *p12* only ~15% of *pJ* activity. Dibbens (1990) had previously noted a similar inequality of transcription from the *pV* and *p12* promoters. It seems likely that these differences do not reflect differences in B binding but in other steps in promoter activity: RNAP binding, contact with B, opening of the DNA helix, or initiation of elongation. Presumably the variation in late transcript levels produced from each promoter reflects a requirement for different levels of the particular morphogenetic functions encoded (though protein levels could also be regulated at later stages of expression,

including message stability and translation). This is readily apparent for some 186 structural genes – for example, the major head protein is expressed from the strongly activated promoter, *pV*, and tail tube and sheath proteins also required in large numbers are transcribed from *pJ*. However, it is not so obvious why the 186 *p32* promoter is required. Transcription from the upstream late promoter, 186 *pV*, would be expected to continue rightward across *orf32* and downstream genes, since no transcriptional terminators separate these regions (see Figure 1.2). The P2 phage has a divergently transcribed gene, *orf30*, immediately upstream of the promoter equivalent to 186 *p32* (see Figure 1.2), which could prevent upstream late transcription from continuing through the *orf32*-analogous operon, and it may be that the 186 promoter is a remnant from a P2-like predecessor phage. However, the 186 *p32* promoter sequence shows little identity with its P2 equivalent, P2 *pV* (see Figure 1.7) – much less than within the 186 *orf32* and P2 *V* genes themselves (Dibbens and Egan, 1992) – suggesting that the promoter has been retained in 186 despite divergence of the nucleotide sequence. Presumably, therefore, the presence of a B-activated promoter upstream of *orf32* and the following genes is specifically required to express the necessary levels of these tail fibre, baseplate and assembly proteins.

The confirmation of 186 *p32* as a B-activated promoter also suggests that there could be other B binding sites in the 186 genome, perhaps upstream of other B-activated promoters. Such promoter sequences may be difficult to identify, as they could score poorly using the weight matrix of Portelli (1998), and would probably show little similarity to the consensus sequences for RNAP sigma70 recognition.

5.C. THE ROLE OF CI REPRESSION OF B TRANSCRIPTION.

The third aim of my project was to determine the purpose of 186 CI protein repression of transcription of the 186 late gene activator, from *pB*.

During lysogenic development of temperate double-stranded DNA phages, the phage genome is generally inserted in single copy into the bacterial chromosome, and a repressor protein or function is produced which inhibits the transcription of lytic genes. This prevents the expression of proteins which may damage the host, for example by initiating phage replication, interacting with the bacterial cellular machinery, or even lysing the cell. Not surprisingly therefore, excessive production of the activator of 186 morphogenetic gene transcription, in a 186 lysogenic cell, can retard the growth of the cell (as seen here) or even result in cell death (Dibbens and Egan, 1992).

However, when the 186 late activator is expressed from a single copy of its native promoter and gene (on a plasmid), little activation of the 186 late promoters is seen (Figures 3.5 and 3.9). In addition, the expression of sufficient late functions to assemble and release progeny phages also appears to require multiple copies of the late genes (see Section 3.B.5.). Therefore, it is not readily apparent why transcription of the late activator gene on a prophage, which is in single copy in the chromosome, is repressed by the 186 CI protein. In order to test this directly, the CI binding site over *pB* was mutated on the phage to remove repression by lysogenic levels of CI and retain a similar level of intrinsic promoter activity (Figure 4.2). The effect of this mutation on different stages of lysogenic development was then assayed. As expected, this mutation did not reduce the growth rate or stability of the lysogen (Figure 4.3), suggesting that any late gene expression from the prophage was insufficient to affect the cell. However, when the frequency of lysogenization of the mutant phage was determined it was found that ~3-fold fewer lysogenic cells were established when CI was unable to prevent *B* gene transcription from the phage (Figure 4.5). Similar results were seen with a wildtype 186 phage and B protein expressed from a plasmid, where the severity of the effect was dependent on the level of activator protein produced. Thus, when a plasmid carrying a single copy of the *B* gene was present, the establishment of 186+ lysogens was reduced ~2-fold, while B from 3-4 copies of the *B* gene reduced lysogen formation by ~4-fold compared to that in the absence of B expression (Figure 4.5). This implies that B protein expression specifically reduces the establishment of 186 lysogens.

CI protein molecules bound to the *pR*/*pL* promoter region and to recognition sites ~300bp away appear to interact by looping out of the intervening DNA, in order to enhance repression of lytic gene expression from the *pR* promoter (see Section 1.B.3.2.). In addition, the CI CTD has been shown to interact over as much as 3.8kb in a reporter assay (Ian Dodd, personal communication). Therefore, it seemed possible that CI recognition sequences at the B promoter could play an additional role in the phage, namely, to interact with CI protein bound to the *pR* operators and improve inhibition of *pR* transcription. However, in experiments performed here, inclusion of the CI operators from *pB* ~3.8kb downstream of the *pR* promoter on a transcriptional *lacZ* reporter, had no significant effect on CI repression of the promoter (Figure 4.4). It therefore seems unlikely that cooperative binding of CI protein to the *pB* and *pR* operators is important for CI repression of *pR*. A

converse effect of the *pR* operators on CI repression of *pB* transcription was not assayed, but also seems unlikely.

How the expression of a very low level of B protein is detrimental to the establishment of a 186 lysogenic cell is not known. It is possible that B plays an unexpected role in influencing whether lytic or lysogenic development will be followed after phage infection. It could do this by interfering with CI expression or repression of the *pR* promoter – perhaps by activating an as yet undiscovered promoter within the switch region which biases the cell towards lytic development, or even by interacting with the CI protein itself to inhibit its activity. To initially investigate these possibilities, B protein binding to this section of DNA could be measured by gel-shift experiments; and the effect of B protein on transcription and CI or CII regulation of the switch region, could be tested using reporter assays.

It seems more likely that low levels of B protein expressed in a 186-infected cell prior to the establishment of a lysogen result in a low level of late gene expression, which ultimately kills the cell. The holin and endolysin proteins, which normally cause cell lysis during infection, are obvious candidates for the lethal phage genes in this case. Mutation of these functions on the phage should therefore also restore the number of 186 lysogens formed in the presence of B protein. Since *B* and late gene expression from a single copy of the 186 genome is not lethal (e.g. from a 186 *cI*ts *OB1* prophage – Figure 4.3), this result also suggests that B is activating late gene expression from multiple copies of the genome, in order to kill a potential lysogen. Thus, replication of the phage genome may occur in some infections which eventually develop lysogenically, prior to CI expression and/or integration of the phage DNA; and if B expression is unrepressed in these infections, sufficient late gene expression may occur to kill the cell. It would therefore be interesting to see if unrepressed B protein expression has any effect on the formation 186 *A*am lysogens in a non-suppressor host, where the phage is unable to replicate due to mutation of the replicase gene, *A*.

5.D. OVERVIEW OF LATE GENE REGULATION IN TEMPERATE PHAGES 186, P2 AND LAMBDA

All bacteriophages face the problem of regulating the expression of structural and lysis phage genes during lytic development such that high levels of a number of proteins are

produced but only late in the phage infection, after replication of the genome has occurred. In coliphage 186, there are three processes that combine to produce a very rapid increase in morphogenetic functions with time, after phage replication has begun: (1) a replication-stimulated increase in late activator levels due to increased gene dosage; (2) an ultrasensitive response of activation of late transcription to increasing activator levels; and (3) a replication-dependent increase in the late gene dosage. The P2 and lambda coliphages also appear to employ most, or all, of these process, suggesting that they may be commonly utilized.

As for 186, replication of the P2 late genes is required to give normal expression of the structural and lysis proteins and phage production. Little P2 late transcription is seen during infection by a non-replicating P2 phage, P2 A^-, (Geisselsoder *et al.*, 1973; Christie and Calendar, 1983), and with A being *cis*-acting, there is insufficient production of late functions from this phage to complement a co-infecting P2 phage defective in an essential late gene (Lindahl, 1970). It was originally suggested that this was due to Ogr protein requiring a replicating template to activate promoter transcription (Bertani and Six, 1988; Birkeland *et al.*, 1991), but this seems unlikely as Ogr can activate a chromosomal reporter of the P4 *psid* promoter to high levels (Reiter *et al.*, 1998). Interestingly, the late promoter activator of the satellite phage, P4, is able to express sufficient P2 or 186 structural genes for P4 progeny formation, from a replication-defective helper phage (Sauer *et al.*, 1982; Lindqvist *et al.*, 1993). Presumably, this is due to the covalent dimeric nature of the P4 Delta protein allowing more efficient activation of the helper phage late promoters. Ogr protein probably also acts in a cooperative manner to activate the P2 late promoters, since a reporter of the P4 *psid* promoter responds non-linearly to increasing Ogr protein levels *in vitro* (Lee and Christie, 1990). However, it has been suggested that replication of the *ogr* gene is not absolutely necessary to enhance activator expression, since Ogr expression from a P2 A^- phage is sufficient to complement a co-infecting phage in which the *ogr* gene has been deleted (Halling and Calendar, 1990; Birkeland *et al.*, 1991). Instead, Ogr protein levels appears to be enhanced later in infection by an unknown early lytic protein which stimulates transcription from the intrinsically weak *pogr* promoter (Birkeland *et al.*, 1991).

For coliphage lambda, antitermination of late transcription by the Q protein also shows ultrasensitivity indicative of cooperative protein action (Yang *et al.*, 1987; Kobiler *et al.*, 2005). It also seems likely that lambda phage replication is important to increase the gene dosage (and therefore protein expression) of the late genes and the Q gene itself. During

infection by a replication-defective lambda phage, only a low level of late transcription was seen (Oda *et al.*, 1969); and infection of a lysogen by a hetero-immune phage defective in a late gene showed little phage production, which increased slightly when two lambda prophages were present (Thomas, 1970). In addition, premature Q activity and late gene transcription (during infection by a lambda CII^- phage – McMacken *et al.*, 1970; Kobiler *et al.*, 2005) had little effect on the size of the phage burst produced (Folkmanis *et al.*, 1977), supporting the idea that late gene replication is important for phage production. Similarly, Q protein expressed from a non-replicating phage gave little stimulation of endolysin protein expression from a co-infecting Q^- phage (Dambly *et al.*, 1968); however, this result was complicated by a low level of Q-independent transcription of the late genes, initiating at the lambda *pR* promoter.

Thus, as originally propsed for lambda in the 1960s (Dove, 1966, 1968) and shown here directly for 186, an ultrasensitive response of late transcription to the late regulator, the dependence of enhanced late transcription on increased levels of the late activator provided by replication and the amplification of late gene dosage provided by replication, ensures that late gene expression in these phages increases sharply and dramatically, after (or during) phage replication. Some of these processes also appear important for expression of late functions in coliphage P2. The mechanism of cooperativity in B and Q (and probably Ogr) action is not fully understood and is a question for future work.

For each of these temperate phage, 186, P2 and λ, replication proteins are encoded by early lytic transcripts (*pR*, *pE* and *pR* respectively) whose expression is directly repressed in a lysogen by the immunity repressor protein (186 CI, P2 C and λ CI). This would prevent increased late gene dosage and therefore significant late gene transcription in the lysogen. Expression of active levels of the late regulator protein from the prophage is also prevented in 186 by the lack of replication, in lambda by the inhibition of *N* gene expression and in P2 by repression of transcription of the putative *pogr* activator. However, some phage replication may occur in cells which are destined to become lysogens, prior to the formation of the lysogen. In this situation, additional mechanisms may be required in order to prevent this leading to potentially lethal late gene expression. In 186, as seen here, direct CI repression of B protein expression is necessary for the normal formation of 186 lysogens. Similarly in lambda, Q production is reduced by the CII protein. Lambda CII activates an oppositely orientated promoter, *paQ*, within the *Q* gene, which inhibits Q expression (Ho and Rosenberg, 1985; Hoopes and McClure, 1985; Stephenson, 1985), and

mutation of the CII binding sites at this promoter results in a clear plaque-forming phage (Kobiler *et al.*, 2005). Establishment of lysogeny is also reduced by lambda phage mutations which result in constitutive Q (and therefore, late gene) production. Low Q expression from a promoter created by the *byp* mutation reduces the frequency of lysogeny by ~3-6-fold (Sternberg and Enquist, 1979; Costantino *et al.*, 1990), while lysogeny is abolished by the *qin101* mutation which forms a highly active promoter upstream of *Q* (Dambly *et al.*, 1968). The formation of λ *qin101* lysogens can be partially restored by additional mutations which disrupt the holin (*R*) and endolysin (*S*) genes; and further mutation of the *A* gene (which encodes the large terminase subunit) results in a wildtype frequency of lysogeny (Dambly *et al.*, 1968). This suggests that the lambda lysis proteins (and possibly other late functions) are responsible for the reduction in lysogen formation seen in the presence of Q protein expression. Ogr expression from P2 does not appear to be directly repressed by the immunity repressor or other mechanisms, so P2 presumably relies on the C protein preventing expression of the *pogr* activator protein during the establishment of P2 lysogens.

These results suggest that formation of a lysogen is a developmental option imposed on a default pathway of replicative development, which will automatically initiate lytic gene expression, leading to replication of the phage genome and production of morphogenetic functions. Expression of the phage lysis genes in particular may initiate an irreversible process leading to the timed death of the cell. In order to successfully establish a lysogen it is therefore be important to prevent expression of the late regulator protein from early during the phage infection. Thus, the extreme sensitivity of late gene expression to phage replication, that allows a large and rapid increase in expression during lytic development, also means that, at least for 186 and lambda, expression of lethal late products must be directly repressed in order for lysogenic cells to be formed.

CHAPTER 6

MATERIALS AND METHODS.

6.A. MATERIALS.

6.A.1. BACTERIAL STRAINS.

Strain	Genotype	Use (Source)	Glycerol no.[a]
C600	*thr*-1 *leuB*6 *thi*-1 *lacY*1 *glnV*44 *rfbD*1 *fhuA*21	Suppressor (*supE*) strain used as a control indicator host in phage assays.	E508
DH5α	*endA*1 *hsdR*17(r_K^- m_K^+) *glnV*44 *thi*-1 *recA*1 *gyrA*96(Nal^R) *relA*1 Δ(*lacZYA-argF*)U169 *deoR* (ϕ80 Δ(*lacZ*)M15	Used for routine cloning. (Bethesda Research Laboratories)	E4241
DPB1562	XL1-blue MRA Δ(*lacZ*)M15	Used as host for cloning of mini-F origin plasmids (pZF-B and pZC-B). (Shi and Biek, 1995)	E4490
E251	*galK*2 *galT*22 IN(*rrnD-rrnE*)1 *strA*748	Non-suppressor strain used as a host for phage assays. (Hocking and Egan, 1982d)	E251
E251 *lacZ*::Km^R	E251 *lacZ*::Tn5(Km^R)	*lacZ*::Tn5(Km^R) from E4481, transduced into E251 recipient strain. Used as a host for some LacZ reporter assays.	R410
E251 *rpoA*109	E251 *zhc*::Tn10(Tc^R) *rpoA*109	E251 carrying the RNA polymerase α-subunit mutation *rpoA*109 (which gives L290H substitution).	E4310
E251 *rpoA*109 *lacZ*::Km^R	E251 *zhc*::Tn10(Tc^R) *rpoA*109 *lacZ*::Tn5(Km^R)	*lacZ*::Tn5(Km^R) from E4481 transduced into E4310 recipient strain.	R435
E251 *rpoZ*::Cm^R	E251 *rpoZ*::Cm^R	*rpoZ*::Cm^R from E4466 transduced into E251 recipient strain.	R381a
E251 *rpoZ*::Cm^R 186^R (HM2)	E251 *rpoZ*::Cm^R 186^R (HM2)	*rpoZ*::Cm^R and 186^R mutation from HM2 *rpoZ*::Cm^R donor strain transduced into E251.	R381
E4412	MG1655 *zia*-3077::Tn10(Km^R)	Used as a donor strain for P1 transduction mapping of 186^R mutation in Host Mutants. (Singer *et al.*, 1989)	E4412

E4419	MG1655 *zid*-501::Tn10(Tc^R)	Used as a donor strain for P1 transduction mapping of 186^R mutation in Host Mutants. (Singer *et al.*, 1989)	E4419
E4465	MG1655 Δ*gltS*::Km^R	Used as a donor strain for P1 transduction mapping of 186^R mutation in Host Mutants.	E4465
E4466	W3110 *rpoZ*::Cm^R	Used as a donor strain for P1 transduction of *rpoZ*::Cm^R to E251 and HM2-6 strains.	E4466
E4479	*cysE*::Tn5(Km^R) *araD*139 *lac*4169 *lam malE*1 *lacZ fus*72	Used as a donor strain for P1 transduction mapping of 186^R mutation in Host Mutants.	E4479
E4481	*galK lacI*^{q} Str^R *lacZ*::Tn5(Km^R)	Used as a donor strain for P1 transduction of *lacZ*::Tn5(Km^R) to E251 and HM2-6 strains. (K3093 – Retallack *et al.*, 1994)	E4481
HM2		Host Mutant 2 – mutagenized E251 strain resistant to Kill plasmid selection and 186 infection. Cured of plasmids. (Ravi Tiwari, unpublished – see Section 2.A.1.2.)	E4469
HM2 *lacZ*::Km^R	HM2 *lacZ*::Tn5(Km^R)	*lacZ*::Tn5(Km^R) from E4481 transduced into HM2 strain.	R414
HM2 *rpoZ*::Cm^R	HM2 *rpoZ*::Cm^R	*rpoZ*::Cm^R from E4466 transduced into HM2 recipient strain, which remains resistant to 186 infection.	R378
HM2 *rpoZ*::Cm^R 186^S	HM2 *rpoZ*::Cm^R 186^S	*rpoZ*::Cm^R from E4466 transduced into HM2 recipient, to give a strain no longer resistant to 186 infection.	R382
HM3		Host Mutant 3 – isolated as for HM2.	E4470
HM3 *lacZ*::Km^R	HM3 *lacZ*::Tn5(Km^R)	*lacZ*::Tn5(Km^R) from E4481 transduced into HM3 strain.	R419
HM3 *rpoZ*::Cm^R 186^S	HM3 *rpoZ*::Cm^R 186^S	*rpoZ*::Cm^R from E4466 transduced into HM3 recipient, to give a strain no longer resistant to 186 infection.	R383
HM4		Host Mutant 4 – isolated as for HM2.	E4471
HM4 *lacZ*::Km^R	HM4 *lacZ*::Tn5(Km^R)	*lacZ*::Tn5(Km^R) from E4481 transduced into HM4 strain.	R421

HM4 *rpoZ*::Cm^R 186^S	HM4 *rpoZ*::Cm^R 186^S	*rpoZ*::Cm^R from E4466 transduced into HM4 recipient, to give a strain no longer resistant to 186 infection.	R384
LE392	e14$^-$ (McrA$^-$) *hsdR*514(r_K^- m_K^+) *glnV*44 *tyrT*58 Δ(*lacIZY*)6 *galK*2 *galT*2 *metB*1 *trpR*55	Used to purify plasmids to allow transfer from m_K^- strains (e.g. DPB1562) to r_K^+ strains (e.g. E251).	E519
MC1061	*araD*139 Δ(*ara-leu*)7697 Δ(*lacIZYA*)X74 *galE*15 *galK*16 *hsdR*2(r_K^- m_K^+) *mcrB*1 *mcrA* *rpsL*150	Used as a host for routine cloning and *in vivo* recombination of promoter-*lacZ* fustions.	E901
NK7049	Δ(*lacIZYA*)X74 *galOP*308 *rpsL*	$RecA^+$ Lac^- strain used as a host for *in vivo* recombination of promoter-*lacZ* fustions, and LacZ reporter assays. (Simons *et al.*, 1987)	E4300
waaB::Tn5	*waaB(rfa*B7*)*::Tn5(Km^R *lacZ*$^-$) Δ*lac thr*-1 *araC*14 *leuB*6 Δ(*gpt-proA*) *lacY*1 *tsx*-33 *qsr*'-0 *glnV*44 *galK*2 Rac-0 *hisG*4 *rfbD*1 *mgl*-51 *rpoS*396 *rpsL*31 *kdgK*51 *xylA*5 *mtl*-1 *argE*3 *thi*-1	Non-polar Tn5 insertion into *waaB* (formerly *rfaB*) gene. Used to characterize LPS requirements for 186 infection. (CS2274 – Pradel *et al.*, 1992) (Obtained from John Klena, University of Auckland, New Zealand)	E4510
waaL::Tn5	*waaL*::Tn5(Km^R *lacZ*$^-$) *thr*-1 *araC*14 *leuB*6 Δ(*gpt-proA*) *lacY*1 *tsx*-33 *qsr*'-0 *glnV*44 *galK*2 Rac-0 *hisG*4 *rfbD*1 *mgl*-51 *rpoS*396 *rpsL*31 *kdgK*51 *xylA*5 *mtl*-1 *argE*3 *thi*-1	Non-polar Tn5 insertion into *waaL* (formerly *rfaL*) gene. Used to characterize LPS requirements for 186 infection. (CS2334 – obtained from John Klena, University of Auckland, New Zealand)	E4512
waaO:: Tn5	*waaO(rfaI*23*)*::Tn5(Km^R *lacZ*$^-$) *thr*-1 *araC*14 *leuB*6 *lacY*1 *tsx*-33 *glnV*44 *galK*2 *hisG*4 *rpsL*31 *xylA*5 *mtl*-1 *argE*3 *thi*-1 *proA*2 *recB*21 *recC*22 *sbcB*15 *sbcC*201 Δ(*arg-lac*)U619 Δ(*trpEA*)2	Non-polar Tn5 insertion into *waaO* (formerly *rfaI*) gene. Used to characterize LPS requirements for 186 infection. (CS2327 – Pradel *et al.*, 1992) (Obtained from John Klena, University of Auckland, New Zealand)	E4511

waaQ:: Tn5	*waaQ*::Tn5(Km^R) Δ*lac thr*-1 *araC*14 *leuB*6 Δ(*gpt-proA*) *lacY*1 *tsx*-33 *qsr*'-0 *glnV*44 *galK*2 Rac-0 *hisG*4 *rfbD*1 *mgl*-51 *rpoS*396 *rpsL*31 *kdgK*51 *xylA*5 *mtl*-1 *argE*3 *thi*-1	Non-polar Tn5 insertion into *waaQ* (formerly *rfaQ*) gene. Used to characterize LPS requirements for 186 infection. (CS2774 – Klena *et al.*, 1992) (Obtained from John Klena, University of Auckland, New Zealand)	E4492
waaS:: Tn5	*waaS*::Tn5(Km^R) Δ*lac thr*-1 *araC*14 *leuB*6 Δ(*gpt-proA*) *lacY*1 *tsx*-33 *qsr*'-0 *glnV*44 *galK*2 Rac-0 *hisG*4 *rfbD*1 *mgl*-51 *rpoS*396 *rpsL*31 *kdgK*51 *xylA*5 *mtl*-1 *argE*3 *thi*-1	Non-polar Tn5 insertion into *waaS* (formerly *rfaS*) gene. Used to characterize LPS requirements for 186 infection. (CS2775 – Klena *et al.*, 1992) (Obtained from John Klena, University of Auckland, New Zealand)	E4493
XL1-Blue	F'::Tn10 *proA*$^+$*B*$^+$ *lacI*^{q} Δ(*lacZ*)M15/ *recA*1 *endA*1 *gyrA*96(Nal^R) *thi hsdR*17(r_K^- m_K^+) *glnV*44 *relA*1 *lac*	Used for routine cloning. (Stratagene)	E4240
Δ*waaL*	C600 Δ*waaL*	In-frame deletion of *waaL* gene. Used to characterize LPS requirements for 186 infection. (Renato Morona, University of Adelaide)	R753
Δ*waaOB*	C600 Δ*waaOB*	In-frame deletion of *waaOB* genes. Used to characterize LPS requirements for 186 infection. (Renato Morona, University of Adelaide)	R750
Δ*waaR*	C600 Δ*waaR*	In-frame deletion of *waaR* gene. Used to characterize LPS requirements for 186 infection. (Renato Morona, University of Adelaide)	R751
Δ*waaU*	C600 Δ*waaU*	In-frame deletion of *waaU* gene. Used to characterize LPS requirements for 186 infection. (Renato Morona, University of Adelaide)	R752

[a] E* glycerol numbers refer to Egan lab stocks, R* glycerol numbers refer to personal lab stocks

6.A.2. BACTERIOPHAGE.

Phage	Description	Lysogen[b] – Glycerol no.[a]
186 phage:		
186$^+$	Wildtype 186 phage.	E – E635
186 *cI*10	186 phage with lytic mutation (22684.AT→G) which results in a frame-shift mutation in CI, giving 108 wildtype residues (and additional 33 amino acids). (Lamont *et al.*, 1993)	
186 *cI*ts	186 phage with temperature-sensitive mutation in the lysogenic repressor gene, *cI* (G22R: 22948.T→C mutation). Lytic development can be induced by transfer of a lysogen to 39°C. (Baldwin *et al.*, 1966)	E – E252
186 *cI*ts *B*am17	186 *cI*ts phage also carrying an amber mutation in the *B* gene, 20744.G→T, giving a 45-residue truncated protein in a non-suppressor host. Used as 186 B$^-$ control in phage experiments. (Hocking and Egan, 1982c)	E – E264
186 *cI*ts *B*am57	186 *cI*ts phage also carrying an amber mutation in the *B* gene, 20690.C→T, giving a 27-residue truncated protein in a non-suppressor host. Used to recombine the *OB1* mutation onto the 186 phage. (Hocking and Egan, 1982c)	E – E285
186 *cI*ts *OB1*	186 *cI*ts with the *OB1* mutation and wildtype *B* gene transferred from pBS-pB OB1 onto the 186 *cI*ts *B*am57 phage. (*OB1* mutation changes all basepairs in one half-site of a CI operator at *pB* to complementary bases – see Section 4.B.2.1. for details.)	E – R747
Lambda reporter phage:		
λ*p12.lacZYA*	Transcriptional fusion of 186$^+$ *p12* promoter fragment (–260 to +228) to *lacZ* gene from pMRR9R-p12 transferred to λRS45.	N – R314
λ*p32.lacZYA*	Transcriptional fusion of 186$^+$ *p32* promoter fragment (–260 to +230) to *lacZ* gene from pMRR9R-p32 transferred to λRS45.	N – R318
λpB_{64}.*lacZ*	Transcriptional fusion of 186$^+$ *pB* promoter fragment (–176 to +64) to *lacZ* gene from pTL61T-pB_{64} transferred to λRS45Δ*YA*.	Z – R539
λ*pB.lacZ*	Transcriptional fusion of 186$^+$ *pB* promoter fragment (–213 to +178) to *lacZ* gene from pTL61T-pB transferred to λRS45Δ*YA*.	Z – R733

λ*pB OB1.lacZ*	Transcriptional fusion of 186 *pB OB1* mutant promoter fragment (–213 to +178) to *lacZ* gene from pTL61T-pB OB1 transferred to λRS45Δ*YA*.	Z – R735
λ*pJ.lacZYA*	Transcriptional fusion of 186$^+$ *pJ* promoter fragment (–259 to +230) to *lacZ* gene from pMRR9R-pJ transferred to λRS45.	N – R316
λ*pL.lacZ*	Transcriptional fusion of 186 *pL* HincII-SnaBI promoter fragment (with *pR*$^-$, *FL*$^-$, *cI*ts and *apl*am mutations) to *lacZ* gene from pTL61T-pL, transferred to λRS45Δ*YA*. (Ian Dodd, unpublished)	
λ*pR.lacZ*	Transcriptional fusion of 186 *pR* HincII-SnaBI promoter fragment to *lacZ* reporter from pBC2-pR HS, transferred to λRS45Δ*YA*. (Dodd and Egan, 2002)	N – R754
λ*pR.lacZ.OB*$^+$	Transcriptional fusion of 186 *pR* HincII-SnaBI promoter fragment to *lacZ* reporter from pBC2-pR HS transferred to λRS45Δ*YA.OB*$^+$. (see Figure 6.1)	N – R755
λ*pR.lacZ.OB1*	Transcriptional fusion of 186 *pR* promoter fragment to *lacZ* reporter from pBC2-pR HS transferred to λRS45Δ*YA.OB1*. (see Figure 6.1)	N – R756
λ*ptet.lacZYA*	Transcriptional fusion of the *ptet* promoter to *lacZ* in pMRR9, transferred to λRS45. (Dodd *et al.*, 2001) Used to transfer *OB* sequence from pRS308-OB$^+$/OB1 onto reporter phage. (see Figure 6.1)	N – R101
λ*pV.lacZ*	Transcriptional fusion of 186$^+$ *pV* promoter fragment (–260 to +229) to *lacZ* reporter from pTL61T-pV transferred to λRS45Δ*YA*. (see Figure 6.2)	Z – R442 N – R508
λ*pV.lacZYA*	Transcriptional fusion of 186$^+$ *pV* promoter fragment (–260 to +229) to *lacZ* reporter from pMRR9R-pV transferred to λRS45.	N – R312
λpV_{AA}.*lacZYA*	Transcriptional fusion of 186$^+$ *pV* AluI-AluI promoter fragment (–262 to +229) to *lacZ* reporter from pMRR9-pV_{AA} transferred to λRS45. (Tina Rathjen, unpublished)	
λpV_{103}.*lacZYA*	Transcriptional fusion of 186$^+$ *pV* promoter fragment (–260 to +103) to *lacZ* reporter from pMRR9R-pV_{103} transferred to λRS45.	N – R310
λpV_{AHa}.*lacZYA*	Transcriptional fusion of 186$^+$ *pV* AluI-HaeIII promoter fragment (–262 to +125) to *lacZ* reporter from pMRR9-pV_{AHa} transferred to λRS45. (Tina Rathjen, unpublished)	

λRS45	Lambda phage derivative used to create single-copy promoter-*lacZ* fusions. (Simons *et al.*, 1987)	
λRS45Δ*YA*	Derivative of λRS45 with SgrAI-BsrGI deletion of *lacY* and *lacA* sequences. Also used to create single-copy *lacZ* fusions. (Dodd *et al.*, 2001) (see Figure 6.2)	
λRS45Δ*YA.OB*$^+$	Derivative of λRS45 with *tB-OB*$^+$ sequence inserted into SgrAI-BsrGI sites within *lacY* and *lacA* sequences (also gives Δ*YA* deletion). Used to create λ*pR.lacZ.OB*$^+$ reporter phage. (see Figure 6.1)	
λRS45Δ*YA.OB1*	Derivative of λRS45 with *tB-OB1* sequence inserted into SgrAI-BsrGI sites within *lacY* and *lacA* sequences (also gives Δ*YA* deletion). Used to create λ*pR.lacZ.OB1* reporter phage. (see Figure 6.1)	
Other phage:		
P1	Used for transduction of selectable markers between bacterial strains.	
P2 vir24	P2 phage with virulent deletion mutation. (Ljungquist *et al.*, 1984) Stock made on E. coli K-12 host, E251, used in plating assays on waa mutants.	

[a] E* glycerol numbers refer to Egan lab stocks, R* glycerol numbers refer to personal lab stocks

[b] lysogens are of: E251 (E), NK7049 (N), E251 *lacZ*::Km^R (Z)

6.A.3. PLASMIDS.

This table lists the plasmids used in this study in the chapters that they were used, or under 'for general use' when used in several places; and are listed in alphabetical order in each section. References are given in the description for plasmids not constructed in this study. Those constructed here were made by cloning of PCR products or restriction enzyme digests as indicated, and were checked by PCR using appropriate primers and/or restriction enzyme digestion. Inserts derived from PCR products were also sequenced in their entirety.

Plasmid	**Description (Source)**	**Glycerol no.[a]**
For general use:		
pBC1	Has the tandem repeats of the rrnB *T1T2* terminator region $(T1T2)_2$ either side of the pUC19 polylinker, with ampicillin-resistance gene (Ap^R) and ColE1 origin. (Callen *et al.*, 2004) (see Figure 6.3)	
pBS KS$^+$	pBluescript KS$^+$ used for general cloning. (Stratagene)	E2807

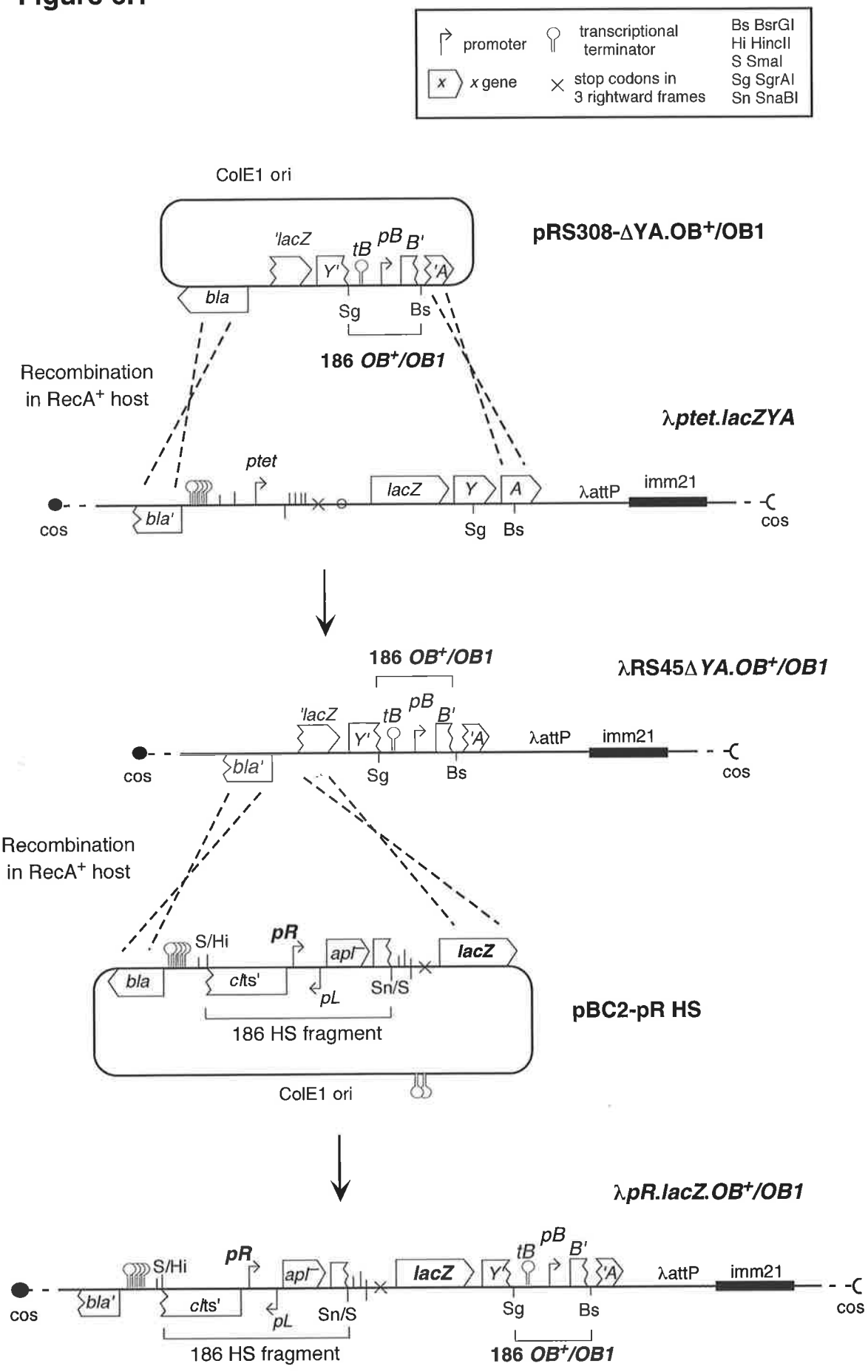
Figure 6.1. Construction of the λ*pR.lacZ.OB* reporter phages.

This figure shows the steps used to construct the λ*pR.lacZ.OB r*eporter phages, for measuring 186 *pR* repression by CI in the presence of the CI operators from 186 *pB*. Relevant promoters, genes and transcriptional terminators are shown, with truncated genes having jagged ends. Restriction enzyme sites used in cloning are also indicated in the key. For lambda phage derivatives, the attP and immunity21 sites are also indicated.

The wildtype or *OB1* mutant *pB* operator sequence from 20527 to 20633 (or –57 to +50 of the promoter) was amplified by PCR with primers #429 and #430, cut with SgrAI and BsrGI and inserted into the SgrAI-BsrGI sites of pRS308. The primers included the *B* terminator sequence (*tB*, from 20 843 to 20 868) upstream of the *B* operators, and the first 8 codons of the wildtype *B* gene (*B'*). The cloning also resulted in deletion of most of the *lacY* and *lacA* genes on the plasmid (Δ*YA*). The pRS308-ΔYA.OB plasmids were recombined *in vivo* in a RecA$^+$ host (NK7049) with a λRS45 reporter phage carrying a *ptet.lacZ* transcriptional fusion (which gives blue plaques when plating with X-gal). Recombination between homologous *bla* and *lac* sequences resulted in recombinant phages which lacked the promoter-*lacZ* fusion (and gave colourless plaques on X-gal) and also carried the 186 *OB* insertion (λRS45Δ*YA.OB*$^+$/*OB1*).

These phages were then plated on the NK7049 cells carrying the pBC2-pR HS plasmid, which contains the 186 HincII.22547-SnaBI.23550 *cI*ts.*apl*am fragment with *lacZ* transcription from the *pR* promoter. Homologous recombination between *bla* and *lacZ* sequences resulted in transfer of the *pR.lacZ* fusion onto the reporter phages, to give the λ*pR.lacZ.OB*$^+$/*OB1* reporters (which formed blue plaques on X-gal).

Figure 6.1

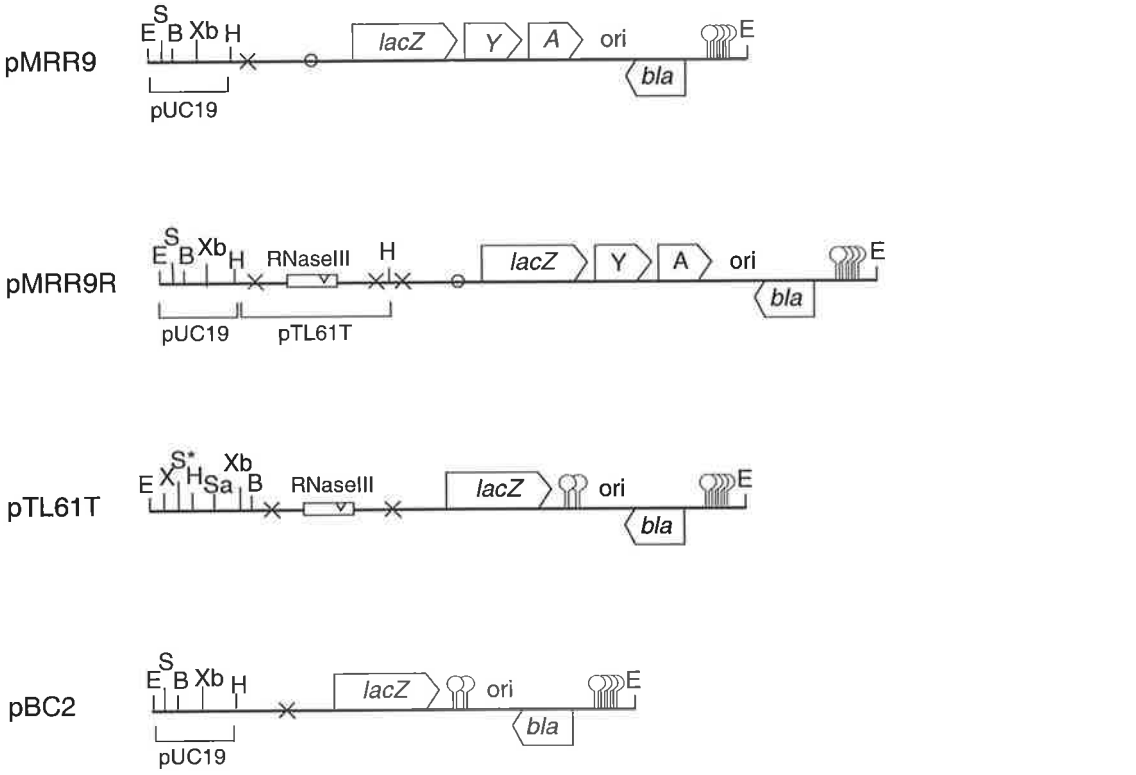

promoter
transcriptional terminator
x gene
stop codons in 3 rightward frames
Bs BsrGI
Hi HincII
S SmaI
Sg SgrAI
Sn SnaBI
ColE1 ori
'lacZ
tB
pB
B'
Y'
'A
bla
Sg
Bs
pRS308-ΔYA.OB+/OB1
186 OB+/OB1
Recombination in RecA+ host
λptet.lacZYA
ptet
lacZ
Y
A
λattP
imm21
cos
bla'
186 OB+/OB1
λRS45ΔYA.OB+/OB1
Recombination in RecA+ host
S/Hi
pR
apI
lacZ
cIts'
pL
Sn/S
186 HS fragment
pBC2-pR HS
ColE1 ori
λpR.lacZ.OB+/OB1

pBS KS$^-$	pBluescript KS$^-$ used for cloning pEC628opp. (Stratagene)	E2808
pDM1.1	A p15A plasmid, resistant to kanamycin, which expresses LacI from the strong *lacI*^{q} promoter. Used to control expression of genes from LacI-repressed promoters on compatible plasmids. (Obtained from H. Bujard, Heidelberg University, Germany)	E2397
pEC628opp	186 *pB* HinPI.20408-HinPI.20647 fragment (–176 to +64) in ClaI site of pBS KS$^-$, such that *pB* transcribes towards *lacZ*α. (Ian Dodd, unpublished)	
pET3a-B	186 *B* coding sequence (20609-20830) inserted into NdeI-BamHI sites of pET3a. (pKS1 – Pountney *et al.*, 1997)	E2817
pMRR9	LacZ reporter plasmid for transferring transcriptional promoter-*lacZ* fusions to lambda reporter phages. (Reed, 1994) Described by Dodd and Egan (2002). (see Figure 6.2)	
pMRR9R	Derivative of pMRR9 containing the RNaseIII cleavage site inserted between polylinker and *lacZ* gene (Dodd and Egan, 2002). Used as for pMRR9. (see Figure 6.2)	R95
pRS308	Used to make *lacZ* reporter phage λRS45Δ*YA.OB*$^+$*/OB1*. (Simons *et al.*, 1987) (see Figure 6.1)	E2832
pTL61T	LacZ reporter vector for transfer of transcriptional promoter-*lacZ* fusions to lambda reporter phages. (Linn and St Pierre, 1990) (see Figure 6.2)	E2862
pUHA1	Identical to pDM1.1, except that LacI is expressed from the wildtype *lacI* promoter. Used to control expression of genes from LacI-repressed promoters on compatible plasmids. (Dodd *et al.*, 2001)	E2396
pUHS43-30Pd	Modular plasmid with spectinomycin-resistance gene (Sp^R), pSC101 origin and *pN25* promoter expressing the tetracycline-resistance gene (Tet^R). Maintained at 10-12 copies per chromosome in the cell. (Lutz and Bujard, 1997)	E2876
pZA31-luc	Modular plasmid with p15A origin, chloramphenicol-resistance gene (Cm^R) gene and *pLtetO-1* promoter driving luciferase gene expression. Maintained at 20-30 copies per chromosome in the cell. (Lutz and Bujard, 1997)	E2866

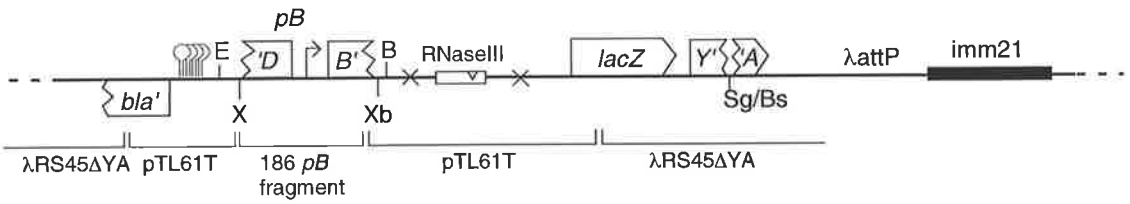
Figure 6.2. LacZ reporter plasmids and phages.

LacZ reporter plasmids pMRR9, pMRR9R, pTL61T and pBC2, used to create single-copy transcriptional promoter-*lacZ* fusions (adapted from Dodd and Egan, 2002). Transcriptional *lacZ* fusions created in the plasmids were transferred by homologous recombination between flanking *bla* and *lac* sequences onto the modified lambda phage, λRS45 or λRS45Δ*YA*, and the resultant recombinant phage were lysogenized in a Lac^- host strain. Plasmids are shown linearized at the EcoRI site. Each has a multiple cloning site (MCS), separated from the *lacZ* (or *lacZYA)* genes by stop codons in three rightward frames and an RNaseIII cleavage site in pMRR9R and pTL61T. A number of restriction enzyme sites in the MCS are shown (SmaI* represents four repeats of the SmaI site), and a small circle upstream of *lacZ* in pMRR9 and pMRR9R represents the *trpt* terminator sequence. Each carries the *bla* gene for ampicillin-resistance and a ColE1 origin of replication. The representative reporter phage (λ*pB.lacZ*) shows the 186 *pB* promoter fragment (from –213 to +178) cloned into the XhoI-XbaI sites of pTL61T and transferred to the λRS45Δ*YA* phage.

Figure 6.2



λ*pB.lacZ* (λRS45Δ*YA* x pTL61T-pB)



B BamHI	S* SmaI repeat	promoter	transcriptional terminator
Bs BsrGI	Sa SalI		
E EcoRI	Sg SgrAI	x gene	stop codons in 3 rightward frames
H HindIII	X XhoI		
Hi HincII	Xb XbaI		
S SmaI			

pZC320	Mini-F plasmid, with ampicillin-resistance gene (Ap^R) and wildtype *lac* promoter preceding a multiple cloning site (MCS) and *lacZα* gene. Maintained at 1-2 copies per chromosome in the cell. (Shi and Biek, 1995)	E2858
pZE15	*plac* and MCS from pZC320 inserted into pZE12 plasmid (Lutz and Bujard, 1997), with Ap^R gene and ColE1 origin. (Dodd *et al.*, 2001)	
pZE21-MCS-1	Modular plasmid with ColE1 origin, kanamycin-resistance gene (Km^R) and *pLtetO-1* promoter upstream of a MCS. Maintained at 50-70 copies per chromosome in the cell. (Lutz and Bujard, 1997)	E2868
pZS*24-MCS-1	Modular plasmid with derivative of the pSC101 origin, pSC101*, Km^R gene and *plac/ara-1* promoter upstream of a MCS. Maintained at 3-4 copies per chromosome in the cell. (Lutz and Bujard, 1997)	E2870

For Chapter 2:

pEC428	490bp AluI.3603-AluI-4093 fragment from 186 *cI*ts containing the *pV* promoter (–262 to +229), inserted into SmaI site of pKO2, with *pV* directing *galK* transcription. (Dibbens, 1990)	E2418
pKO2	GalK transcription fusion vector carrying the Ap^R gene and ColE1 origin. (de Boer, 1984) (Obtained from Genentech, California)	E2184
pKO2-B	pKO2 carrying the native *B* gene (*pB-B-tB*), with *pB* transcription towards *galK*. (pEC465 – Justin Dibbens, unpublished)	E2435
pKO2-Bam	pKO2 carrying the *B*am17 gene (*pB-B*am17-*tB*), with *pB* transcription towards *galK*. (pEC466 – Justin Dibbens, unpublished)	E2436
pMRR9-pV_{AA}	HindIII-EcoRI fragment from pEC428, containing the186 *pV* promoter sequence from AluI.3603-AluI-4093 (–262 to +229), inserted into the HindIII-EcoRI sites of pMRR9. *pV* controls *lacZ* transcription. (Tina Rathjen, unpublished)	E2615
pMRR9-pV_{AH}	HindIII-EcoRI fragment from pUC19-pV_{AH}, containing the186 *pV* promoter sequence from AluI.3603-HphI-3950 (–262 to +86), inserted into the HindIII-EcoRI sites of pMRR9. *pV* controls *lacZ* transcription. (Tina Rathjen, unpublished)	E2617
pMRR9-pV_{AHa}	~390bp EcoRI-HaeIII fragment from pMRR9-pV_{AA}, containing the 186 *pV* promoter sequence from AluI.3603-HaeIII.3989 (–262 to +125) inserted into pMRR9. *pV* controls *lacZ* transcription. (Tina Rathjen, unpublished)	E2618

pTL61T-pL	HincII.22547-SnaBI.23550 186 fragment carrying *c*Its, *apl*am, pR^-, and FL^- mutations inserted into pTL61T, such that *pL* transcription drives *lacZ*. (Ian Dodd, unpublished)	
pTMC10	BsrBI fragment containing ribosome binding site (GGAGG) and polylinker from pEC611 (Brumby, 1994) inserted into EcoRI-BamHI sites of pMRR14, downstream of $pT7_{A1\text{-}la}$ promoter, controlled by LacI. Also has *lacI* gene expressed from $placI^q$, Km^R gene and p15A origin. (Tina Rathjen, unpublished)	E2600
pTMC10-B	As for pTMC10, except that BsrBI fragment is from pEC611-B (or pEC462 – Justin Dibbens, unpublished) and also contains the *B* coding sequence (20609-20830), which is expressed from $pT7_{A1\text{-}lac}$ promoter. (Tina Rathjen, unpublished)	E2601
pUC19-pV_{AH}	EcoRI-HphI(end-filled) fragment from pEC428, containing the 186 *pV* promoter sequence from AluI.3603-HphI-3950 (–262 to +86), ligated to pUC19 cut with EcoRI/SmaI. (Tina Rathjen, unpublished)	E2593

For Chapter 3:

pBC1-B	Native *B* gene sequence (*pB-B-tB*) from 20371-20948 amplified from a 186^+ phage template by PCR with primers #136 and #66, cut with EcoRI/XhoI and inserted into EcoRI-SalI sites in polylinker of pBC1. (see Figure 6.3)	R397
pBC1-Bam	As for pBC1-B, except that a 186 *c*Its *B*am17 phage template was used to clone the *pB-B*am17-*tB* sequence into pBC1.	R416
pBS-B	PvuII T2T1-*pB-B-tB*-T1T2 fragment from pBC1-B ligated to HincII-cut pBS KS^+, such that *pB* points towards ColE1 origin. (see Figure 6.4)	R604
pBS-Bam	As for pBS-B except PvuII T2T1-*pB-B*am17-*tB*-T1T2 fragment from pBC1-Bam was inserted into pBS KS^+.	R606
pMRR9R-p12	186 *p12* promoter fragment from 4013-3526 (–260 to +228), amplified by PCR with primers #423 and #422 from a 186^+ phage template, cut and inserted into the KpnI-XbaI sites of pMRR9R, to give a transcriptional fusion with the *lacZ* reporter gene.	R304
pMRR9R-p32	186 *p32* promoter fragment from 9467-9953 (–260 to +230), amplified by PCR with primers #416 and #415 from a 186^+ phage template, cut and inserted into the KpnI-XbaI sites of pMRR9R, to give a transcriptional fusion with the *lacZ* reporter gene.	R308

pMRR9R-pJ	186 *pJ* promoter fragment from 13900-14388 (–259 to +230), amplified by PCR with primers #418 and #417 from a 186^+ phage template, cut and inserted into the KpnI-XbaI sites of pMRR9R, to give a transcriptional fusion with the *lacZ* reporter gene.	R306
pMRR9R-pV	186 *pV* promoter fragment from 3605-4093 (–260 to +229), amplified by PCR with primers #421 and #419 from a 186^+ phage template, cut and inserted into the KpnI-XbaI sites of pMRR9R, to give a transcriptional fusion with the *lacZ* reporter gene.	R302
pMRR9R-pV_{100}	186 *pV* promoter fragment from 3605-3967 (–260 to +103), amplified by PCR with primers #421 and #420 from a 186^+ phage template, cut and inserted into the KpnI-XbaI sites of pMRR9R, to give a transcriptional fusion with the *lacZ* reporter gene.	R300
pTL61T-B1	HindIII T2T1-*pB-B-tB* fragment from pBS-B inserted into HindIII site of pTL61T-pV_{XB}, such that *pB* transcription is towards the Ap^R gene. (see Figure 6.4)	R622
pTL61T-B2	~1kb XhoI-XbaI fragment from pTL61T-B1 containing *B* gene cassette (T2T1-*pB-B-tB*), inserted into SalI-XbaI sites of pTL61T-B1. (see Figure 6.4)	R645
pTL61T-B3	~1kb XhoI-XbaI fragment from pTL61T-B1 containing *B* gene cassette (T2T1-*pB-B-tB*), inserted into SalI-XbaI sites of pTL61T-B2.	R649
pTL61T-B4	~2kb XhoI-XbaI fragment containing two tandem copies of the *B* gene cassette from pTL61T-B2, inserted into SalI-XbaI sites of pTL61T-B2.	R653
pTL61T-Bam1	HindIII T2T1-*pB-B*am17-*tB* fragment of pBS-Bam inserted into pTL61T-pV_{XB}, as for pTL61T-B1.	R623
pTL61T-Bam2	~1kb XhoI-XbaI fragment from pTL61T-Bam1 containing *B*am17 gene cassette (T2T1-*pB-B*am17-*tB*), inserted into SalI-XbaI sites of pTL61T-Bam1, as for pTL61T-B2.	R647
pTL61T-Bam3	~1kb XhoI-XbaI fragment from pTL61T-Bam1 containing *B*am17 gene cassette (T2T1-*pB-B*am17-*tB*), inserted into SalI-XbaI sites of pTL61T-Bam2, as for pTL61T-B3.	R651
pTL61T-Bam4	~2kb XhoI-XbaI fragment containing two tandem copies of the *B*am17 gene cassette from pTL61T-Bam2, inserted into SalI-XbaI sites of pTL61T-Bam2, as for pTL61T-B4.	R655

pTL61T-pB_{64}	186 *pB* fragment (–176 to +64) amplified from pEC628opp with RSP/USP, cut with EcoRI/XhoI and inserted into EcoRI-XhoI sites of pTL61T. Then cut with SmaI and religated to remove SmaI repeat sequences.	R528
pTL61T-pV	186 *pV* promoter fragment from 3605-4093 (–260 to +229), amplified by PCR with primers #421 and #419 from a 186^+ phage template, end-filled and ligated to SmaI-cut pTL61T plasmid. (Contains additional GG basepairs immediately upstream of *pV* fragment.)	R394
pTL61T-pV_{XB}	186 *pV* promoter fragment from 3605-4093 (–260 to +229), amplified by PCR with primers #425 and #426 from a 186^+ phage template, cut and inserted into the XbaI-BamHI sites of pTL61T. Used to clone pZC-B plasmids (see Figure 6.4).	R 611
pZA-B	pZE-B plasmid with AvrII-SacI ColE1 origin fragment replaced by AvrII-SacI p15A origin fragment from pZA31-luc. (see Figure 6.3)	R404
pZA-Bam	pZE-Bam plasmid with ColE1 origin replaced by p15A origin as for pZA-B.	R427
pZC320-Δplac	pZC320 with *lac* promoter sequence deleted. Made in two steps: (i) BamHI-XbaI small fragment of pZC320 was subcloned into BamHI-XbaI sites of pBS KS^+, cut with BsaBI/NheI, endfilled and religated to delete the *plac* sequence and restore the NheI site (ii) the Δ*plac* BamHI-XbaI fragment was inserted back into BamHI-XbaI sites of pZC320. (see Figure 6.3)	R665
pZC-B1	XhoI-XbaI fragment from pTL61T-B1 containing the *B* gene cassette, inserted into XhoI-NheI sites of pZC320-Δplac, with *pB* transcription towards the Ap^R gene. (see Figure 6.4)	R667
pZC-B2	XhoI-XbaI fragment from pTL61T-B2 containing two copies of the *B* gene cassette, inserted into pZC320-Δplac as for pZC-B1. (see Figure 6.4)	R669
pZC-B3	XhoI-XbaI fragment from pTL61T-B3 containing three copies of the *B* gene cassette, inserted into pZC320-Δplac as for pZC-B1. (see Figure 6.4)	R671
pZC-B4	XhoI-XbaI fragment from pTL61T-B4 containing four copies of the *B* gene cassette, inserted into pZC320-Δplac as for pZC-B1. (see Figure 6.4)	R673

pZC-Bam1	XhoI-XbaI fragment from pTL61T-Bam1 containing the *B*am17 gene cassette, inserted into XhoI-NheI sites of pZC320-Δplac, with *pB* transcription towards the Ap^R gene (as for pZC-B1).	R675
pZC-Bam2	XhoI-XbaI fragment from pTL61T-Bam2 containing two copies of the *B*am17 gene cassette inserted into pZC320-Δplac, as for pZC-Bam1.	R677
pZC-Bam3	XhoI-XbaI fragment from pTL61T-Bam3 containing three copies of the *B*am17 gene cassette inserted into pZC320-Δplac, as for pZC-Bam1.	R679
pZC-Bam4	XhoI-XbaI fragment from pTL61T-Bam4 containing four copies of the *B*am17 gene cassette inserted into pZC320-Δplac, as for pZC-Bam1.	R681
pZE15-B	*B* gene PCR from pET3a-B with primers #164 and #75, cut with HindIII/BamHI and inserted into HindIII-BamHI sites of pZE15. *B* gene is expressed from IPTG-inducible *plac* promoter.	R451
pZE41	pZE21-MCS-1 with SacI-AatII Km^R gene replaced with SacI-AatII SpR gene from pUHS43-30Pd. Used to make pZE-B plasmid.	R400
pZE-B	PvuII T2T1-pB-B-tB-T1T2 fragment from pBC1-B inserted into cut and end-filled XhoI sites of pZE41. Orientated such that pB transcribes towards plasmid origin. (see Figure 6.3)	R402
pZE-Bam	As for pZE-B except that the PvuII T2T1-pB-Bam17-tB-T1T2 fragment from pBC1-Bam was inserted into pZE41.	R425
pZF-B	pZE-B with SalI-SacI ColE1 origin fragment replaced by SalI-SacI mini-F origin fragment from pZC320-Δplac. (see Figure 6.3)	R438
pZF-Bam	pZE-Bam plasmid with ColE1 origin replaced by mini-F origin as for pZF-B.	R439
pZS*-B	pZE-B with AvrII-SacI ColE1 origin fragment replaced by AvrII-SacI pSC101* origin fragment from pZS*24-MCS. (see Figure 6.3)	R408
pZS*-Bam	pZE-Bam plasmid with ColE1 origin replaced by pSC101* origin as for pZS*-B.	R431
pZS-B	pZE-B with AvrII-SacI ColE1 origin fragment replaced by AvrII-SacI pSC101 origin fragment from pUHS43-30Pd. (see Figure 6.3)	R406
pZS-Bam	pZE-Bam plasmid with ColE1 origin replaced by pSC101 origin as for pZS-B.	R429

Figure 6.3. Construction of the pZE-B, pZA-B, pZS-B, pZS*-B and pZF-B plasmids.

Promoters, genes and transcriptional terminators are shown, along with restriction enzyme sites used in cloning. Plasmids are shown from the sequence position start points given by Callen *et al.* (2004) for pBC1, by Lutz and Bujard (1997) for pZE21-MCS-1 (for pZE41), and by Shi and Biek (1995) for pZC320 (for pZC320-Δplac). The *bla* gene for ampicillin resistance (Ap^R) and the spectinomycin-resistance (Sp^R) genes carried by the plasmids are also shown.

To construct plasmids of increasing copy number expressing B from its native gene, the wildtype *B* promoter, gene and terminator (from 20371 to 20948 of the 186 sequence – *pB-B-tB*) were PCR amplified from a 186^+ phage template with primers #136 and #88. The PCR product was cut with EcoRI/XhoI and inserted into the EcoRI-SalI sites of pBC1, which has tandem repeats of the *rrnB T1T2* terminator sequence flanking the pUC19 MCS ($(T2T1)_2$ or $(T1T2)_2$). The PvuII fragment from pBC1-B containing the *B* cassette (promoter, gene and terminator flanked by *T1T2* sequences) was then ligated with the XhoI-cut and end-filled pZE41 plasmid (the pZE21-MCS-1 plasmid of Bujard with Ap^R gene replaced with a Sp^R gene). Plasmids of lower copy number were created by replacing the ColE1 origin with different origin sequences. pZA-B, pZS-B or pZS*-B were created by replacing the AvrII-SacI origin fragment with AvrII-SacI fragments containing the p15A, pSC101 or pSC101* origins respectively (from pZA31-luc, pUHS43-30Pd or pZS*24-MCS-1 respectively). The SalI-SacI ColE1 origin fragment of pZE-B was also replaced with the SalI-SacI fragment of pZC320-Δplac which contains the mini-F origin sequence, to give pZF-B.

Figure 6.3

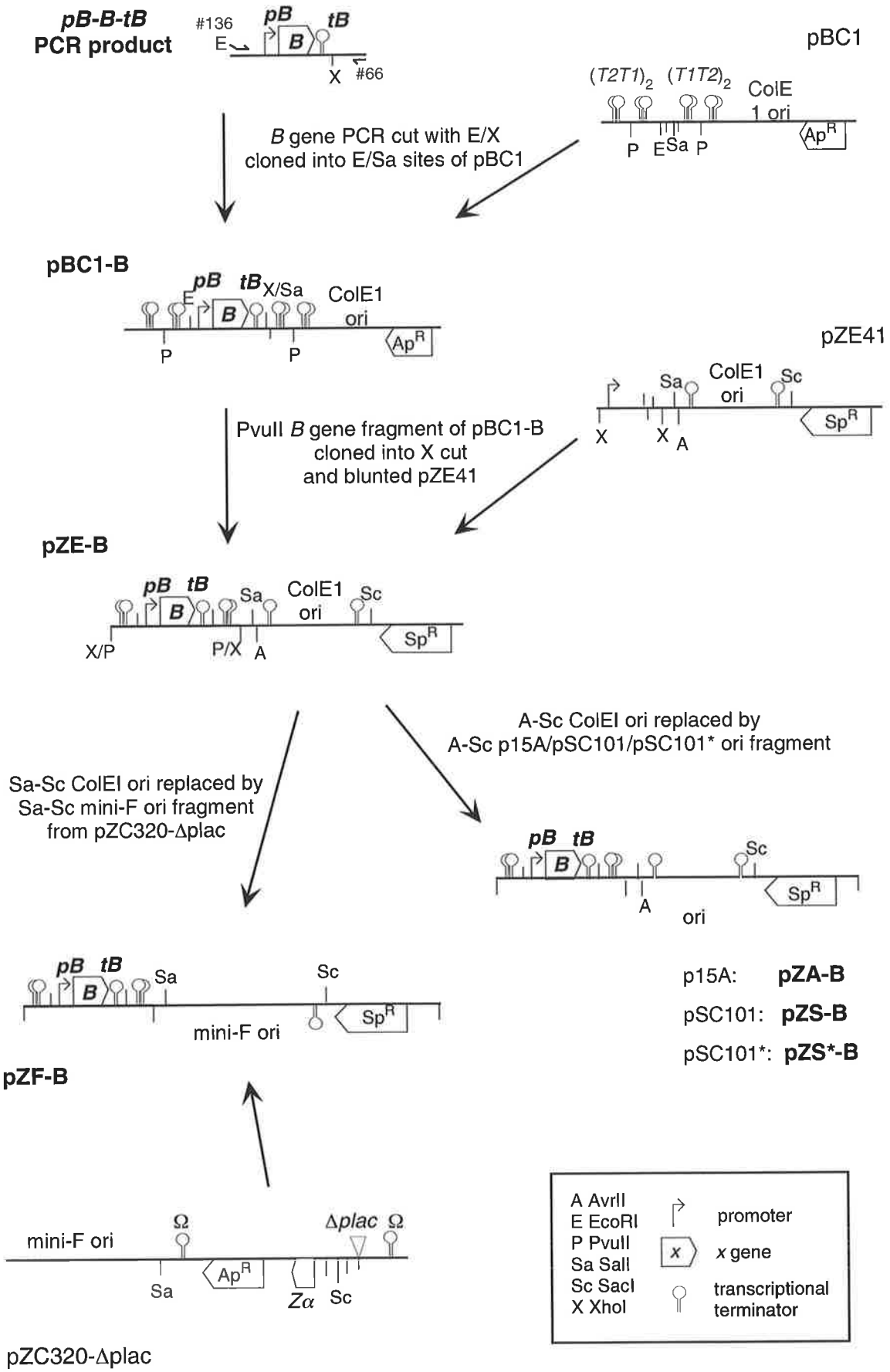
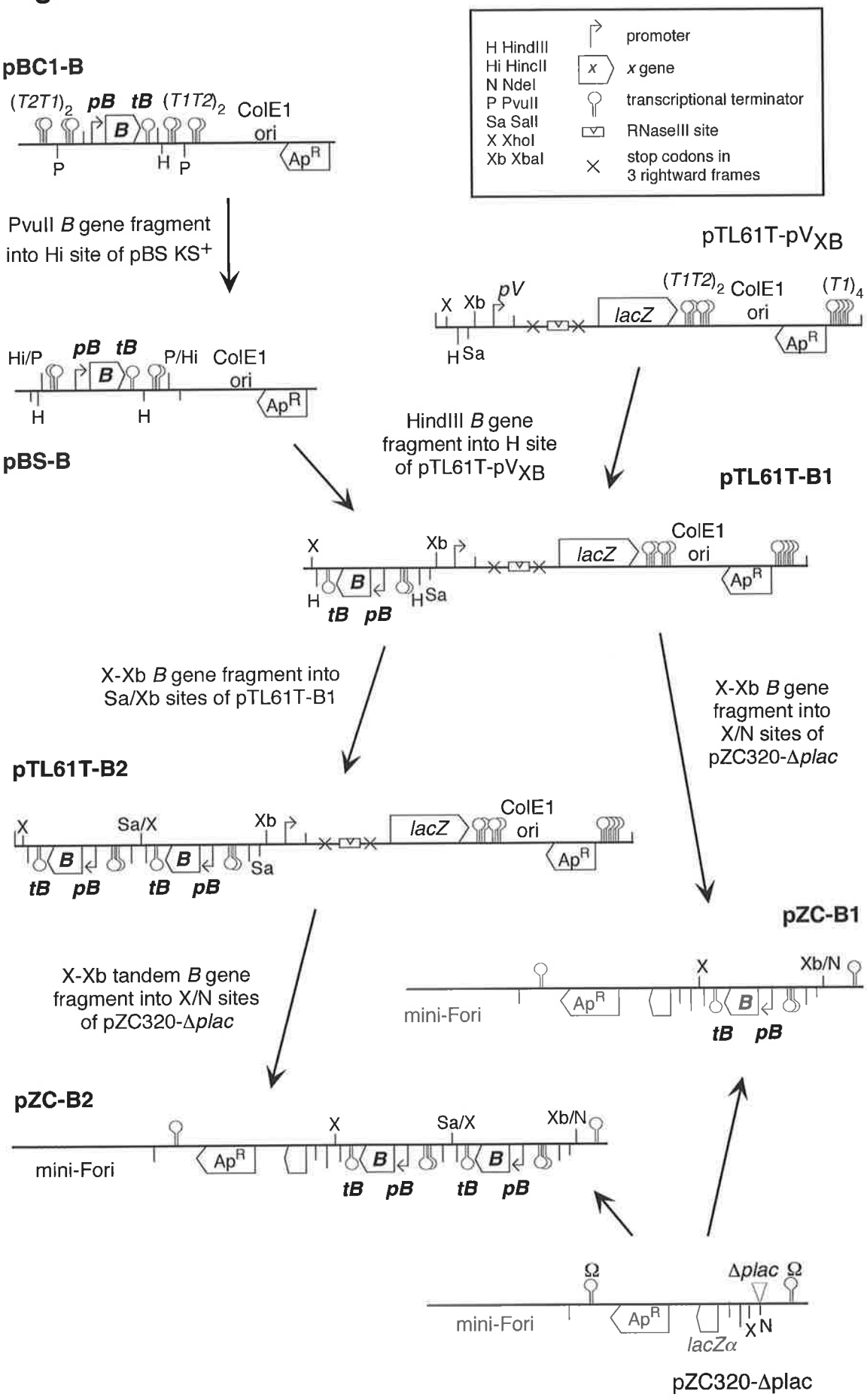

pB-B-tB
PCR product
#136
E
pB
B
tB
X
#66
pBC1
(T2T1)2
(T1T2)2
ColE
1 ori
P
ESa
P
ApR
B gene PCR cut with E/X
cloned into E/Sa sites of pBC1
pBC1-B
pB
tB
X/Sa
E
ColE1
ori
P
P
ApR
pZE41
Sa
ColE1
ori
Sc
X
X
A
SpR
PvuII B gene fragment of pBC1-B
cloned into X cut
and blunted pZE41
pZE-B
pB
tB
Sa
ColE1
ori
Sc
X/P
P/X
A
SpR
A-Sc ColEI ori replaced by
A-Sc p15A/pSC101/pSC101* ori fragment
Sa-Sc ColEI ori replaced by
Sa-Sc mini-F ori fragment
from pZC320-Δplac
pB
tB
Sc
A
ori
SpR
p15A: pZA-B
pSC101: pZS-B
pSC101*: pZS*-B
pB
tB
Sa
Sc
mini-F ori
SpR
pZF-B
mini-F ori
Ω
Δplac
Ω
Sa
ApR
Zα
Sc
A AvrII
E EcoRI
P PvuII
Sa SalI
Sc SacI
X XhoI
promoter
x
x gene
transcriptional
terminator
pZC320-Δplac

Figure 6.4. Construction of the pZC-B1 and pZC-B2 plasmids.

Promoters, genes and transcriptional terminators are shown, along with restriction enzyme sites used in cloning. Plasmids are shown from the sequence position start points given by Callen *et al.* (2004) for pBC1, by Stratagene for pBS KS$^+$ (for pBS-B), by Linn and St Pierre (1990) for pTL61T (for pTL61T-pV$_{XB}$) and by Shi and Biek (1995) for pZC320 (for pZC320-Δplac). The *bla* gene for ampicillin resistance (Ap^R) is also shown.

The PvuII fragment from pBC1-B containing the *B* gene flanked by *T1T2* terminator sequences (see Figure 6.3) was initially ligated with HincII-cut pBS KS$^+$ plasmid. The HindIII fragment from the resultant plasmid (pBS-B) containing the *B* gene and upstream *T1T2* sequence was then inserted into the HindIII site of pTL61T-pV$_{XB}$ (the pTL61T plasmid with 186 *pV* cloned into XbaI-BamHI sites), to give pTL61T-B1. Two copies of the *B* gene and terminators were cloned by isolating the XhoI-XbaI fragment from pTL61T-B1, and ligating it to pTL61T-B1 cut with SalI and XhoI, to give pTL61T-B2. (A similar process was used to create the pTL61T-B3 and pTL61T-B4 plasmids – see Section 6.A.3.) XhoI-XbaI fragments from pTL61T-B1 or pTL61T-B2, containing one or two copies of the *B* gene and terminators, were then inserted into the XhoI-NheI sites of pZC320-Δplac, to give pZC-B1 or pZC-B2 respectively.

Figure 6.4


H HindIII
Hi HincII
N NdeI
P PvuII
Sa SalI
X XhoI
Xb XbaI
promoter
x gene
transcriptional terminator
RNaseIII site
stop codons in 3 rightward frames
pBC1-B
(T2T1)2 pB tB (T1T2)2 ColE1 ori
P H P ApR
PvuII B gene fragment into Hi site of pBS KS+
pBS-B
Hi/P pB tB P/Hi ColE1 ori
H H ApR
pTL61T-pVXB
X Xb pV (T1T2)2 ColE1 ori (T1)4
lacZ
H Sa ApR
HindIII B gene fragment into H site of pTL61T-pVXB
pTL61T-B1
X Xb lacZ ColE1 ori
H Sa ApR
tB pB
X-Xb B gene fragment into Sa/Xb sites of pTL61T-B1
X-Xb B gene fragment into X/N sites of pZC320-Δplac
pTL61T-B2
X Sa/X Xb lacZ ColE1 ori
Sa ApR
tB pB tB pB
pZC-B1
mini-Fori
X Xb/N ApR
tB pB
X-Xb tandem B gene fragment into X/N sites of pZC320-Δplac
pZC-B2
mini-Fori
X Sa/X Xb/N ApR
tB pB tB pB
Ω Δplac Ω
mini-Fori ApR
X N
lacZα
pZC320-Δplac

For Chapter 4:		
pBC2-pR HS	HincII.22547-SnaBI.23550 fragment of 186 (containing *cI*ts and *apl*am mutations) inserted into *lacZ* reporter plasmid pBC2, such that the *pR* promoter directs *lacZ* transcription. (Dodd and Egan, 2002)	
pBS-pB	186^{+} *B* promoter sequence from 20371-20761 (–213 to +178) amplified by PCR with primers #136 and #107 (including P52* truncating mutation in *B* gene), cut with EcoRI-BamHI and inserted into EcoRI/BamHI sites of pBS KS^{+}. For Quickchange mutagenesis of CI binding site at *pB* (*OB1* mutation).	R608
pBS-pB OB1	Quickchange mutagenesis with primers #427 and #428 on pBS-pB^{+} to give *OB1* mutation in *pB*: 20559-20564.GTGAAT→CACTTA.	R612
pRS308-Δ*YA.*OB^{+}	Wildtype CI operators from *pB* inserted into pRS308 downstream of *lacZ* gene. Made in two steps: (i) Wildtype 186 *OB* sequence from 20527-20633 (–57 to +50 of *pB*) was amplified from 186 *cI*ts phage with primers #429 and #430 (including upstream *tB* transcriptional terminator and flanking SgrAI and BsrGI sites) and ligated with EcoRV-cut pBS KS+. (ii) SgrAI-BsrGI *tB-OB* fragment was then inserted into SgrAI-BsrGI sites within the *lacYA* sequence of pRS308. Used to make the λRS45Δ*YA.*OB^{+} reporter phage. (Figure 6.1)	R743
pRS308-Δ*YA.OB1*	CI operators from *pB* with *OB1* mutation inserted into pRS308 downstream of the *lacZ* gene. Made as for pRS308-Δ*YA.*OB^{+}, except that the 186 *cI*ts *OB1* phage was used as a template instead of 186 *cI*ts. Used to make the λRS45Δ*YA.OB1* reporter phage. (Figure 6.1)	R745
pTL61T-pB	XhoI-XbaI fragment of pBS-pB containing wildtype *pB* sequence (–213 to +178), inserted into XhoI-XbaI sites of pTL61T, such that *pB* directs *lacZ* transcription.	R614
pTL61T-pB OB1	XhoI-XbaI fragment of pBS-pB OB1 containing *pB OB1* sequence (–213 to +178), inserted into XhoI-XbaI sites of pTL61T, such that *pB* directs *lacZ* transcription.	R616
pZC320-cI	186 *cI* gene, from 22403 to 23030, inserted into HindIII-BamHI sites of pZC320, such that *plac* directs *cI* transcription. (Dodd and Egan, 2002) Used to assay 186 CI repression of *pR* transcription.	R616

[a] E* glycerol numbers refer to Egan lab stocks, R* glycerol numbers refer to personal (RS) lab stocks.

6.A.4. PRIMERS.

All primers used in this study are detailed in Figure 6.5.

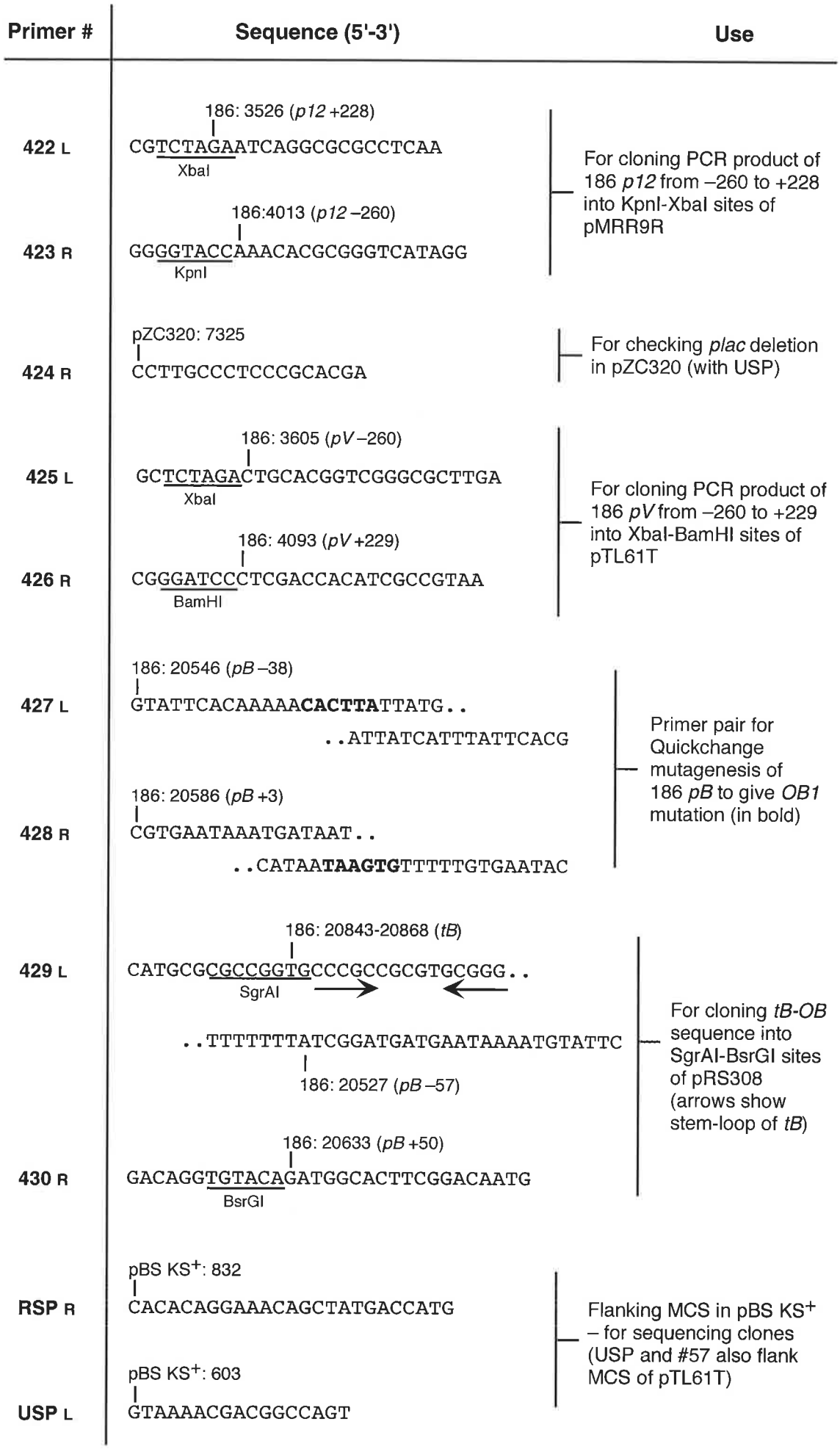
Figure 6.5

The 5' to 3' sequences of DNA oligonucleotides used in this thesis are given, along with the lab stock number (primer #) and the position of the sequence in *E. coli* K-12 (Genbank accession number AE000407 or AE000123), 186 phage (NC_001317) or indicated plasmids. L and R refer to the strand of the bacterial, phage or plasmid sequence that the primer is identical to, where L indicates the strand 5'-3' from left to right according to the coordinates given in the Genbank database. Primers were purchased from GeneWorks (Australia).

Primer #	Sequence (5'-3')	Use
56 R	pMRR9R: 411 GCTCTAGACTCGCCACCGATTGCCA	Flanking MCS of pMRR9R – for sequencing inserts (#57 and USP also flank MCS of pTL61T)
57 L	pMRR9R: 11100 GGGGTACCAGCGATAGTGCGGCGGTGG	
66 R	186: 21173 GCGATGGTTCTGAGTAA	Downstream of 186 *tB* – with #136 for cloning native *B* or *B*am17 gene into pBC1
75 L	pET3a: 447 GCTAGTTATTGCTCAGCGG	Upstream of MCS in pET3a – with #164 for PCR of *B* gene from pET3a-B
88 R	186: 20830 CGGGATCCTTATCAGAGCCAC BamHI * * 72	At 3' end of *B* coding sequence – for checking *B* gene cloning
110 R	*E. coli* K-12 (AE000407): 9131 CAAGTAGATCTTAGTACCAAAG	For sequencing RNA polymerase α-subunit gene (*rpoA*) of *E.coli* K-12
111 L	*E. coli* K-12 (AE000407): 8076 GACCGGATCCTTCTCAGTAAAAC	
107 R	186: 20761 (*pB* +178) CGGGATCCTTAAGTAACAATAAAGCGCTCT BamHI * 51	For cloning PCR product of 186 *pB* from –213 to +178 into pBS KS$^+$ (with P52* mutation in B)
136 L	186: 20371 (*pB* –213) GGAATTCCCTGAGACACCGG EcoRI	
154 L	TTTAATATATTGATATTTATATC.. ..ATTTTACGTTTCTCGTTC	Primers in lambda attP, attB and *int* gene – for differentiating single and multiple lambda lysogens (Powell *et al.*, 1994)
155 L	GAGGTACCAGCGCGGTTTGATC	
156 R	ACTCGTCGCGAACCGCTTTC	

Primer #	Sequence (5'-3')	Use
164 R	pET3a: 578 CGGAAGCTTAAGTTTAACTTTAAGAAGGAGA HindIII	Downstream of MCS in pET3a – with #75 for PCR of *B* gene from pET3a-B
187 L	pZA31-luc: 2144 CTCGTGCGCTCTCCTGTTCCT	For checking cloning of p15A origin
188 R	pZA31-luc: 2559 GCGCGTAATCTCTTGCTCTGA	
189 L	pZS*24-MCS-1: 2044 TACCGTCCGTTCTTTCCTTGT	For checking cloning of pSC101 or pSC101* origin
190 R	pZS*24-MCS-1: 2746 ACGGGCTTTTCTTGTATTATG	
206 L	pZE41: 2352 ACGTCTAAGAAACCATTATTATCATGAC	For checking *B* cassette cloning into pZE41
207 R	pZE41: 189 TAGCACGCGTACCATGGGATC	
250 R	pBC1: 1126 AGTTCCCAAGCTTGCATGCC	With #136 for sequencing native *B* or *B*am17 gene in pBC1 (Callen *et al.*, 2004)
289 R	pZE15: 418 ACTCAGGAGAGCGTTCACCG	Flanking MCS in pZE15 – for sequencing *B* gene cloning
290 L	pZE15: 2335 TAAACAAATAGGGGTTCCGC	
299 L	pRS308: 1261 CCCGATTTGGCTACATGAC	For sequencing *OB* inserts in SgrAI-BsrGI sites of pRS308
300 R	pRS308: 3019 AACGCCAGCCGCCACGAC	

Primer #	Sequence (5'-3')	Use
409 R	*E. coli* K-12 (AE000407): 8647 TTATGTGCCGGCTTCTACC	For sequencing RNA polymerase α-subunit gene (*rpoA*) of *E.coli* K-12
410 L	*E. coli* K-12 (AE000407): 8558 TCCACAGGGCTGTAGCATG	
411 R	*E. coli* K-12 (AE000123): 9567 GTCATGCTGAGCTATGATTAG	For sequencing Poly(A) polymerase gene (*pcnB*) of *E.coli* K-12
412 R	*E. coli* K-12 (AE000123): 8854 TGCCGCCAAATTGGGTATG	
413 L	*E. coli* K-12 (AE000123): 8764 GCGTGCCGGTGGGATATCG	
414 L	*E. coli* K-12 (AE000123): 8061 GGAGAGGCCAGATTGCTGCC	
415 R	186: 9953 (*p32* +230) GCTCTAGACTCGCCACCGATTGCCA XbaI	For cloning PCR product of 186 *p32* from –260 to +230 into KpnI-XbaI sites of pMRR9R (with *p32* +1 at 9728 and including 1bp deletion at –5 – see Section 3.B.3.1.)
416 L	186: 9467 (*p32* –260) GGGGTACCAGCGATAGTGCGGCGGTGG KpnI	
417 R	186: 14388 (*pJ* +230) GCTCTAGAAGCTGACAGAGTGCCT XbaI	For cloning PCR product of 186 *pJ* from –259 to +230 into KpnI-XbaI sites of pMRR9R
418 L	186: 13900 (*pJ* –259) GGGGTACCTGAGGCGGAGGCAACAA KpnI	
419 R	186: 4093 (*pV* +229) GCTCTAGACTCGACCACATCGCCGTAA XbaI	For cloning PCR product of 186 *pV* from –260 to +229 or +103 into KpnI-XbaI sites of pMRR9R or SmaI site of pTL61T
420 R	186: 3967 (*pV* +103) GCTCTAGAGCTGATAATGCGCCCGTC XbaI	
421 L	186: 3605 (*pV* –260) GGGGTACCTGCACGGTCGGGCGCTTGA KpnI	


Primer #
Sequence (5'-3')
Use
186: 3526 (p12 +228)
422 L
CGTCTAGAATCAGGCGCGCCTCAA
XbaI
186:4013 (p12 –260)
423 R
GGGGTACCAAACACGCGGGTCATAGG
KpnI
For cloning PCR product of 186 p12 from –260 to +228 into KpnI-XbaI sites of pMRR9R
pZC320: 7325
424 R
CCTTGCCCTCCCGCACGA
For checking plac deletion in pZC320 (with USP)
186: 3605 (pV –260)
425 L
GCTCTAGACTGCACGGTCGGGCGCTTGA
XbaI
186: 4093 (pV +229)
426 R
CGGGATCCCTCGACCACATCGCCGTAA
BamHI
For cloning PCR product of 186 pV from –260 to +229 into XbaI-BamHI sites of pTL61T
186: 20546 (pB –38)
427 L
GTATTCACAAAAACACTTATTATG..
..ATTATCATTTATTCACG
186: 20586 (pB +3)
428 R
CGTGAATAAATGATAAT..
..CATAATAAGTGTTTTTGTGAATAC
Primer pair for Quickchange mutagenesis of 186 pB to give OB1 mutation (in bold)
186: 20843-20868 (tB)
429 L
CATGCGCGCCGGTGCCCGCCGCGTGCGGG..
SgrAI
..TTTTTTTATCGGATGATGAATAAAATGTATTC
186: 20527 (pB –57)
186: 20633 (pB +50)
430 R
GACAGGTGTACAGATGGCACTTCGGACAATG
BsrGI
For cloning tB-OB sequence into SgrAI-BsrGI sites of pRS308 (arrows show stem-loop of tB)
pBS KS+: 832
RSP R
CACACAGGAAACAGCTATGACCATG
pBS KS+: 603
USP L
GTAAAACGACGGCCAGT
Flanking MCS in pBS KS+ – for sequencing clones (USP and #57 also flank MCS of pTL61T)

6.A.5. REAGENTS.

6.A.5.1. Enzymes.

Big Dye Version 3 Ready Mix: Perkin-Elmer ABI.

Lysozyme: Sigma Chemical Co.

Restriction Endonucleases: New England Biolabs, Boehringer Mannheim or Pharmacia.

Shrimp Alkaline Phosphatase (SAP): USB.

T4 DNA ligase: GeneWorks or Promega.

T4 DNA polymerase: New England Biolabs.

Taq DNA polymerase: Fisher Biotech International (Australia).

Pfu Turbo DNA polymerase: Stratagene.

6.A.5.2. Chemicals.

All chemicals were of analytical grade or of the highest purity available.

5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal): Sigma Chemical Co. Stock solutions at 30mg/ml in dimethyl formamide were kept at –20°C.

Acetic acid: B.D.H. Labs., Australia.

Agarose: Sigma Chemical Co.

Ammonium acetate: B.D.H. Labs., Australia.

Ampicillin (Ap): Sigma Chemical Co. Stock solutions of sodium salt (25-100mg/ml in H_2O) were millipore filtered and stored at –20°C.

β-Mercaptoethanol (βME): Sigma Chemical Co.

Bacto-tryptone, yeast extract and Bacto-agar: Difco Labs., U.S.A.

Boric acid: B.D.H. Labs., Australia.

Bovine serum albumin (BSA): Sigma Chemical Co. Kept as a 10mg/ml solution in H_2O at –20°C.

Bromophenol blue: B.D.H. Labs., Australia.

Caesium chloride (CsCl): Bethesda Research Labs.

Calcium chloride ($CaCl_2$): Sigma Chemical Co.

Carbenicillin (Cb): Sigma Chemical Co. Stock solutions (100mg/ml in H_2O) were millipore filtered and stored at –20°C.

Chloramphenicol (Cm): Sigma Chemical Co. Stock solutions (30mg/ml in ethanol) were stored at –20°C.

Chloroform: B.D.H. Labs., Australia.

Deoxyribonucleoside triphosphates (dNTP): Sigma Chemical Co. Stock solutions at 20mM (in 5mM Tris-HCl, pH 8.0, 0.1mM EDTA) were kept at –20°C.

Di-potassium hydrogen orthophosphate (K_2HPO_4): B.D.H. Labs., Australia.

Di-sodium hydrogen orthophosphate (Na_2HPO_4): B.D.H. Labs., Australia.

Ethanol (95%): Crown Scientific. RNase-free.

Ethidium bromide: Sigma Chemical Co. Stored as a 10mg/ml solution in H_2O in the dark at 4°C.

Ethylenediaminetetraacetic acid (Disodium salt) (EDTA): Sigma Chemical Co.

Gelatin: Sigma Chemical Co.

Glucose: Ajax.

Glycerol: B.D.H. Labs., Australia.

Glycogen: Boehringer Mannheim.

Hydrochloric acid (HCl): B.D.H. Labs., Australia.

Isopropanol: May and Baker Ltd.

Isopropyl-β-D-thiogalactopyranoside (IPTG): Sigma Chemical Co. Stock solutions (1M in H_2O) were millipore filtered and stored at –20°C.

Kanamycin (Km): Sigma Chemical Co. Stock solutions (50mg/ml in H_2O) were millipore filtered and stored at –20°C.

Magnesium chloride ($MgCl_2$): Ajax.

Magnesium sulfate ($MgSO_4$): Ajax.

O-nitrophenyl-β-D-galactopyranoside (ONPG): Diagnostic Chemicals Ltd. Used as a freshly made 4mg/ml solution in 0.2M phosphate buffer, pH 7.0 (for Miller LacZ assays) or in TZ8 buffer (for microtitre plate LacZ assays).

Polymyxin-B sulfate: Sigma Chemical Co. Stored as 20mg/ml solution in H_20 at –20°C.

Potassium acetate (KAc): B.D.H. Labs., Australia.

Potassium chloride (KCl): B.D.H. Labs., Australia.

Sodium acetate (NaAc): B.D.H. Labs., Australia.

Sodium chloride (NaCl): B.D.H. Labs., Australia.

Sodium citrate (Na_3 citrate): B.D.H. Labs., Australia.

Sodium dihydrogen phosphate (NaH_2PO_4): May and Baker Ltd.

Sodium dodecyl sulphate (SDS): Sigma Chemical Co.

Sodium hydroxide (NaOH): Ajax.

Spectinomycin (Sp): Sigma Chemical Co. Stock solutions (50mg/ml in H_2O) were millipore filtered and stored at –20°C.

Tetracycline (Tc): Upjohn Pty Ltd. Stock solutions (10mg/ml in ethanol) were stored at –20°C.

Tris acetate: B.D.H. Labs., Australia.

Xylene cyanol: Sigma Chemical Co.

6.A.6. MEDIA AND BUFFERS.

6.A.6.1. Growth Media.

a. Liquid media.

Luria broth (LB)
1% Bacto-tryptone, 0.5% yeast extract, 1% NaCl, pH 7.0.

Tryptone broth (TB)
1% Bacto-tryptone, 0.5% NaCl, pH 7.0.

All media were prepared in glass distilled H_2O and were sterilised by autoclaving for 25 minutes at 120°C and 120kPa. Antibiotics were routinely added to growth medium at the following concentrations: ampicillin or carbenicillin at 100μg/ml; chloramphenicol at 30μg/ml; kanamycin at 50μg/ml; spectinomycin at 50μg/ml and tetracycline at 10μg/ml. When selecting for plasmids with mini-F origins carbenicillin at 30μg/ml (for pZC-B plasmids) and spectinomycin at 20-25μg/ml (for pZF-B plasmids) were used.

b. Solid media.

L plates
1.5% Bacto-agar was added to L broth, autoclaved and kept molten at 45°C. Plates were poured from ~30ml of the appropriate medium, dried overnight at 37°C and stored at 4°C.

T plates
1.5% Bacto-agar was added to T broth, autoclaved and kept molten at 45°C. Plates were poured from ~30ml of the appropriate medium, dried overnight at 37°C and stored at 4°C.

Soft agar overlay (soft agar)
0.7% Bacto-agar.

Antibiotics were added to the medium before pouring or were spread onto plates as follows: ampicillin or carbenicillin at 100μg/ml; chloramphenicol at 30μg/ml; kanamycin; spectinomycin at 50μg/ml and tetracycline at 10μg/ml. When selecting for plasmids with mini-F origins carbenicillin at 30μg/ml (for pZC-B plasmids) and spectinomycin at 20-25μg/ml (for pZF-B plasmids) were used. When selecting for putative clones or strains expressing a functional LacZ protein, colonies were grown on L plates containing the appropriate antibiotics and supplemented with 20-60μg/ml X-Gal.

6.A.6.2. Buffers and solutions.

Big Dye dilution buffer
200mM Tris-HCl pH 9.0, 5mM $MgCl_2$.

Citrate buffer
0.1M citric acid, 0.1M sodium citrate.

Cloned Pfu DNA polymerase reaction buffer (10x)
200mM Tris-HCl pH 8.8, 20mM $MgSO_4$, 100mM KCl, 100mM $(NH_4)_2SO_4$, 1% Triton X-100, 1mg/ml nuclease-free BSA. (Stratagene)

Glycerol loading buffer (10x)
50% (v/v) glycerol, 0.40% (w/v) Bromophenol Blue, 0.20% (w/v) Xylene Cyanol, 10mM EDTA.

GTE buffer
50mM glucose, 25mM Tris-HCl pH 8.0, 10mM EDTA pH 8.0.

Ligation buffer (10x)
500mM Tris-HCl pH 7.5, 100mM $MgCl_2$, 10mM ATP, 100mM DTT, 250µg/ml BSA. (New England Biolabs)

Phage Storage Buffer (PSB)
10mM Tris-HCl pH 7.4, 10mM $MgCl_2$, 100mM NaCl, 0.05% gelatin.

TAE (10x)
0.4M Tris-acetate, 0.2M NaAc, 10mM EDTA, pH 8.2.

Taq DNA polymerase reaction buffer (10x)
670nM Tris-HCl pH 8.8, 166mM $(NH_4)_2SO_4$, 0.45% Triton X-100, 2mg/ml gelatin. (Biotech International)

TE
10mM Tris-HCl pH 8.0, 1mM EDTA.

TBE (10Xx)
0.89M Tris-HCl, 0.89M boric acid, 2.7mM EDTA, pH 8.3.

TZ8 buffer
100mM Tris-HCl pH 8.0, 1mM $MgSO_4$, 10mM KCl.

TZ8+P50
TZ8 buffer with 2.7µl/ml β-ME and 50µg/ml polymyxin-B added.

Z buffer
100mM Na_2PO_4 buffer pH 7.0, 10mM KCl, 1mM $MgSO_4$, 2.7µl/ml β-ME

6.A.7. DNA MARKERS.

DNA size markers were all purchased from GeneWorks (Australia) and 250ng or 500ng were routinely loaded on an agarose gel.

HpaII digest of pUC19 DNA at 500ng/µl. Fragment sizes in bp: 501, 489, 404, 331, 242, 190, 147, 111, 110, 67, 34, 34, 26.

EcoRI digest of phage SPP-1 DNA at 500ng/µl. Fragment sizes in kb: 8.51, 7.35, 6.11, 4.84, 3.59, 2.81, 1.95, 1.86, 1.51, 1.39, 1.16, 0.98, 0.72, 0.48, 0.36.

6.A.8. DNA PURIFICATION KITS.

All kits were used according to the manufacturers specifications, using the buffers, solutions and materials contained within the kit. Elution buffer was 10mM Tris pH 8.0.
QIAfilter Plasmid Midiprep Kit: Qiagen.
QIAGEN Plasmid Midiprep Kit: Qiagen.
QIAprep Spin Miniprep Kit: Qiagen.
QIAquick Gel Extraction Kit: Qiagen.
QIAquick PCR purification Kit: Qiagen.
Ultra Clean PCR Clean-up DNA purification Kit: MO BIO Laboratories.

6.B. METHODS.

6.B.1. BACTERIAL PROCEDURES.

6.B.1.1. Storage of bacterial stocks.

Bacterial colonies were maintained in the short term on the appropriate plates at 4°C.
A final concentration of 40% glycerol was added to bacterial cultures for long term storage at –80°C.

6.B.1.2. Growth of bacterial strains.

All cultures were routinely grown at 37°C, except for lysogens of the 186 phage carrying a *c*Its mutation, which were grown at 30°C. Stationary phase bacterial cultures for plasmid purification were prepared by inoculating broth with a single colony of bacteria from a plate stock and incubating overnight with aeration at the appropriate temperature. Log-phase cultures and indicator bacteria were prepared by diluting a fresh stationary phase

culture or adding a colony of bacteria into sterile broth and incubating with aeration at the appropriate temperature, until the required cell density was reached. Cell density was measured by observing the optical density at 600nm of the culture (or OD_{600}) using a Gilford 300 T-1 Spectrophotometer. The number of colony-forming units per ml (or cfu/ml) in a culture was determined by plating 100µl of culture diluted in LB with 3ml soft agar onto T or L plates and incubating at the appropriate temperature overnight.

6.B.1.3. Preparation and Transformation of $CaCl_2$ competent cells.

Bacterial cells competent for DNA transformation were prepared from fresh cultures grown with aeration at the appropriate temperature to an OD_{600} 0.5-0.7. The culture was chilled on ice and the cells harvested by centrifugation (5-10 minutes, 5000rpm, 4°C). Cells were then resuspended in one tenth of the original culture volume of cold 0.1M $CaCl_2$ and left on ice for between 1 hour and several days, before being transformed. Competent cells were sometimes stored at –80°C after adding glycerol to a final concentration of ~15%. (Frozen cells were thawed on ice before use.)

Chemically competent cells were transformed by the 5 minute method of Pope and Kent (1996). 100-200µl of cells were aliquotted into Eppendorf tubes on ice and 1-10µl of DNA solution (usually 5-10ng DNA) added. The mixture was incubated on ice for 1-5 minutes, then spread directly onto selective agar plates pre-warmed to 37°C, which were incubated overnight at 37°C. When cells carrying a 186 *c*Its or 186 *c*Its *B*am17 prophage were transformed, plates were pre-warmed and incubated at 30°C. Cells were transformed with 1-10ng pBS KS^+ DNA to determine cell competence and transformation efficiency. Colonies containing recombinant plasmids were identified by PCR with primers internal to or flanking the insert. Alternatively, or in addition, plasmid DNA of putative clones was extracted and analyzed by restriction enzyme digestion.

6.B.1.4. Determining the generation time of bacterial cultures.

The generation or doubling times of 186 lysogenic and non-lysogenic cultures was determined after following the optical density at 620nm of cultures grown in 96-well microtitre plates. For cultures carrying the pZE15-B plasmid, cells were grown at 37°C in selective LB media in the presence of IPTG to induce B protein expression. Single colonies were resuspended in 150µl of media and used to inoculate 2ml of media containing IPTG, which was grown overnight at 37°C with rotation. Overnight cultures were diluted to approximately the same cell density and 2µl of each was subcultured into 98µl of media containing the same IPTG concentration, in a 96-well microtitre plate, and incubated at

37°C with rotation. Lysogens of 186 *cI*ts or 186 *cI*ts *OB1* were grown at 30°C in the absence of antibiotics or IPTG (i.e.in LB only). Single colonies were resuspended in 200µl LB in a 96-well microtitre plate, which was shaken for 15 seconds, sealed and incubated at 30°C overnight without rotation. The next day, 2µl of overnight culture was added to 98µl LB in a 96-well microtitre plate, shaken and incubated at 30°C with rotation.

The optical density at 620nm (OD_{620}) of subcultures was measured using a Labsystems Multiskan Ascent plate reader with a 620nm filter, every 30 minutes until cultures reached an OD_{620} ~0.3. OD_{620} measurements were converted to equivalent optical density at 600nm (OD_{600}) using an empirical relationship described by Dodd *et al.* (2001), and the background OD_{600} of LB media alone was subtracted. Log_{10} derivatives of these values were calculated and the slope (K) of the graph of $log_{10}(OD_{600})$ values plotted against time was determined in Microsoft Excel using linear regression, for OD_{600} readings between 0.05 and 1.0 (i.e. log-phase growth). Generation times were then calculated as log2/K (Miller, 1972).

6.B.2. PHAGE PROCEDURES.

6.B.2.1. Phage stocks.

Low titre phage stocks were made by eluting 1-3 plaques in 100µl PSB or by taking the supernatant of overnight cultures of lysogenic strains. A few drops of chloroform were added and stocks were stored at 4°C.

High titre phage stocks were made by plating 10^4-10^5 pfu onto indicator bacteria, for confluent lysis of the indicator lawn. After overnight incubation at 37°C the top agar was scraped into 2-3ml of LB, shaken and left at room temperature for ~15 minutes for phages to elute. After centrifugation at 5000rpm (5K) for 10 minutes at 4°C, the supernatant was removed, a few drops of chloroform added and the stock stored at 4°C. When making P2 high titre stocks, 1mM EDTA was added to the scraped top agar in 2ml LB or PSB. When making high titre P1 phage stocks, 1-5x10^6 pfu of P1 were plated, and stocks were treated with chloroform then removed to a new tube and stored (in the absence of chloroform) at 4°C.

6.B.2.2. Plating and assaying phage stocks.

186 phages were plated by adding 100µl of phage solution diluted in PSB to 200µl of log-phase indicator bacterial culture, mixing with 3ml of molten soft agar overlay (soft agar)

and pouring onto L or T plates. Plates were incubated overnight at 37°C, and plaques were counted and scored as plaque-forming units per ml (pfu/ml). P1 and P2 stocks were plated as for 186 except that 5mM $CaCl_2$ was added to the LB medium, L plates and soft agar. When plating λ stocks 10mM $MgCl_2$ or $MgSO_4$ was added to the soft agar and L plates were always used. For screening of lambda reporter phages carrying a promoter-*lacZ* fusion or functional *lacZ* gene, up to 60μg/ml of X-Gal was also added to the soft agar prior to pouring.

6.B.2.3. P1 transductions.

P1 transductions were used for the transfer of a chromosomal antibiotic-resistance marker gene and the surrounding DNA to a new bacterial strain. A P1 stock was prepared by passaging twice on the transduction donor strain to be used. For transduction, a fresh turbid or overnight culture (OD_{600}>1) of the recipient strain was grown in LB plus 5mM $CaCl_2$, and 500μl of culture was pelleted and resuspended in the same volume of 100mM $MgCl_2$, 5mM $CaCl_2$ solution. 50μl of this solution was then added to 50μl of P1 stock containing ~10^8-10^9 pfu (i.e. neat or diluted10^{-1} or 10^{-2} in PSB). To control for contaminating antibiotic-resistant cells, 50μl of PSB was added to the recipient solution, and 50μl of 100mM $MgCl_2$, 5mM $CaCl_2$ solution was added to 50μl of P1 stock containing ~10^9 phages. Infections were placed at 37°C for 20 minutes before 100μl of citrate buffer was added to prevent further P1 infections. The mixtures were then spread immediately onto selective L plates and incubated overnight at 37°C. X-gal was spread onto selective plates when transducing the *lacZ*::Km^R insertion into a Lac^+ recipient.

*6.B.2.4. Construction of chromosomal promoter-*lacZ *reporters.*

The method of Simons *et al.* (1987) was used to make a single-copy chromosomal transcriptional fusion of a promoter to a *lacZ* reporter gene. Firstly, the promoter sequence to be assayed was cloned into the polylinker of a *lacZ* reporter plasmid, like pMRR9R or pTL61T, such that the promoter controls *lacZ* transcription. This fusion was then recombined *in vivo* with the modified lambda phage, λRS45 (or derivative λRS45ΔYA), which has homologous *bla* and *lac* operon sequences flanking the polylinker and promoter sequence, and the resultant phage carrying the promoter-*lacZ* fusion was lysogenized in a Lac^- host strain.

Transfer of the promoter-*lacZ* fusion was achieved by plating or spotting 5-10μl of a high titre λRS45 (or λRS45ΔYA) phage stock onto $RecA^+$ indicator bacteria (NK7049 or MC1061) carrying the reporter plasmid. The cleared area was scraped into PSB and the

supernatant treated with chloroform. The resultant stock was plated onto a NK7049 lawn containing up to 60μg/ml X-Gal and single blue plaques formed by recombinant phages expressing the *lacZ* gene were selected, and purified several times. To isolate lysogens, 5-10μl of the reporter phage stock was spotted or spread onto a lawn of the recipient strain, and the centre of a turbid plaque was streaked out for colonies onto L plates spread with up to 40μg/ml X-Gal. Blue colonies carrying the lambda reporter prophage were purified several times by restreaking, and single or multiple lambda lysogens were distinguished by the PCR analysis method of Powell *et al.* (1994). In this method, primers within and around the lambda attachment site (#155, #156, #157) give a single product of 501bp for single lysogens, and an additional product of 379bp for multiple lysogens. Colonies to be tested were washed several times in water to remove free phages, which can also give a 379bp PCR product with these primers. Purified single lysogens were kept for analysis.

Usually two independent recombinant lambda phages were purified, lysogenized and assayed, to ensure that spontaneous mutation within the promoter or *lacZ* sequence had not occurred. The promoter to be assayed was also often sequenced from a PCR product of the chromosomal reporter.

6.B.2.5. Construction of 186 lysogens.

To isolate 186 lysogens, a dilution of the 186 phage was plated or 5-10μl of neat stock spotted onto a lawn of the bacterial strain to be lysogenized and the plates incubated overnight at the appropriate temperature. The centre of a turbid plaque was then streaked out for single colonies on an L plate and incubated overnight. Single colonies were tested for immunity to 186 infection by streaking across a dried line of 30μl 186 *cI*10 phage stock on an L plate, and/or PCR tested for the 186 prophage. Lysogens thus identified were purified at least twice by streaking for single colonies, and the presence of the prophage was confirmed before making a glycerol stock.

6.B.2.6. Assaying free phages in the supernatant of 186 lysogenic cultures.

Cultures of 186 lysogens carrying the *cI*ts mutation were grown from single colonies in 2ml LB at 30°C with rotation, until an OD_{600} ~0.6 was reached. An aliquot of each culture was diluted and 100μl of a 10^{-5} dilution plated for colonies (cfu/ml) at 30°C. 1ml aliquots of each culture were also centrifuged in Eppendorf tubes at 12K for several minutes to pellet cells. The supernatants were removed, treated with chloroform and diluted in PSB and 100μl of a 10^{-3} dilution was plated with C600 indicator bacteria for free phages (pfu/ml).

The ratio of pfu/ml to cfu/ml was determined for each culture and multiplied by 100 to give the number of phages released per 100 bacterial cells.

6.B.2.7. Temperature induction of 186 cI*ts lysogens.*

Temperature inductions were performed on lysogens of 186 which carry a temperature sensitive mutation in the *cI* gene (and sometimes other phage mutations) and were transformed with plasmids as described. Single colonies were grown in 2ml LB plus appropriate antibiotics at 30°C to an OD_{600} ~0.6, before being diluted 10^{-2} to 10^{-4} into 50ml of the same medium in a shaking 39°C waterbath, for temperature induction. To determine the number of infectious centres present, aliquots taken at various times after induction were diluted immediately in PSB, and plated with C600 indicator on T plates. Plates were incubated overnight at 37°C. To determine the number of free phages present, aliquots taken at different timepoints were added to 10µl chloroform and kept on ice until the end of the assay. After a short spin, samples were then diluted in PSB and plated with C600 indicator on T plates, which were then incubated at 37°C overnight.

The burst size – or average number of phages released per cell – was calculated as the average number of free phages present after the burst (or phages released by the burst), divided by the average number of infectious centres (or lysogenic cells) present prior to the burst. The timing of the phage burst was quantitated by determining the time taken to reach the mid-point of the rise in free phages (on a logarithmic scale), or mid-rise time. This was calculated by taking the average level of free phages present prior to the burst (pre-burst) and following the burst (post-burst). The log of pre-burst and post-burst levels of free phages was determined and the average taken (to one decimal place). The time taken to reach this value was then determined to within 0.1 minute from a graph of log(free phage) against time for the assay.

Temperature inductions of 186 *cI*ts lysogens (or derivatives) also carrying a lambda *lacZ* reporter prophage were performed in the same way except that cultures were induced by transfer to a 39°C waterbath without dilution. LacZ activity of the cultures with time after induction was assayed as described in Section 6.B.4.2.

6.B.2.8. 186^{+} phage infection.

For 186 infection, 186^{+} phages were added at a multiplicity of addition of 0.1-0.7 to 200µl of a log-phase bacterial culture of OD_{600} ~0.6 (where E251 cells at OD_{600} 0.6 gave ~$2x10^{8}$ cfu/ml). The volume was made up to 250µl with PSB and the mixture was incubated at

37°C for 10 minutes to allow for phage adsorption and infection. 186^+ phages were then neutralized by the addition of 0.5µl 186-antiserum and incubation at 37°C for 4 minutes; and the infection was diluted 10^{-2} or 10^{-3} into 20-50ml of selective LB medium and incubated at 37°C with shaking. Aliquots taken at different times after infection were directly diluted in PSB and plated with C600 indicator on T plates to assay the number of infectious centres present. Aliquots were also taken into 10µl of chloroform on ice, and after the assay were subjected to a quick spin, diluted in PSB and plated with C600 indicator on T plates, to measure the number of free phages present. The burst sizes and mid-rise times were calculated as described for temperature induction assays.

6.B.2.9. Frequency of lysogeny assays at 30°C.

Cultures of the host strain were grown in LB (with appropriate antibiotics) at 37°C to an OD_{600}~0.6, before cells were harvested and resuspended in fresh LB to an OD_{600}~1.2. 186 *cI*ts or 186 *cI*ts *OB1* phages (or PSB as a control) were added to 1ml of concentrated host culture at an approximate multiplicity of addition of 0.25, and the volume made up to 1.25ml total with PSB. Infection mixtures were incubated at 30°C without shaking for 10 minutes to allow adsorption to occur. To measure the number of lysogens present in the infection, 200µl of 10^{-4} dilution (in LB) of the infection mix was added to ~10^9 pfu of 186 *cI*10 phages (to kill non-lysogenic cells). 3ml of molten soft agar was then added and the mixture plated onto T plates which were incubated at 30°C overnight. To assay the number of phages added in the infection 100µl of a 10^{-5} dilution of the infection mix was plated with C600 indicator bacteria onto T plates and incubated at 37°C overnight. To measure the total number of infections, 0.5µl of 186 antiserum was added to a 250µl aliquot of the infection mix (neat), and placed at 30°C for 4 minutes, to allow antibody binding and neutralization of free phages. 100µl of a 10^{-4} dilution (in LB) of this mixture was then plated with C600 indicator onto T plates, and incubated at 37°C overnight. Concentrated cultures were also assayed for cfu/ml by diluting in LB and plating 100µl of 10^{-5} dilution with soft agar onto T plates which were incubated overnight at 30°C. Generally, plates were left at room temperature for another night prior to counting to allow small colonies and plaques to become larger, and therefore easier to count.

The numbers of colonies and plaques counted were used to calculate the number of lysogenic colonies, phages added or cells infected (respectively) in the original infection mix. The PSB control generally gave a few colonies (0-5 colonies) at 30°C, due to 186 resistant cells (186^R) in the 186 *cI*10 stock. These were subtracted from the total number of 186^R colonies given by the phage infection on that day, to give the total number of lysogens

formed by the phage. The yield of lysogens was calculated as the total number of lysogens formed expressed as a percentage of the total number of phages added. The frequency of lysogeny was determined as the total number of lysogens formed as a percentage of the total number of infections.

6.B.3. DNA MANIPULATION.

6.B.3.1. Plasmid DNA minipreps.

Small-scale plasmid DNA preparations were performed using QIAprep Spin Plasmid Miniprep Kit (Qiagen) according to the manufacturers instructions. DNA was eluted using either 30µl or 50µl of 10mM Tris pH 8.0, and was routinely stored at –20°C.

6.B.3.2. Large-scale plasmid purification of high copy number plasmids.

Large-scale preparations of plasmid DNA for high copy number plasmids, were obtained either by using a Qiagen Midiprep kit or by alkaline extraction followed by sedimentation on a caesium chloride (CsCl) gradient. The resultant DNA was stored at –20°C.

Midiprep DNA from 50ml overnight cultures was prepared using the QIAGEN or QIAfilter Midiprep Kit (Qiagen) according to the manufacturers instructions. Purified DNA was resuspended in a final volume of 200µl 10mM Tris pH 8.0.

For alkaline extraction, fresh overnight cultures grown in 50ml selective LB media at 37°C with aeration, were harvested by centrifugation at 5-10K for 10 minutes at 4°C. The cell pellet was resuspended in 5ml GTE buffer plus 4mg/ml lysozyme (when available) and left at room temperature for 10 minutes. 10ml 0.2M NaOH, 1% SDS solution was then added, mixed gently and placed on ice for 10 minutes, before 7.5ml ice-cold KAc solution (3M KAc plus glacial acetic acid) was added. After 10 minutes on ice, precipitated cell proteins and debri were pelleted (15K, 15 minutes, 4°C) and the supernatant warmed to room temperature. The DNA was isopropanol precipitated and resuspended in 1.5ml TE. For CsCl purification, 1g/ml of solid CsCl was dissolved in 1.4ml of the DNA solution, and 20µl of ethidium bromide (10mg/ml) added. This solution was transferred to a Beckmann quickseal tube, overlaid with mineral oil and centrifuged overnight at 80K, at 20°C. The supercoiled plasmid DNA band (below that of nicked or chromosomal DNA) was visualized under UV light, and collected with a syringe. Ethidium bromide in the solution was removed by extraction with isopropanol saturated with 5M NaCl, 10mM Tris pH 8.0, 1mM EDTA pH 8.0. Plasmid DNA was then diluted with 2 volumes of TE, precipitated

with 7 volumes of 95% ethanol and the pellet resuspended in TE. Further contaminants (such as salt) were removed by re-precipitating with ethanol and the DNA was resuspended in a final volume of 50-100μl TE for storage.

6.B.3.3. Large-scale plasmid purification for very low copy number plasmids.

Large-scale preparations of plasmid DNA for very low copy number plasmids (with mini-F or pSC101* origins) were obtained either by using the QIAGEN Midiprep Kit (Qiagen) protocol for very low copy number plasmids; or by using a larger scale method for alkaline extraction and CsCl gradient purification.

Midiprep DNA was extracted from 500ml overnight cultures according to the protocol for very low copy number plasmids. Purified DNA was resuspended in 200μl 10mM Tris pH 8.0 and stored at –20°C.

For larger scale CsCl gradient purification, essentially the same protocol was used with the following changes. 400ml of overnight culture was harvested and resuspended in 8ml of GTE buffer plus lysozyme, before a 10 minute incubation on ice. 16ml NaOH/SDS solution was added and the solution incubated on ice for 5 minutes, before the addition of 12ml KAc solution. The mix was placed on ice for at least 15 minutes before being pelleted. After isopropanol precipitation of the supernatant, the nucleic acid pellet was resuspended in 4ml TE, and subjected to a second spin (5K, 5 minutes, 4°C) to remove insoluble material. CsCl (at ~1g/ml) and up to 500μl of ethidium bromide were then added and the solution centrifuged overnight at 80K as previously described. Plasmid DNA was removed and ethanol precipitated as above (Section 6.B.3.2.), except that Eppendorf tubes were used for the precipitation and incubation was at –20°C for at least an hour. Plasmid DNA was then re-precipitated with ethanol and resuspended in a final volume of ~150μl TE, to be stored at –20°C.

6.B.3.4. Nucleic acid precipitation.

Ethanol or isopropanol precipitation of plasmid DNA (or a PCR product) was routinely used to remove salt and other contaminants, or to concentrate the DNA. DNA was routinely stored at –20°C.

For ethanol precipitation, 0.1 volumes of 3M NaAc pH 5.2, 2 volumes 95% cold ethanol (RNase-free) and sometimes 1μl glycogen were added to the DNA. The solution was mixed and incubated on ice for 5-20 minutes. Nucleic acids were pelleted by centrifugation at 12K

for 15 minutes at room temperature, and the pellet was washed with 70% ethanol, dried and resuspended in 10mM Tris pH 8.0 or 1xTE.

For isopropanol precipitation, 0.6 volumes of isopropanol were added, mixed and incubated at room temperature for at least 15 minutes. The solution was then centrifuged at 12-15K for 15-20 minutes at 20°C. The pellet was rinsed with 70% ethanol, dried and resuspended in 10mM Tris pH 8.0 or 1xTE.

6.B.3.5. Agarose gel electrophoresis.

DNA purity, size and quantity were determined by agarose gel electrophoresis in horizontal minigels. DNA samples were combined with Glycerol loading buffer, loaded onto 1-2% agarose gels and electrophoresis performed in 1xTAE buffer at 90-120 Volts. DNA bands were visualized after staining with low concentration ethidium bromide solution, and photographed under short wavelength UV light. Comparison of band intensity with that of molecular weight markers of known concentrations, allowed approximate DNA concentrations to be determined.

6.B.3.6. Precise determination of DNA concentration.

The DNA concentration of plasmid solutions to be used in Quickchange reactions were determined accurately by spectrophotometric measurement of the adsorption at 260nm (or A_{260}). Plasmid DNA was diluted in 10mM Tris pH 8.0 and the A_{260} was measured using a Cary 3 Bio UV-visible spectrophotometer. The concentration of the DNA was then calculated using the assumption that 50μg/ml of double-stranded DNA gives an A_{260} measurement of 1.

6.B.3.7. Restriction enzyme digestion.

Restriction enzyme digests were performed in conditions recommended by the manufacturers, in 10-50μl reaction volume for 1 hour to overnight at the recommended temperature. Digestion was checked by agarose gel electrophoresis.

6.B.3.8. Isolation of DNA fragments from agarose gels.

Digested DNA fragments or plasmids required for cloning were run on an agarose gel, visualized under long wavelength UV light using EtBr staining, and excised by cutting with a scalpel blade. DNA was isolated from the agarose using the QIAquick Gel Extraction Kit (Qiagen) as specified by the manufacturers.

6.B.3.9. Reactions with alkaline phosphatase.

Linearised vectors with compatible ends were treated with Shrimp alkaline phosphotase (SAP) to reduce background religation. 1-2units of SAP were added to a restriction digest and incubated for 1 hour at 37°C. The enzyme was inactivated by heating to 65°C for 20 minutes. The efficiency of the SAP reaction was checked by transformation of ligation reactions containing the same concentration of vector DNA without the addition of DNA ligase.

6.B.3.10. Blunt ending 5' overhangs.

T4 DNA polymerase was used to fill-in 5' overhangs. 1-3units of T4 DNA polymerase was added to a restriction enzyme digest supplemented with 100μg/ml BSA and 100μM of each dNTP. The reaction was incubated at 16°C for 30 minutes, before the enzyme was heat-inactivated at 75°C for 10 minutes.

6.B.3.11. Polymerase Chain Reaction (PCR).

Polymerase Chain Reactions for screening of clones or amplification of a template for sequencing were routinely performed 10μl reactions containing 1x Taq DNA polymerase reaction buffer, 2.5mM $MgCl_2$, 0.2mM each dNTP, 10ng of each primer and 0.5units of Taq DNA polymerase. To amplify from plasmid template ~0.1-10ng of DNA was added to the reaction, and to amplify from phage DNA ~10^4-10^6 pfu were added. To screen colonies or sequence from a prophage, a colony was picked and swirled into the reaction mixture, or resuspended in 100μl of water, and 1μl of the resuspension added to the reaction. (Heating of the bacterial cells or phages during the temperature cycles results in lysis and release of the template DNA.) PCR reactions were performed in a Rapidcycler from Idaho Technology in thin-walled 0.2ml PCR tubes. Temperature cycles routinely used for PCR products of <1kb were 98°C for 10 seconds, then 30 cycles of (98°C for 10 seconds, 46°C for 10 seconds and 74°C for 15 seconds). Longer extension times at 74°C (of up to 2 minutes) were used to generate longer PCR products.

Pfu Turbo DNA polymerase was used in PCR reactions to amplify DNA for cloning. 10μl reactions contained 1x cloned Pfu DNA polymerase reaction buffer, 0.2mM each dNTP, 10ng of each primer and 0.5-1unit of Pfu Turbo DNA polymerase. Template DNA was added and an Idaho Rapidcycler used as above, except that the extension times used for Pfu Turbo DNA polymerase reactions were twice as long as those used for Taq DNA polymerase reactions. If PCR products were to be used in further enzymatic reactions, a

number of PCR reactions were pooled and purified using the QIAquick PCR purification kit or Ultra Clean PCR Clean-up DNA purification Kit.

6.B.3.12. DNA ligation reactions.

Ligations were performed in 10µl reactions containing 1x Ligation buffer, 0.5-2units T4 DNA ligase and approximate 3:1 ratio of insert to vector DNA (10-100ng). A control was usually made up in the same way but lacking the insert DNA, to determine the number of background colonies from the vector DNA alone. The reactions were incubated for 1-16 hours at 16°C or room temperature, before half of the ligation mix was used to transform calcium chloride competent cells.

6.B.3.13. Site-directed mutagenesis.

The QuickChange method of Stratagene was used to generate a mutation, *OB1,* in the 186 *B* promoter fragment cloned into pBS KS^+. Complementary primers were designed which contained the mutation and enough wildtype sequence flanking the mutation to allow stable annealing of the primers to the vector. Quickchange mutagenesis was performed in a 50µl reaction, containing 1x cloned Pfu DNA polymerase reaction buffer, 100µM each dNTP, 125ng of each primer, 20ng of plasmid DNA template and 2.5units of Pfu Turbo DNA polymerase. A control reaction was identical except for the omission of the polymerase. The reactions were overlaid with 30µl mineral oil and kept on ice before placing in a MJ Research, Inc. PTC-100™ Programmable Thermal Controller already heated to 90°C. Reactions were incubated at 95°C for 30 seconds, then subjected to 18 cycles of (95°C for 30 seconds, 55°C for 1 minute and 68°C for 9 minutes). Agarose gel electrophoresis of a small amount of the reactions showed a plasmid DNA band in the Quickchange reaction but not the control tube. To digest the template DNA, 10units of the methylation-dependent DpnI restriction enzyme was added to each reaction mixture, and the digest placed at 37°C for 2 hours. 5µl of each reaction was then transformed into calcium chloride competent cells. No background colonies were seen for the control reaction. Sequencing was used to confirm the presence of the mutation.

6.B.3.14. Big Dye Sequencing reactions.

DNA sequencing was performed using Perkin-Elmer ABI PRISM Big Dye Version 3 with a purified PCR product as a template. Reactions contained 2-8µl of Big Dye V3 Ready Mix plus Big Dye dilution buffer to make a total volume of 8µl, 30-180ng of template and 3.2pmol of primer in a total volume of 20µl. Sequencing reactions were incubated in thin-walled 0.2ml PCR tubes in an Idaho Technology Rapidcycler, at 96°C for 20 seconds, then

25 cycles of (96°C for 30 seconds, 50°C for 30 seconds and 60°C for 4 minutes). To precipitate sequencing reaction products 80µl of 75% isopropanol was added and the mixture vortexed briefly before being incubated at room temperature for at least 15 minutes. Products were collected by centrifugation at 12K for 20 minutes at room temperature. The pellet was then washed with 250µl 75% isopropanol, centrifuged for a further 5 minutes and the supernatant removed. Samples were dried and analyzed at the Institute of Medical and Veterinary Science Sequencing Centre. The resultant chromatograph files were viewed using EditView 1.0.1 and compared to wildtype sequence using a Blast nucleotide search of database files or DNA Strider 1.3.

6.B.4. LacZ ASSAYS.

6.B.4.1. Miller LacZ assays.

β-galactosidase (or LacZ) activity was initially measured using the method of Miller (1972). Single colonies were cultured overnight in 2ml of selective LB media at 37°C with aeration. The following day cells were diluted 200-fold into 2ml of the same medium and grown at 37°C to an OD_{600} between ~0.3 and 0.9. The background OD_{600} of the LB medium without culture was also determined. 0.6ml of the culture was then added to 0.6ml of Z buffer containing 35µl chloroform and the mixture placed on ice. Tubes were vortexed for 10 seconds and placed in a 28°C waterbath for 5-10 minutes to warm, before the reaction was started by the addition of 0.24ml of prewarmed 4mg/ml ONPG solution (freshly made in 0.1M Na_2PO_4 pH 7.0). At an appropriate time, 0.6ml of 1M Na_2CO_3 solution was added to stop the reaction, cell debri was pelleted by centrifugation at 5K for 5 minutes and the OD_{420} of the reaction solution was determined. LacZ units were calculated as OD_{420} value multiplied by 1000, divided by the reaction time (in minutes), the culture volume as a fraction of the total reaction volume (i.e. 0.5) and the difference between the OD_{600} of the culture and LB.

6.B.4.2. Microtitre plate LacZ assays.

LacZ activity in a culture was generally determined by a kinetic assay in microtitre plates described by Dodd *et al.* (2001). For this assay, single colonies were resuspended in 200µl of selective LB media in 96-well microtitre plates, which were sealed and incubated overnight at 37°C. The following day, cells were subcultured 1 in 50 into 100µl of the same medium, shaken for 15 seconds using a Labsystems Multiskan Ascent plate reader and incubated at 37°C with rotation. Optical density of the cultures was assayed using the plate reader and a 620nm filter, and OD_{620} measurements were converted to equivalent OD_{600}

values by the method of Dodd *et al.* (2001). Aliquots of up to 50μl of culture at OD_{600} ~0.2-1.0, were then combined with 150μl of TZ8+P50 buffer (which included polymyxin-B to permeabilize the cells) and LB added to make a total volume of 200μl. Plates were shaken for 15 seconds in the plate reader and placed at 30°C for 15 minutes to warm. 40μl of pre-warmed 4mg/ml ONPG solution (in TZ8 buffer) was then added and a kinetic LacZ assay performed. In brief, plates were incubated at 28°C in the plate reader, and OD_{414} readings taken every 2 minutes for 1 hour. The slope of OD_{414} versus time was determined and LacZ units were calculated as 200 000 multiplied by the slope and divided by the OD_{600} and the volume (in μl) of the culture assayed. These units were roughly equivalent to LacZ units determined by the Miller assay (Dodd *et al.*, 2001).

When measuring activity of a promoter-*lacZ* reporter in the presence of a regulatory protein whose expression was induced with IPTG (see Figures 4.1 and 4.4), colonies were suspended in 100-200μl LB and 1-2μl diluted into 200μl of selective media containing a range of IPTG concentrations for overnight incubation. Cells were subcultured into the same medium containing IPTG. When assaying 186 *pR* repression by CI (see Section 4.B.3.1.), overnight cultures (in IPTG) were diluted 1μl into 199μl of selective media containing IPTG and incubated overnight for a second time before subculturing. (This was required to remove stable LacZ protein expressed from pR – Dodd and Egan, 2002.)

When assaying 186 *pV* reporter activity in 186 *cI*ts or 186 *cI*ts *OB1* lysogenic cells (see Section 4.B.2.3.), microtitre plate assays were performed as above, except that cultures were grown at 30°C, and cells were subcultured by a 1/25 dilution or 1/50 dilution of the overnight cultures into 100μl of media.

When assaying activity of a chromosomal *lacZ* reporter during temperature induction of a 186 *cI*ts (or 186 *cI*ts *B*am17) prophage (see Section 6.B.2.7.), 50μl aliquots of the culture taken at various times after transfer to 39°C were added (in duplicate) to 150μl TZ8+P50 pre-aliquotted into a 96-well microtitre plate on ice. After all timepoints were taken, the plate was shaken for 15 seconds and placed at 30°C for 30 minutes to warm. ONPG solution was then added and a kinetic LacZ assay performed as described above.

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