Studies on the *ftsW* and *mraY* genes of *Escherichia coli.*

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To my mother and late father

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Declaration

I declare that the composition of this thesis was all my own work, except where stated.

David S. Boyle

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Acknowledgements

ABSTRACT

The morphogenes are genes required for cell growth and division in *Escherichia coli*. The morphogenes are often grouped as clusters in different regions of the chromosome. The *mra* region, located at 2 minutes on the *E. coli* chromosome is the largest cluster of morphogenes. It contains sixteen open reading frames from which fourteen genes have been identified as encoding for proteins involved in either cell division or the biosynthesis of the cell wall. The organisation of the cluster appears to be complex with the genes tightly packed and often overlapping. Many of the genes appear to share promoters for their expression. However, several of these genes have been incompletely characterized. The predicted proteins encoded by *ftsW*, a cell division gene, and *mraY*, a gene required for murein synthesis, have not yet been identified. One of the aims of this study was to identify the peptides produced from *ftsW* and *mraY*. The mutant phenotypes of these genes are poorly characterized. At the time of this study there was one temperature sensitive *ftsW* allele and there was no mutant allele of *mraY*. In this study null mutants were made for both *ftsW* and *mraY*. This allowed a more accurate characterization of their roles in cell division and cell growth, respectively. The isolation of the mutants allowed studies on their complementation. The promoter regions required for the independent expression of *ftsW* and *mraY* were then identified.

The close proximity of genes in the *mra* region, the presence of overlaps and the sharing of promoters has provoked speculation that several of these genes may be translationally coupled. A previous study on three genes of the *mra* cluster revealed no translational coupling between the three genes but showed differences in the efficiency of translation of each gene product. Part of the work presented in this thesis studied the relationship between the expression of a further six genes of the *mra* region to determine if these genes were translationally coupled and their relative translational efficiencies.

Abbreviations

aa	Amino acid
ADP	Adenosine-5'-diphosphate
Amp	Ampicillin
APS	Ammonium persulphate
ara	Arabinose
ATP	Adenosine-5'-triphosphate
bp	Base pair(s)
BSA	Bovine serum albumen
cAMP	3',5'-cyclic adenosine monophosphate
CAT	Chloramphenicol acetyl transferase
cm	Centimetre
Cmp	Chloramphenicol
cps	Counts per second
CTAB	Hexadecyltrimethyl ammonium bromide
CTP	Cytidine-5'-triphosphate
(d)dATP	2'(3'-di)-deoxyadenosine-5'-triphosphate
(d)dCTP	2'(3'-di)-deoxycytidine-5'-triphosphate
(d)dGTP	2'(3'-di)-deoxyguanosine-5'-triphosphate
(d)dTTP	2'(3'-di)-deoxythymidine-5'-triphosphate
(d)dNTP	2'(3'-di)-deoxynucleoside-5'-triphosphate
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dsDNA	Double-stranded deoxyribonucleic acid
EDTA	Diaminoethanetetra-acetic acid
GTP	Guanosine-5'-triphosphate
HEPES	N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulphonic acid)
IPTG	Isopropyl-β-D-thiogalactoside
Kan	Kanamycin
kb	Kilobase pair(s)
kDa	Kilodalton
mm	Millimetres
moi	Multiplicity of infection
MOPS	Morpholinopropanesulphonic acid
mRNA	Messenger ribonucleic acid
NAcGluc	N-acetylglucosamine
NAcMur	N-acetylmuramic acid
Nal	Nalidixic acid
NCBI	National Center for Biotechnology Information
nm	Nano metre
OD	Optical density
OLB	Oligonucleotide labelling buffer
ONPG	o-nitrophenyl-β-D-galactoside
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
Pi	Inorganic phosphate

PIPES	Piperazine-N,N'bis (2-ethanesulphonic acid)
PIPS	Penicillin insensitive peptidoglycan synthesis
ppGpp	Guanosine 5'-diphosphate 3'-diphosphate
R	Resistant
rbs	Ribosome binding site
Rif	Rifampicin
RNA	Ribonucleic acid
RNase	Ribonuclease
rpm	Revolutions per minute
S	Sensitive
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
Spc	Spectinomycin
STE	Salt Tris-EDTA
SSC	Salt sodium citrate
ssDNA	Single-stranded deoxyribonucleic acid
Str	Streptomycin
TAE	Tris-acetate/EDTA
TE	Tris-EDTA
TEMED	N,N,N',N'-tetramethyl-1,2-diaminoethane
Tet	Tetracycline
Thesit	Dodecylpoly(ethyleneglycolether)$_n$
Tmp	Trimethoprim
TOE	Thermo-oscillatory enrichment
Triton X-100	Octylphenoxypolyethoxyethanol
Tris	2-amino-2-hydroxymethyl-1,3-propandiol
tRNA	Transfer ribonucleic acid
ts	Temperature-sensitive
TTP	Thymidine-5'-triphosphate
UDP	Uridine diphosphate
UTP	Uridine-5'-triphosphate
UV	Ultraviolet
v/v	Volume by volume
wt	Wild-type
w/v	Weight by volume
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactoside

Chapter 1.

Introduction.

Chapter 1. Introduction.

The shape of the *Escherichia coli* cell is determined by the shape of the peptidoglycan or murein sacculus, which is a hollow cylinder with two hemispherical polar caps. The cell cycle is essentially a simple process. During the cell cycle two processes alternate, one is the extension of the lateral cell wall and the other the invagination at the cell midpoint to produce two new hemispherical poles. Cleavage of the two caps results in two identical daughter cells. In addition to the growth and division cycle, the chromosome replicates and each daughter cell receives a copy before the cell divides. The order of these processes is known but the interaction and timing of the processes in relation to each other are unclear. Discussed below are the genetic and biochemical factors thought to have roles in the functions of saccular synthesis, cell division and its control, chromosomal replication and transfer and the relationship between cell shape and division. Also discussed is the control and function of genes in the *mra* locus, a large cluster of morphogenes, all of which appear to be essential in either cell growth or division.

1.1. 1. Murein analysis.

The structure responsible for the cell shape and protection against physical damage and osmotic lysis is the peptidoglycan or murein sacculus. The internal osmotic pressure of the cell has been estimated to be between 2-5 Atmospheres (Mitchell and Moyle, 1957). Therefore, without the presence of a structure enveloping the cell, lysis would occur or the rod-shape of the cell would be compromised if the cells were not in an isotonic solution. The murein layer encompasses the cytoplasmic membrane and is itself covered by the outer membrane of the cell. The sacculus is bound to both the cytoplasmic membrane where new murein is being synthesised (Höltje and Glauner, 1990) and to the outer membrane by peptide cross links with lipoproteins (Braun and Höltje, 1974).

The sacculus is a flexible non crystalline layer (Naumann *et al.*, 1982) formed from repeating subunits of murein. The maximum thickness of the sacculus was determined to be 7 nm +/-0.5 nm by electron microscopy of the hydrated murein (Hobot *et al.*, 1984; Glauner *et al.*, 1988). Studies on the thickness of this layer by small angle scattering revealed that 75-80% is a

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monolayer and only 20-25% is covered with an additional two layers (Labischinski *et al.*, 1991). The position of the triple layer 'patches' is not located to specific areas of the cell, that is triple layers are found in both the cylindrical wall and at the polar caps (Labischinski *et al.*, 1993).

CH2OH
O OH
OH
OH
NHAc
NAcGluc

CH2OH
O OH
O
OH
NHAc
CHCH3
COOH
NAcMur
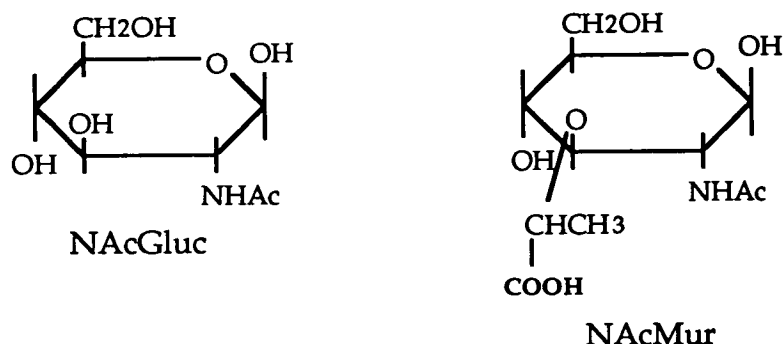
Figure 1.1.1. The structure of the two glycan molecules, N-Acetyl glutamic acid (NAcGluc) and N-Acetyl muramic acid (NAcMur) which together form the glycan polymer of murein strands. The carboxyl group of NAcMur where L-alanine is bound is highlighted in bold.

The murein is a heteropolymer consisting of glycan chains which are linked together by short peptide bridges. The glycan chain consists of two alternating amino sugars, *N*-acetylglucosamine (GlucNAc) and *N*-acetylmuramic acid (MurNAc). The MurNAc residue at the end of the chain is not reducing. Instead, a 1-6 anhydromuramic acid residue is located at the 'reducing' end of the sugar chain (Figure 1.1.2.). The two sugar residues are linked by a β1-4 glycosidic bond. When the disaccharide's are polymerised the bonding is also by β1-4 glycosidic bonds. Bound to the carboxyl group of each MurNAc residue via an amide bond with L alanine is a pentapeptide side chain (Figure 1.1.1.). The peptide is L-alanyl-D-glutamyl-D-meso-diaminopimelyl-D-alanyl-D-alanine. In mature murein these are shortened to tri- or tetrapeptides after enzymatic hydrolysis by DD- and LD carboxypeptidases (Höltje and Glauner, 1990). The precursor for the murein strand is the disaccharide pentapeptide N-acetylglucosamyl-N-acetylmuramyl-pentapeptide. This is incorporated into the nascent strand of murein via a lipid carrier, bactoprenol phosphate (Umbreit and Strominger, 1972; Bupp and van Heijenoort, 1993). The disaccharide pentapeptide is bound to the pre-existing murein strand by a transglycosylation reaction. At a later stage the pentapeptide side chain is modified and bound to other peptide side chains from adjacent glycan strands by carboxypeptidases and

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transpeptidases. The reducing end with MurNAc is made non-reducing by the dansylation of 4C to the carboxyl group (Figure 1.1.2.) The crosslinking of the peptide residues from adjacent murein strands creates a vast macromolecule which encompasses the entire cell; this is the murein sacculus.
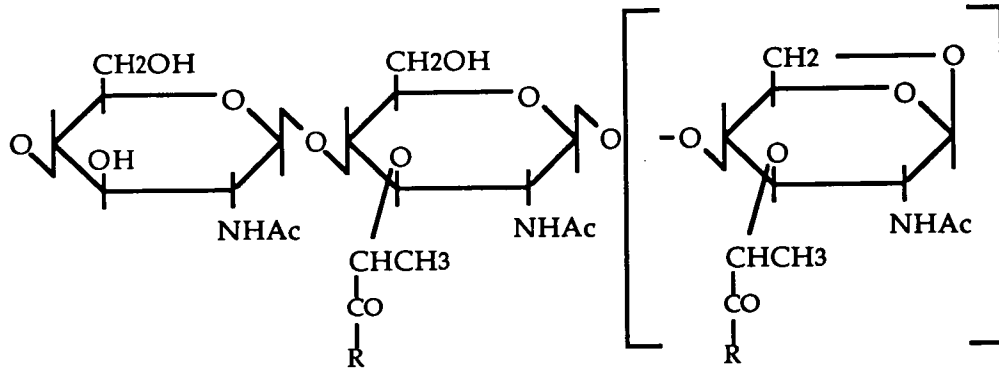
Figure 1.1.2. The structure of the disaccharide residue showing the $\beta1$-4 glycosidic bond linking NAcGluc and NAcMur pentapeptide. Also shown on the right is the dansyl form of NAcMur, this is located at the 'reducing' end of the murein polymer. R indicates the peptide side group. Adapted from Höltje and Glauner (1990).

The average length of each glycan polymer is 30 disaccharide subunits (Glauner *et al.*, 1988). Examination of murein sacculi where the amide bonds have been sheared by either enzymatic or mechanical cleavage reveals that the glycan strands are oriented in parallel, perpendicular to the poles. Schwartz (1993) predicted that 100 glycan chains of an average length of 30 disaccharide subunits are necessary to span the circumference of the bacterial cell; this is if the average circumference is 2µm. Data from HPLC analysis of digested sacculi (Höltje and Glauner, 1990) showed that the mode of the glycan chains was 10 subunits and therefore they concluded that the short chains must be interspersed with longer chains to encircle the cell. X-ray analysis of the glycan strands revealed that the glycan strands were arranged as a helix which rotates 360° every 4.5 glycan subunits (Labischinski *et al.*, 1985). For each rotation through 360° of the glycan strand, there are two peptide side chains, separated by one subunit, which are positioned such that each can cross link to an adjacent glycan strand

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peptide side chain. The remaining two are oriented with one positioned towards the outer membrane and the other towards the inner membrane.

The HPLC analysis of sacculi digested with muramidase by Glauner *et al.* (1988) produced a spectrum of more than 80 different muropeptides. The two most common species isolated were dimers linked by tetra-tri (D-ala-DAP) and tri-tri (DAP-DAP) peptide cross links. Mature murein, that is from cells in stationary phase, differs from nascent murein with respect to the greater degree of peptide cross-linking between glycan strands and also with lipoprotein (Glauner *et al.*, 1988). 4 minutes after insertion into new murein 96% of the pentapeptide side chains are modified by the removal of the terminal D-alanine residue (de Jonge *et al.*, 1989). 3-4% of the muropeptides form cross links with lipoprotein in a growing culture (de Jonge *et al*, 1989).

1.1.1. The synthesis of the lipid-linked precursor molecule for murein biosynthesis.

Knowledge of the subsequent steps in the biosynthesis of the murein precursor Bactoprenol phosphate GlcNAc-MurNAc-pentapeptide (Lipid II) is now almost complete (van Heijenoort *et al.*, 1993). The process can be divided into cytoplasmic and membrane stages. The precursor sugar moiety, UDP-N-acetyl glucosamine (UDP-Gluc-NAc) is utilised for the biosynthesis of murein, lipoprotein and the enterobacterial common antigen (van Heijenoort *et al.*, 1993). It is synthesised in a four stage reaction from fructose-6-phosphate. The biosynthesis of UDP-*N*-acetyl-muramic acid requires UDP-GlucNAc and phosphenolpyruvate (PEP). The condensation reaction of UDP-GlucNAc and PEP is catalysed by the MurA protein to form UDP-N-acetylglucosamine-enolpyruvate (Doublet *et al.*, 1993). This is subsequently reduced to UDP-N-acetyl-muramic acid (UDP-MurNAc) by the MurB protein (Miyakawa *et al*, 1972; Pucci *et al.*, 1992; Doublet *et al.*, 1993). Interestingly the genes for both MurB and MurI are in the same region of the chromosome, the *mrb* (murein region B) region (Miyakawa *et al*, 1972; Doublet *et al.*, 1993).

The sequential addition of the amino acids to form the pentapeptide side chain is performed by a series of proteins whose genes are all located in the same operon, the *mra* region. All stages require one molecule of ATP to energise the subsequent addition of the amino acid by condensation (Figure

1.1.3.). The first amino acid residue added is L-alanine. This reaction is catalysed by MurC. The sequential addition of D-glutamate and *meso*-diaminopimelic acid (DAP) residues is performed by MurD and MurE proteins respectively (Mengin-Lecreulx *et al*, 1989a; Maruyama *et al.*, 1988; Tao and Ishiguro, 1989)
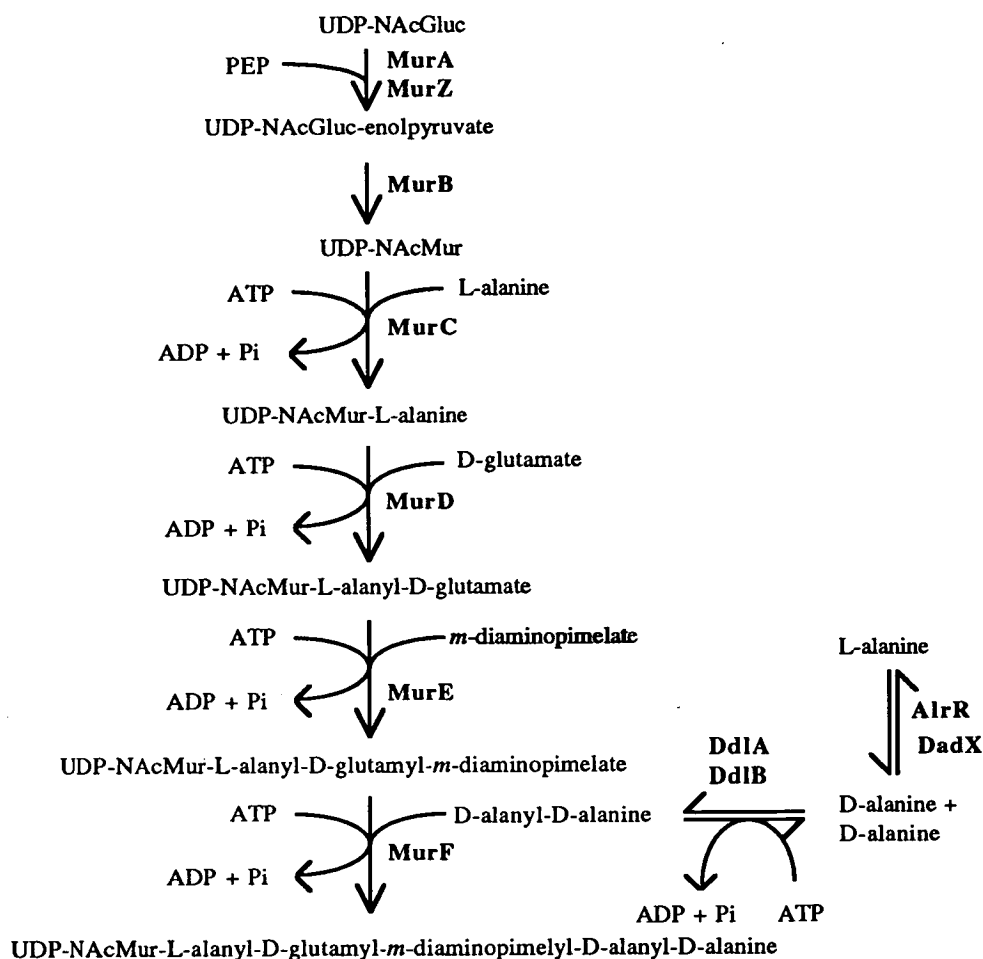
Figure 1.1.3. The stepwise sequence of reactions in the biosynthesis of UDP-NAc-Mur-pentapeptide (adapted from Walsh, 1989).

The D-alanine residues are not added sequentially to UDP-MurNAc-L-alanyl-D-glutamyl-*m*-diaminopimelate; but as a dipeptide, D-alanyl-D-alanine (Figure 1.1.3.). The addition of the dipeptide is performed by the MurF protein (Lugtenberg and van Schinjdel van Dam, 1972; Maruyama *et al.*, 1988). D-alanine is made from its stereoisomer L-alanine by an alanine racemase. Two alanine racemase genes, *alrR* and *dadX*, have been identified in *E. coli.*. Either enzyme can convert L-alanine to D-alanine and it is not

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known if either of the enzymes is specific for cell growth (Wijsman, 1972; Wild *et al.*, 1985; deRoubin *et al.*, 1992). This reaction is reversible. The condensation of two D-alanine residues is performed by D-alanine: D-alanine ligase of which there are two in *E. coli*, both of which are capable of hydrolysing/condensing the dipeptide (Figure 1.1.3.; Lugtenberg and van Schinjdel-van Dam, 1973; Zawadzke *et al.*, 1991). These reactions require the hydrolysis of ATP. It is interesting to ask why *E. coli* (and indeed *Salmonella typhimurium*) requires two D-alanine ligases, either of which complements the *ddl* double mutant ST640 (Zawadzke *et al.*, 1991). The presence of two alanine racemases in *S. typhimurium* may mean that *E.coli* also has a second racemase (Zawadzke *et al.*, 1991) The other pathways in the formation of the UDP-Mur-NAc-pentapeptide have also been studied, and include the production of *m*-diamino-pimelate involving MurZ (Cohen and Saint-Girons, 1987) and the formation of D-glutamate by MurI (Doublet *et al* ., 1992).

The next stage in murein biosynthesis involves the formation of the disaccharide pentapeptide on a carrier lipid located in the cytoplasmic membrane (Figure 1.1.4.). All the enzyme-catalysed events occur on the inner face of the membrane (van Heijenoort *et al.*, 1992). The carrier molecule is undecaprenol (bactoprenol) phosphate, a C_{55} isoprenoid lipid (Wright *et al.*, 1967). The reaction is a three stage process where the phospho-MurNAc-pentapeptide moiety of UDP-MurNAc-pentapeptide is bound to undecaprenol phosphate in a reaction catalysed by the MraY protein (Ikeda *et al.*, 1991). The undecaprenol-pyrophosphate-Mur-NAc-pentapeptide (Lipid I) is the acceptor for the NAc-gluc moiety from UDP-NAc-gluc in a reaction catalysed by the MurG protein (Mengin-Lecreulx *et al.*, 1991) to create a lipid-linked disaccharide pentapeptide, undecaprenol-pyrophosphate-NAcGluc-NAcMur-pentapeptide (Lipid II). Lipid II is the donor of disaccharide-pentapeptide subunits required for murein synthesis. The enzyme required for the translocation step where Lipid II is turned in the membrane to face the periplasm is not known. Dai and Ishiguro (1988) isolated a ts mutant where cells grown at the restrictive temperature were unable to transfer DAP from the cytoplasm to the periplasm. The locus of the mutant gene was found to be at 90.2 minutes. Dai and Ishiguro (1988) named this gene *murH*.

Murein layer ▨▨▨▨▨▨▨▨▨▨▨▨▨▨▨▨▨▨▨▨▨▨▨▨▨▨▨▨▨▨▨▨▨▨▨▨▨▨▨

Periplasm

Cytoplasmic membrane

M r a Y

MurG

MurH?

Cytoplasm

P
+
UDP-MurNAc
p
e
p

UMP

P
P
MurNAc
p
e
p

UMP

P
P
MurNAc-GlcNAc
p
e
p
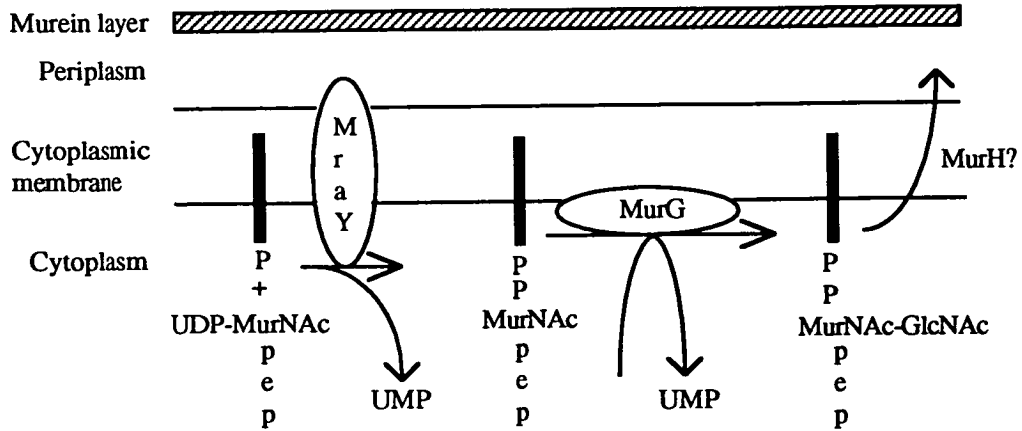
Figure 1.1.4. The lipid stage of murein biosynthesis, involving the formation of Lipid I and Lipid II (adapted from Bupp and van Heijenoort, 1993).

The ratio of UDP-NAcMur-pentapeptide to Lipid I and Lipid II in the cell was calculated to be 300:1:3 (van Heijenoort *et al.*, 1992). Mengin-Lecreulx *et al.* (1989b) calculated a pool of 10^5 molecules of UDP-NAcMur-pentapeptide per cell. The concentration of the Lipid I and Lipid II would therefore be 700 and 1000-2000 molecules per cell, respectively (van Heijenoort *et al.*, 1992). It was also found in their research that UDP-Mur-tripeptide could be bound to form Lipid I. Begg *et al.* (1990) had proposed that division and elongation were related to the type of precursor available. That is tripeptide Lipid II for division and pentapeptide Lipid II for elongation. *In vitro* and *in vivo* studies on polymerisation by glycosylation with the tripeptide Lipid II showed that PBP1b had activity. Therefore the disaccharide tripeptide may be used by the cell for polar cap formation. The same assay using PBP3 showed no polymerisation, *in vitro*, using the tripeptide Lipid II as a substrate (van Heijenoort *et al.*, 1992). Once the disaccharide pentapeptide has been inserted into the nascent murein strand, bactoprenol must first be phosphorylated before again entering the cycle (Park, 1987).

1.1.2. The penicillin binding proteins (PBPs).

Park and Strominger (1957) identified the bacterial cell wall as the target for penicillin. Penicillin is the substrate analogue of D-alanyl-D-alanine, the terminal peptide residues located in the murein precursor, disaccharide pentapeptide (Tipper and Strominger, 1965). Penicillin binds

covalently to a serine residue present in the active site of all the penicillin binding proteins, thereby inhibiting binding to D-alanyl-D-alanine. (Ghuysen, 1991). Spratt and Pardee (1975) showed the presence of distinct PBPs after SDS-PAGE analysis of purified cytoplasmic membranes and labelling with ^{14}C penicillin. Ultimately 8 distinct proteins were found in membrane fractions which bound radiolabelled penicillin (Spratt, 1977). These were classed according to their molecular size. They are numbered PBP1a, 1b, 2, 3, 4, 5, 6, and 7/8, in decreasing order of molecular size.

The PBPs (with the exception of PBP7/8) can be divided into two groups according to their catalytic activity. The higher molecular weight PBPs 1a, 1b, 2 and 3 are dual functioning enzymes. They are all murein synthesising proteins. They have transglycosylase activity (adding new disaccharide-pentapeptide subunits to glycan strands from the lipid linked precursor) and β-lactam sensitive transpeptidase activity (forming peptide cross links between the parallel glycan strands, Tamaki *et al.*, 1977; Ishino *et al.*, 1980; Ishino and Matsuhashi, 1981; Ishino *et al.*, 1986). The active site is located in the centre of the protein and each has a cytoplasmic anchor at the amino-terminus (Broome-Smith *et al.*, 1985; Adachi *et al.*, 1987; Edelman *et al.*, 1987; Spratt and Bowler, 1987). The lower molecular weight PBPs (4, 5 and 6) have carboxypeptidase activity. This removes the fifth amino acid, the D-alanine residue, from the disaccharide pentapeptide in newly synthesised murein (Spratt *et al.*, 1976; Matsuhashi *et al.*, 1979). They are not murein synthesising enzymes. Penicillin inhibits both the carboxypeptidase and transpeptidase activities, thus preventing the crosslinking of new glycan strands, resulting in lysis of growing cells (Izaki *et al.*, 1968).

PBP1a and 1b.

PBP1a and 1b are the two largest PBPs. They have molecular weights of 94 kDa and 89 kDa respectively (Broome-Smith *et al*, 1985). They are encoded by the genes *mrcA* and *mrcB* respectively (Tamaki *et al.*, 1980), these are synonymous with *ponA* and *ponB* respectively (Broome-Smith *et al.*, 1985). The two proteins are homologous (Broome-Smith *et al*, 1985). Inactivation of either protein is not lethal to the cell (Kato *et al.*, 1985; Youssif *et al.*, 1987). Inactivation of both together is lethal (Tamaki *et al.*, 1977). On the basis of this it is proposed that each can compensate for the other and therefore both proteins may have similar functions (Suzuki *et al.*, 1978, Kato

et al., 1987). The cellular levels of PBP1a and 1b were estimated to be 100 and 120 molecules per cell. respectively (Spratt, 1975). Both PBP1a and 1b function in murein synthesis during cell elongation although each may have subtly different functions (Schmidt *et al.,* 1981; del Portillo and dePedro, 1990). There are distinctions between the roles of PBP1a and 1b in that the PBP1b prevents cell lysis upon the inhibition of PBP2 or PBP3. PBP1a activity alone does not rescue the cells (Schmidt *et al.,* 1981; del Portillo and dePedro, 1991).

PBP1b has been proposed to be the major transpeptidase synthase (Spratt *et al.,* 1977). This may be a result of higher catalytic activity of PBP1b or the influence that either may have on autolysin activity (Höltje and Tuomanen, 1991). *In vitro* measurements of the transglycosylase activity of PBP1b using Lipid II pentapeptide and Lipid II tripeptide showed similar rates of activity. It was suggested that PBP1b transglycosylase activity may be coupled with PBP3 transpeptidase activity, particularly if Lipid II tripeptide was the substrate (van Heijenoort *et al.,* 1992). It has been reported that a protein of 38 kDa is associated with PBP1b (Zijderveld *et al.,* 1989). For the synthesis of septal murein PBP1a and/or PBP1b are required (del Portillo *et al.,* 1989). PBP1b was purified from cell extracts using the soluble lytic transglycosylase (Slt) as an affinity ligand (Höltje *et al.,* 1995). This may indicate that PBP1b has a role in septal murein synthesis.

PBP2.

PBP2 has a molecular weight of 66 kDa (Asoh *et al,* 1986). *In vitro* studies have demonstrated that it can catalyse transpeptidation. Transglycosylation requires the presence of a second protein, RodA. This reaction is penicillin insensitive (Ishino *et al.* 1986). The PBP2 protein is encoded by *pbpA.* Interestingly the RodA gene (*rodA*) is located downstream from *pbpA.* (Matsuhashi *et al.,* 1990) and it appears that these genes are cotranscribed (Matsuzawa *et al.,* 1989). The roles of PBP2 and RodA in the *E. coli* cell cycle will be discussed in greater detail later in this study.

The β-lactam antibiotic, mecillinam, binds with a high specificity to PBP2 (Lund and Tybring, 1972; Matsuhashi *et al.,* 1979). Inhibition of its catalytic activity results in cells assuming a spherical morphology (James *et al.,* 1975). Spratt (1978) isolated a mecillinam resistant mutant, SP45. This strain does not possess PBP2 and yet is still viable, but as a coccal form. The

spherical shape of PBP2 mutants or cells treated with mecillinam shows that PBP2 is involved with the maintenance of rod shape. The *rodA* null allele is lethal under optimum growth conditions (Addinall, 1994). In later work by Begg and Donachie (1985) it is also assumed that PBP2 is dispensable to the cell. It appears that the lethality shown by PBP2 inactivation is due to growth conditions for the affected cells. That is, spherical cells cannot survive fast growth rates. Cells can survive without PBP2 activity, by either mecillinam treatment or a null allele, only when cultured in minimal media (K. Begg, pers. comm.). Spratt (1975) estimated there to be 20 copies of PBP2 per cell.

It has been estimated that at least 50%-70% of the murein made in growing cells may be attributed to the activity of PBP2 (Park and Burman, 1973). The role of PBP2 is in initiation of growth (de la Rosa *et al.*, 1985) by building small template strands of murein which are then extended by PBP1a and/or PBP1b (Park, 1993). Therefore a major role of PBP2 is the extension of the lateral cell wall. It has been proposed that PBP2 has a role in the division process as inhibition of PBP2 activity with mecillinam, and also studies on a PBP2 mutant, revealed cultures blocked in division. The mutant divided aberrantly with minicells being produced (James *et al.* 1975; Rodriguez and dePedro, 1990).

PBP3.

PBP3 is the smallest PBP involved in the synthesis of murein. It has a molecular weight of 60000 kDa and there are estimated to be 50 molecules per cell (Spratt, 1975). PBP3 is coded for by *ftsI*, one of a large cluster of morphogenes located at 2 minutes (Nakamura *et al.*, 1983). PBP3 is an essential gene involved in cell division (Spratt, 1975; Botta and Park, 1981; Ishino and Matsuhashi, 1981). Inactivation of PBP3 activity with β-lactam antibiotics prevents cell division but not lateral wall synthesis. Therefore cells grow into long filaments before lysis. This has also been shown with ts alleles of *ftsI* (Spratt, 1977, Begg *et al.*, 1986). A null mutation of *ftsI* proved that PBP3 is an essential gene (Hara and Park, 1993).

PBP3 is anchored in the cytoplasmic membrane by its amino terminus (Bowler and Spratt, 1989). It is synthesised as a precursor and exported to the periplasm where a polypeptide of eleven amino acids is cleaved from the C terminus (Nagasawa *et al.*, 1989). The structure of the N terminus acts as a

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non processed signal peptide permitting PBP3 entry into the cytoplasmic membrane and anchoring it there by the 36 amino acid N-terminus (Bowler and Spratt, 1989; Gomez *et al.*, 1993). A water soluble PBP3 (Fraipont *et al.*, 1994), with removal of the membrane anchor, could not rescue temperature sensitive PBP3 mutants (Fraipont *et al.*, 1995). Therefore the membrane anchor must play an important role in the localisation of PBP3 at the division site. The catalytic domain of the protein is located in the periplasm (Bowler and Spratt, 1989).

PBP3 catalyses both transglycosylation and transpeptidation reactions *in vitro* (Ishino and Matsuhashi, 1981). These reactions are penicillin-sensitive. Woldringh *et al.* (1987) found evidence that PBP2 and PBP3 act alternately during elongation and division. It has been proposed that PBP3 requires the presence of a second protein, FtsW, for its activity (Ikeda *et al.*, 1989; Matsuhashi *et al.*, 1990). They also proposed two synthesising complexes, one responsible for cell elongation and another for division. PBP2 and RodA are proposed for the former. Genetic studies on the timing of roles for PBP3 and FtsW during the formation of a septum suggest that PBP3 is involved at a later stage than FtsW (Begg and Donachie, 1985; Khattar *et al.*, 1994). The Regulation of PBP3 activity appears to involve MreB protein as a negative regulator and under positive control from FtsH (Wachi *et al.*, 1989; Ferreira *et al.*, 1987).

Recent biochemical studies have shown PBP3 to bind to other proteins involved in cell division. FtsZ was shown to bind *in vitro* to the cytoplasmic domain of PBP3 (Bramhill *et al.*, 1995; . FtsZ is the protein responsible for the construction of a cytoplasmic membrane bound ring structure (Bi and Lutkenhaus, 1991), which is thought to constrict at the midpoint of the cell. If PBP3 was bound to this structure then the enzyme complex thought responsible for the synthesis of septal murein (polar caps) would also be pulled inwards as the septum contracts. Other studies (Sanchéz *et al.*, 1994; Romeis and Höltje, 1994a, b) have shown *in vitro* binding of PBP3 to both FtsA and a soluble lytic transglycosylase (Slt). These will be discussed in greater depth at a later stage in this introduction.

PBP4.

PBP4 is a 49 kDa protein and is largest of the class of PBPs without transglycosylase or transpeptidase activity. It is encoded for by the *dacB* gene, located at 69 minutes (Iwaya and Strominger, 1977). PBP4 has both DD-carboxypeptidase and DD-endopeptidase activities (Korat *et al.*, 1991). It cleaves the terminal D-alanine from the pentapeptide side chains in nascent murein strands and it can hydrolyse the peptide cross links between existing murein strands. The PBP4 is present as a soluble protein in the periplasm and loosely associated with the cytoplasmic membrane (Mottl, 1992). There are estimated to be 110 molecules of PBP4 per cell (Spratt, 1975). Inactivation of PBP4 is not lethal to the cell (Broome-Smith, 1985). Due to its periplasmic location and its DD-endopeptidase activity Höltje (1993) proposed that the main function of PBP4 is to hydrolyse the cross-links between murein strands to allow the insertion of newly synthesised murein strands into the sacculus. This hydrolytic activity has also implicated PBP4 in penicillin induced lysis of cells. The DD-carboxypeptidase activity exhibited by PBP4 increases prior to septation (Hakenbeck and Messer, 1977). This suggests that PBP4 may play a role in division by increasing the pool of tripeptide side chains, preferred for the construction of the poles.

PBP5.

PBP5 has a molecular weight of 42 kDa and is the most common PBP with 1800 molecules per cell (Spratt, 1975). It is encoded by *dacA* (Broome-Smith *et al.*, 1988), which is located at 15 minutes, in the *mrd* region (Matsuhashi *et al.*, 1990). The *mrd* region contains a cluster of genes whose products are involved in cell growth. Included among these are the *rodA* and *pbpA* (PBP2) genes. PBP5 is a membrane bound DD-carboxypeptidase, which is presumed to be the major DD-carboxypeptidase as it has 10 times the activity of PBP6 when studied *in vitro* (Glauner, 1986). It is bound to the membrane at the C-terminus (Jackson and Pratt, 1987). Deletion of PBP5 was shown to be non lethal (Spratt, 1980). Analysis of the murein from a PBP5 null mutant revealed a higher proportion of pentapeptide side-chains, the substrate for PBP5 (Glauner, 1986). Overexpressed of PBP5 produces a spherical phenotype which is detrimental to cell viability (Markiewitz *et al.*, 1985). The change in cell shape may be due to the decrease in the preferred

glycan-peptide side-chain substrate for cell elongation with a pentapeptide side-chain (Begg *et al.*, 1990). Overproduction of PBP5 can rescue the PBP3 ts allele TOE23 at the restrictive temperature (Begg *et al.*, 1990). Perhaps PBP5 stabilises murein by removing the terminal D-alanine, thus preventing transpeptidation of the murein strands (de Pedro *et al.*, 1980). The deletion of *dacA* in an *ftsK* ts mutant suppresses the lethal *ftsK* phenotype (Begg *et al.*, 1995). This may be due to a decrease in the donor tripeptide residues which are preferred by PBP3 for the formation of septal murein, as proposed by Begg *et al.* (1990) in their model for substrate control of division and elongation in *E. coli*.

PBP6.

PBP6 is a peptide of 40 kDa encoded by *dacC*. It is also an abundant PBP with 600 molecules per cell (Spratt, 1975). It is a DD-carboxypeptidase, although *in vitro* it exhibits 10 times less activity than PBP5 (Ananuma and Strominger, 1984). PBP6 shares 62% identity with PBP5 (Broome-Smith *et al.*, 1988) and is inserted in the cytoplasmic membrane by its C-terminus (Jackson and Pratt, 1987). *dacC* is not an essential gene (Broome-Smith and Spratt, 1982) and the overexpression of PBP6 does not visibly affect the cell (Van der Linden *et al.*, 1992). However, overexpression of PBP6 is able to suppress the PBP3 mutant, TOE23 (Begg *et al.*, 1990). This is thought to be due to an increase in tripeptide side chains on the nascent murein strands; these are the preferred substrate for PBP3. Analysis of the peptide side chains of the null mutant does not show the appreciable rise in pentapeptides exhibited by the PBP5 null mutant (Glauner, 1984). A proposed role of PBP6 in the cell is thought to be in stationary phase where subtle changes in murein structure occur (Van der Linden *et al.*, 1992). In accordance with this idea PBP6 has been shown to increase in stationary phase (Buchanan and Sowell, 1982). Aldea *et al.* (1989) found PBP6 to be regulated by BolA, a small DNA binding protein. This in turn is regulated by the stationary phase sigma factor RpoS (Lange and Hengge-Arronis, 1991).

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PBP7/8.

The two smallest PBPs, 7 and 8 have now been identified as being the same peptide (Barbas *et al.*, 1986). They were only observed when labelling exponential growth phase cultures with β-lactam radioisotopes (Spratt, 1977). PBP8 is a degraded form of PBP7 (Henderson *et al.*, 1994). PBP7 was mapped at 47 minutes and is encoded by *pbpG* (Henderson *et al.*, 1995). The peptide has a molecular weight of 34.2 kDa (Henderson *et al.*, 1995). Deletion of *pbpG* proved PBP7 and PBP8 are not essential for growth (Henderson *et al.*, 1995). Cleavage of PBP7 by OmpT produces PBP8 which has been shown to have DD-endopeptidase activity (Romeis and Höltje, 1994b). Romeis and Höltje, 1994a have shown that PBP7 also binds to the Slt *in vitro*. This is the third PBP which binds to the Slt in this manner, suggesting the possibility of multienzyme complexes for both cell wall elongation and division (Höltje, 1993; Höltje *et al.*, 1995).

In addition to their differences in size and catalytic function, the deletion of the smaller PBPs is not lethal to the cell. The double deletion of PBP1a and 1b or deletions in PBP2 and 3 are lethal (Hara and Park, 1993). Individual null strains of PBP4, 5, 6 and 7 are not lethal. Indeed cells are not adversely affected with a double deletion of PBP4 and 5 , or a triple deletion of PBP4, 5 and 6 (Edwards and Donachie, 1993). HPLC analysis of the digested sacculi from the double and triple mutants revealed little change in the peptide side chain composition of the residues (Edwards, 1994). The muropeptides from both strains also contained the tripeptide side chain species, indicating DD-carboxypeptidase activity. Assays for DD-carboxypeptidase activity again revealed no significant difference between the double and triple deletion strains, although it was markedly reduced when compared to a wild-type strain (Edwards and Donachie, 1993). Therefore it is probable that other DD-carboxypeptidases exist in *E.coli* which can compensate for the triple deletion of PBP4, 5 and 6.

1.1.3. The penicillin-insensitive enzymes.

Other proteins involved in the biosynthesis, maturation and recycling of murein are insensitive to penicillin. These include the LD-carboxypeptidases, DD-endopeptidases and lytic transglycosylases. LD-carboxypeptidases cleave the terminal D-ala from the tetrapeptide side chain

of UDP-MurNAc. Several LD-carboxypeptidases have already been isolated. These are a cytoplasmic 12 kDa peptide (Metz *et al.*, 1986a, 1986b), a dimeric 43 kDa protein (Beck and Park, 1977) and a 32 kDa periplasmic protein (Ursinus *et al.*, 1992). The presence of such enzymes favours the hypothesis that the concentration of tripeptidyl side chains is the control for cell division or elongation (Begg *et al.*, 1990). The 43 kDa protein was found to be most active at cell division (Beck and Park, 1977). It may be that some of these enzymes are involved in the recycling of murein (Goodell, 1985).

The lytic transglycosylases are either membrane bound (Mlt) or soluble (Slt) (Höltje, 1993). They hydrolyse the β-1-4 glycosidic bond between the murein subunits. There are two membrane associated lytic transglycosylases MltB and MltB (Lommatzsch and Höltje, 1995; Ehlert *et al.*, 1995). The Slt has a molecular weight of 65 kDa and is encoded by *slt*, located at 99.7 on the *E. coli* linkage map (Betzner and Keck, 1989). Slt was found to bind to murein (Romeis and Höltje, 1994b). The use of the Slt as a ligand for affinity chromatography of periplasmic and membrane fractions enriched PBP1b, PBP3 and PBP7 (Romeis and Höltje, 1994a). Höltje *et al.* (1995) suggests that this demonstrates the presence of a murein synthase and hydrolase multienzyme complex with the necessary catalytic activities to produce the septum. For the biosynthesis of murein in the elongation of the cell, a similar multienzyme complex is proposed, with the substitution of PBP3 for PBP2.

Two classes of proteins with DD-endopeptidase activity are known. These are the penicillin-sensitive PBP4 and PBP7/8 and the penicillin-insensitive MepA (Keck *et al.*, 1990). DD-endopeptidases cleave the DD cross bridges of the murein sacculi. A fivefold increase in the levels of MepA had no apparent affect on cell viability, although there was a greater release of peptides into the medium (Keck *et al.*, 1990).

Wientjes and Nanninga (1989) reported the incorporation of [³H] DAP into the leading edge of the septum during the early stages of constriction. This was observed to be independent of PBP3 activity. The period of incorporation was restricted to the early stage of constriction. PBP3 was required after this stage for insertion of nascent murein at the polar caps. This was termed penicillin-insensitive-peptidoglycan synthesis, (PIPS, Nanninga *et al.*, 1990).

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1.1.4. The recycling of murein.

The cleavage of peptide cross bridges between murein strands in the sacculus to allow the incorporation of nascent murein releases peptides into the culture medium (Goodell and Schwartz, 1985). This event has been observed in Gram positive cells which lose up to 50% of their muropeptides from their cell walls during growth. Goodell (1985) described a metabolic pathway in *E.coli* where up to 50% of the muropeptides were recycled (Figure 1.1.5.). The muropeptide is hydrolysed from MurNAc by N-acetylmuramoyl-L-alanine amidase (van Heijenoort *et al.*, 1975; Parquet *et al.*, 1983). This enzyme is based in the periplasm. Goodell (1985) showed the recycling of muropeptides by using [³H] DAP, incorporated in either the tripeptide (L-ala-D-glu-DAP) or the tetrapeptide (L-ala-D-glu-DAP-D-ala). Both peptides were absorbed by the cell although the D-ala of the tetrapeptide was first hydrolysed, presumably by a periplasmic L-D carboxypeptidase. The molecules incorporating the label were found to be the tripeptide, UDP-MurNAc-pentapeptide and DAP. The levels of free [³H] DAP in the cytoplasm were insignificant. On the basis of this result Goodell (1985) proposed that the tripeptide is immediately transferred into the cytoplasm via an oligopeptide permease (OPP) and is bound to NAcMur and thus re-enters the cytoplasmic synthesis cycle of the Lipid II murein precursor (see 1.1.2.). Goodell and Higgins (1987) measured the rate of tripeptide recycling in a *opp*⁻ strain and showed OPP to be essential for recycling of the tripeptide. Park (1993) repeated the experiment of measuring recycling of labelled tripeptides in the *opp*⁻ strain. The recycling was not abolished, with a 50% rate of turnover during lateral wall synthesis. Therefore a second oligopeptide permease may exist in the membrane.
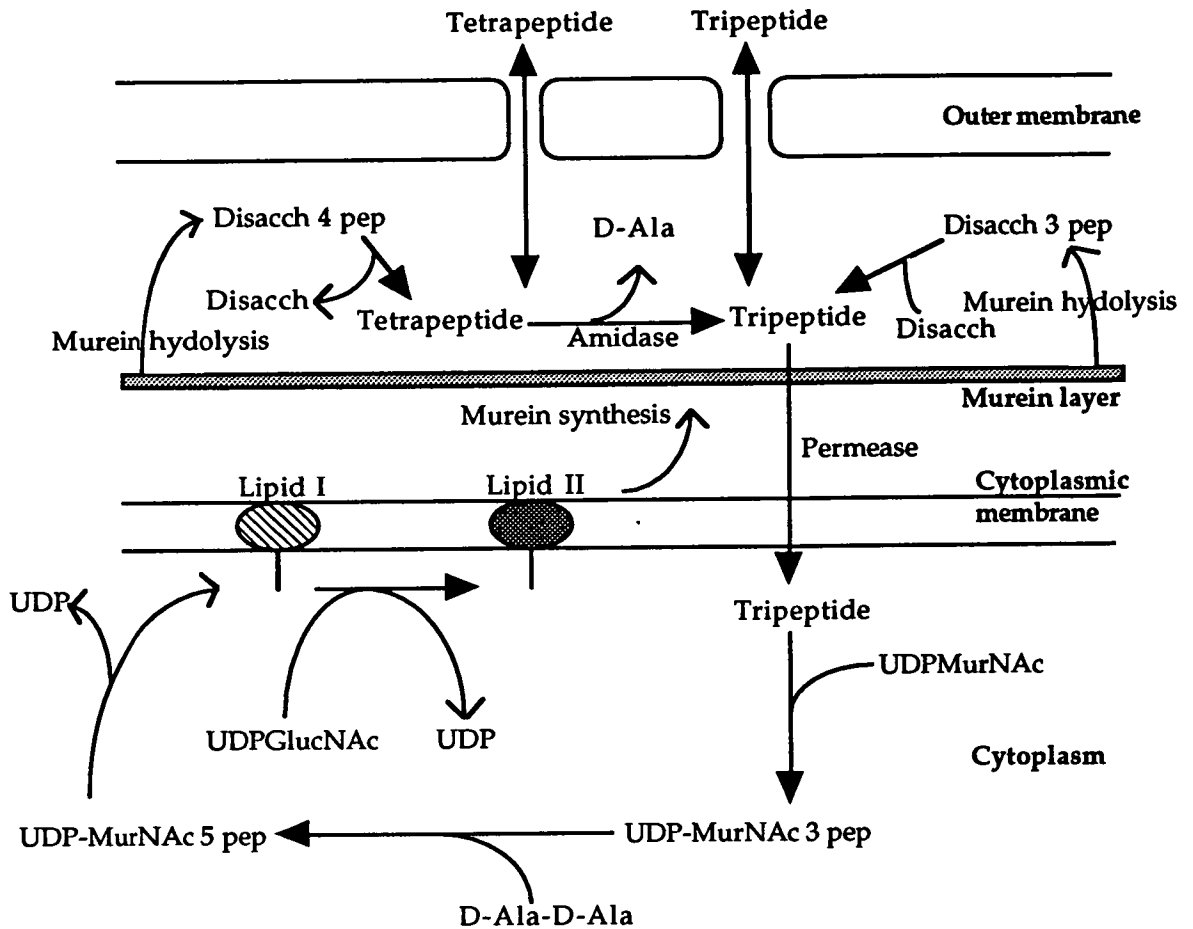
Figure 1.1.5. A schematic diagram of the recycling of muropeptide in *E. coli* after murein hydrolysis (adapted from Goodell, 1985). Key: 5 pep, pentapeptide; 4 pep, tetrapeptide; 3 pep, tripeptide; disacch, GlucNAc MurNAc.

1.2. Cell division.

The study of L-forms (bacteria with no cell wall) showed that cells without cell walls can continue to grow and divide (Onoda *et al.*, 1987). Therefore the process of division is not controlled by the structure of the cell wall. Rather, the process of division is an innate property of the cytoplasm and the inner membrane. The study of division proteins in *E. coli* has centred upon the isolation of conditional mutants where division is arrested. The phenotype of temperature sensitive division mutants is filamentous (*fts*) at the restrictive temperature. The loci of the mutations have been mapped and

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division genes identified and sequenced. The regulation and relationships between division genes has been extensively studied.

It has been shown that division proteins operate in a co-ordinated manner during cell division together with the septum-specific peptidoglycan transglycosylase and transpeptidase PBP3 (Begg and Donachie, 1985). The concerted action of these proteins results in the formation of a covalently cross linked double layered septum across the cell centre. The splitting of the bonds between the two layers, accompanied with the invagination of the outer membrane, completes the process of cell division (Donachie, 1993). The greatest number of division genes are found at 2.4 minutes in the chromosome at the *mra* region (murein region a; Miyakawa *et al.*; Matsuhashi *et al.*, 1990). This contains 7 division genes including *ftsI*, encoding PBP3, the PBP involved in septal murein synthesis (Donachie, 1993).

1.2.1. The cell division proteins.

To date there at least fourteen genes which directly affect cell division in *E. coli*. The roles of the division or morphogenes during septation of the cell is presented below together with a discussion on the controls of the characterised division genes .

FtsZ.   FtsZ is the most abundant of all division proteins with 5000-20000 molecules per cell (Bi and Lutkenhaus, 1991). The 40.3 kDa FtsZ protein is required from the earliest stages of division (Begg and Donachie, 1985). FtsZ is encoded by *ftsZ*, which is the penultimate gene in the *mra* region (Lutkenhaus *et al.*, 1980; Yi and Lutkenhaus, 1985). *ftsZ* has been shown to be an essential division gene (Dai and Lutkenhaus, 1991). During cell growth FtsZ can be detected in the cytoplasm (Bi and Lutkenhaus, 1991). The cell's commitment to division, however, is marked by the oligomerization of FtsZ to form a circumferential ring at the inner membrane before visible invagination of the septum (Bi and Lutkenhaus, 1991; Lutkenhaus, 1993). The ring of FtsZ remains present at the leading edge of constriction during division.

FtsZ contains four regions of similarity with the eukaryotic tubulins (Lutkenhaus, 1993a); amino acid segment (GGGTGTG) has high identity to the eukaryotic tubulin GTP/GDP binding site. Purified FtsZ protein has been shown to bind GTP and to display weak GTPase activity *in vitro* (de

Boer *et al.*, 1992a; RayChaudhuri and Park, 1992; Mukherjee *et al.*, 1993). The GTPase activity does not appear to be essential for FtsZ function (Dai *et al.*, 1994). GTP binding, however, is essential for FtsZ function (Dai *et al.*, 1994). For efficient GTP/GDP binding and GTPase activity *in vitro*, Mg^{2+}, high levels of FtsZ, pre incubation of FtsZ with nucleotides or heating is required (de Boer *et al.*, 1992a; RayChaudhuri and Park, 1992; Mukherjee *et al.*, 1993). The activation of FtsZ requires K^+ or high levels of FtsZ (RayChaudhuri and Park, 1992). This has led to the hypothesis that the oligomerization of FtsZ *in vivo* is dependant on the intracellular concentration of FtsZ, or on the ionic conditions in the cytoplasm (Dai *et al.*, 1994).

Recent *in vitro* studies on FtsZ have shown the FtsZ protein to aggregate into micro tubule-like structures in a GTP-dependant manner (Bramhill and Thomson, 1994; Mukherjee and Lutkenhaus, 1994; Bramhill *et al.*, 1995). Electron microscopy of purified FtsZ protein after suitable incubation with GTP and Mg^{2+} revealed structures including tubules 14-20 nm in diameter with longitudinal arrays of protofilaments (Bramhill and Thomson, 1994; Mukherjee and Lutkenhaus, 1994). In the presence of GTP the micro tubules assemble and in its absence disaggregation occurs (Bramhill and Thomson, 1994). Conflicting results have been obtained concerning FtsZ polymerisation with GDP, which has been shown both to cause the disassembly of FtsZ (Bramhill and Thomson, 1994) and to cause its assembly (Mukherjee and Lutkenhaus, 1994) *in vitro*. *In vitro* studies on the polymerisation of FtsZ mutant proteins showed that an FtsZ protein unable to bind GTP did not form filaments. A mutant FtsZ able to bind GTP but without GTPase activity was able to polymerise (Mukherjee *et al*, 1993; Mukherjee and Lutkenhaus, 1994). Therefore FtsZ GTPase activity is not essential for FtsZ activity. This was shown by Dai *et al.* (1994) who found that two FtsZ mutant proteins with reduced GTPase activity could complement an *ftsZ* ts mutant.

It is not known whether the ingrowth of the septum causes constriction and division of the cytoplasmic membrane or whether the FtsZ ring is contractile and pulls the membrane with it during constriction. Alternatively the FtsZ driven constriction may provide the stimulus for the ingrowth of the septum (Bramhill and Thomson, 1994)

FtsZ has been shown to interact with other proteins, notably PBP3 (Bramhill *et al.*, 1995). This is the protein which makes septal murein (Ishino and Matsuhashi, 1981). The cytoplasmic amino terminus of PBP3 has been

shown to bind FtsZ *in vitro*. It is tempting to envisage a single multienzyme complex spanning the cytoplasmic membrane governing the rate of both cytokinesis and septation. *In vitro* studies on protein interaction has shown that PBP3 may constitute part of a division complex (Romeis and Höltje, 1994a). Another protein has recently been shown to bind to FtsZ *in vitro*; this is a 30.4 kDa membrane associated protein, ZipA (Hale and deBoer, 1995). The function of ZipA is unknown. Increased levels of ZipA caused filamentation. Filamentation was not dependant on the division inhibitors SulA (SfiA) or MinC. Increased levels of FtsZ suppress the filamentation caused by ZipA overproduction, suggesting an *in vivo* interaction between the two proteins (Hale and deBoer, 1995).

Slight overproduction of FtsZ results in the formation of minicells, small anucleate cells formed from division at the polar regions. Therefore division occurs not only at the midpoint of the cell but also at the polar caps; previous division sites from earlier generations are used (Ward and Lutkenhaus, 1985). Tenfold or greater production of FtsZ inhibits cell division and the cells form smooth filaments (Ward and Lutkenhaus, 1985). During normal growth FtsZ will only recognise the site at the cell centre. This is the future division site. FtsZ is inhibited from binding at the old division sites at the polar caps by the products of the *minB* operon at 26 minutes on the *E. coli* linkage map (deBoer *et al.*, 1988). The *minB* operon encodes three proteins MinC, MinD and MinE. Co-expression of *minC/D* in the absence of MinE causes a block in division. The absence of MinC/D or over-expression of *minE* causes non-specific division with minicells being produced. There is a subtle difference between the minicell formation by *ftsZ* over-expression and the MinC/D mutant, *minE* over-expression (Figure 1.2.1., Bi and Lutkenhaus, 1990). The high levels of FtsZ form division sites at the midpoint of the cell and at the polar caps (Ward and Lutkenhaus, 1985). In *min* mutants Min, only one division event occurs, either at a polar cap or in the midpoint of the cell (deBoer *et al.*, 1989).

MinC is the protein responsible for preventing division at the polar caps. The MinD protein is a membrane bound ATPase which activates the MinC protein (deBoer *et al.*, 1991). If *minE* is not present then the activated MinC will block all the division sites (deBoer *et al.*, 1989). The mechanisms as to how MinC operates are not known. It is thought to interact directly with FtsZ. This is because the filamentous morphology of overproducing

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MinC cells can be normalised by raising the levels of FtsZ or by using an altered FtsZ protein (de Boer *et al.*, 1990).

Wild-type   FtsZ overexpression   *min*⁻
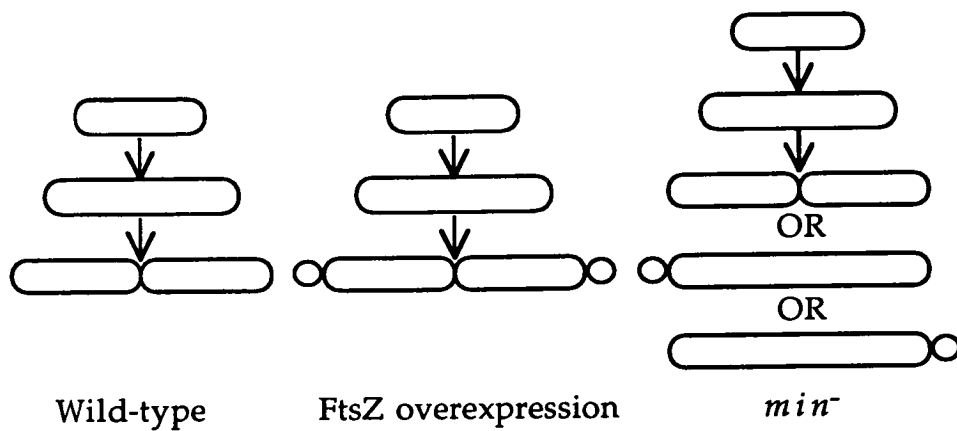
Figure 1.2.1. An example of the difference in phenotypes involving minicell production from increased levels of FtsZ and the *min*⁻ mutant. Adapted from Donachie, 1993.

Another inhibitor of FtsZ activity is SulA (SfiA) which prevents the formation of the FtsZ ring (Bi and Lutkenhaus, 1993). This is one of 15 known genes that are induced, as part of the SOS response, when DNA is damaged. The activity of FtsZ is blocked and the cells grow into long filaments to prevent the formation of anucleate cells (Walker, 1984). The events of the SOS response with respect to inhibition of division will be briefly discussed in Chapter 1.3.4.. A class of mutations resistant to SulA have been located to the *ftsZ* locus (Bi and Lutkenhaus, 1990c; Dai *et al.*, 1994). These mutants are also resistant to the MinCD inhibitors of FtsZ (Dai *et al.*, 1994). The other phenotypes produced by these mutant alleles were varied. *In vitro* analysis of the six mutant FtsZ proteins however showed reduced GTPase activity and in some, reduced GTP binding. The mechanism by which the mutant FtsZ proteins are resistant to SulA is not known. It is not due to altered GTPase activity because there are GTPase mutants of FtsZ with reduced activity which are sensitive to SulA (Bi and Lutkenhaus, 1990c; Dai *et al.*, 1994). SulA may prevent division by binding to either FtsZ itself by competing directly with it for another target. SulA-resistant *ftsZ* alleles may have an increased ability to form multimers or they may compete more actively for a site by having increased affinity for the site (Dai *et al.*, 1994). However, Higashitani *et al.* (1995) showed that SulA bound to FtsZ *in vitro* at a ratio of 1:1 in the presence of GTP and Mg^{2+}. The

presence of GTP was essential for the reaction; use of γS GTP did not allow binding. Therefore hydrolysis of GTP may be required for the reaction of binding SulA to FtsZ (Higashitani *et al.*, 1995). The transcriptional and translational control of *ftsZ* will be discussed later in this chapter.

FtsA.   The FtsA protein is encoded by *ftsA* which is located directly upstream from *ftsZ* (Lutkenhaus and Donachie, 1979; Robinson *et al.*, 1984) The gene encodes a protein with a predicted molecular weight of 45.3 kDa. FtsA is essential for cell division (Lutkenhaus and Donachie, 1979). The protein is membrane bound with the N-terminus of the protein located in the cytoplasm (Chan and Gayda, 1988). It has been calculated that there are 150 molecules of FtsA protein per cell (Wang and Gayda, 1992). High level expression of FtsA causes a block to division (Wang and Gayda, 1990). The ratio of FtsA to FtsZ is critical for successful division since an imbalance between the two proteins produces a block to division (Dai and Lutkenhaus, 1992; Dewar *et al.*, 1992). New FtsA protein is required to be made at each round of division (Donachie *et al.*, 1979).

The phenotype of the ts mutant is the formation of long filamentous cells with evenly spaced blunt constrictions (Donachie *et al.*, 1979). The constrictions are one unit cell length apart (Rothfield *et al.*, 1990). This datum suggests that the FtsZ ring has formed and that division is inactivated at a later stage in septation (Begg and Donachie, 1985). By returning the ts mutant to a non-restrictive temperature the division event is completed at the potential division sites (Rothfield *et al.*, 1990; Cook and Rothfield, 1994). A relationship between FtsA and the levels of PBP3 has been postulated. Tormo *et al.* (1986) isolated a ts allele of *ftsA* which affects the binding of penicillin to PBP3. The overproduction of the *ftsN* product suppresses both *ftsI* and *ftsA* ts alleles (Dai *et al.*, 1993). The C-terminal of the FtsA protein has similarity to the same region in MreB (Doi *et al*, 188). Mutants of *mreB* showed increased levels of PBP3 and a spherical morphology. High levels of MreB produced a filamentous phenotype (Wachi *et al.*, 1987, Doi *et al*, 1988; Wachi *et al.*, 1989). However, Begg and Donachie (1985) showed that the *ftsI* ts allele produces long straight filaments whilst *ftsA* ts phenotype has constrictions. Therefore FtsI is required at an earlier stage in division than FtsA. This may indicate that FtsA plays a regulatory role in division where it is required for the continuation of PBP3 activity in the septum and

not for the initiation of PBP3 activity. Matsuhashi *et al.* (1990) suggested regulatory roles for MreB and FtsA in elongation and division respectively.

The FtsA protein has an actin-like structure and is present in two different forms within the cell, phosphorylated and nonphosphorylated, which can be detected using two dimensional electrophoresis (Sanchéz *et al.*, 1994). The phosphorylated form was found in the cytoplasm and could bind ATP. The nonphosphorylated FtsA was membrane bound and incapable of ATP binding (Sanchéz *et al.* 1994). *In vitro* studies on the effects of mutagenesis of the ATP binding motif and the phosphorylatable serine residue revealed that FtsA had to be in a phosphorylated state before ATP was bound. Mutant alleles of *ftsA* were complemented with mutant forms of FtsA which were inactive for both ATP binding and phosphorylation (Sanchéz *et al.*, 1994). Therefore the phosphorylation and ATP binding of FtsA may not be essential for function although they may play a role in the regulation of FtsA during the formation of the septum (Sanchéz *et al.*, 1994). The control of FtsA expression in relation to FtsZ will be discussed at a later stage in this chapter.

FtsQ. FtsQ is a 31 kDa protein encoded by *ftsQ*, which lies directly upstream from *ftsA* in the 2 minute region (Begg *et al.*, 1980; Robinson *et al.*, 1984; Storts *et al.*, 1989; Carson *et al.*, 1991). The protein is predominantly periplasmic with a short cytoplasmic tail at the N-terminus and a single membrane spanning helix (Carson *et al*, 1991). Such topology has also been reported for FtsL, PBP3 and FtsN (Dai *et al.*, 1993). The protein is thought to be present in 20-40 molecules per cell. The FtsQ protein is essential for cell division (Carson *et al.*, 1991), however, the stage at which FtsQ is required for division has not been determined since some *ftsQ* mutants give rise to smooth filaments and others result in constricted filaments. The transmembrane domain has led to speculation that FtsQ may be involved in the localisation of cytoplasmic or periplasmic proteins at the division site, for example with FtsZ via the cytoplasmic portion of the NH-terminus. The overproduction of FtsQ results in cells forming long septate filaments. This may indicate that the function of FtsQ is at a late stage in the division process (Carson *et al.*, 1991). The formation of constrictions in a *ftsQ rodA* ts double mutant led Begg and Donachie (1985) to predict that FtsQ is required after FtsZ, but before FtsA. Interestingly the filamentous phenotype exhibited with excess FtsQ is different to that observed for PBP3, FtsA and FtsZ. The

constrictions produced are not separated by one unit cell length, as with FtsA, but are larger (Carson *et al.* , 1991). the transcriptional and translational control of *ftsQ* will be discussed later in this thesis.

FtsW.   The *ftsW* gene was identified by Ishino *et al*, 1989. Sequencing of the gene revealed that it encodes a 46 kDa transmembrane protein with striking similarity to the RodA protein of *E. coli* and the SpoVE protein of *Bacillus subtilis* (Ikeda *et al.* 1989). The amount of FtsW protein required for cell division is not known but is probably ~10-50 molecules per cell (M. Khattar, pers. comm.). The FtsW protein is probably essential (Ishino *et al.* 1989; Khattar *et al*, 1994). This result together with the close similarity between FtsW and RodA proteins, led Matsuhashi *et al.* (1990) to speculate that two murein synthesising systems may be controlled by the presence of RodA and PBP2 for elongation of the sacculus and FtsW and PBP3 for the formation of the poles, respectively. The migration of the 46 kDa FtsW protein in SDS-PAGE was shown to be anomalous. FtsW migrated as a protein of 32 kDa (Khattar *et al.*, 1994). RodA also has an unusual migration rate in SDS-PAGE (Stoker *et al.*, 1983). The overproduction of FtsW inhibits cell growth, though it has no visible effect on cell morphology (Khattar *et al.*, 1994). The *ftsW* ts strain isolated by Khattar *et al.* (1994) is hypersensitive to extra copies of *ftsZ* at the permissive temperature. The requirement for FtsW function in septation is known to be early, perhaps before the formation of the FtsZ ring (Khattar *et al.*, 1994).

PBP3.   PBP3 has already been described in depth in Chapter 1.1.3. Deletion of PBP3 is deleterious to the cell. The mutant has a filamentous phenotype. The number of molecules in the cell is very low and the PBP3 protein has a membrane topology similar to that of other membrane associated division proteins such as FtsQ, L and N (Dai *et al.*, 1993). It is thought that PBP3 and FtsW have a concerted action in cell division, although FtsW has been shown to be required by the cell at an earlier stage in division than PBP3 (Begg and Donachie, 1985; Khattar *et al*, 1994).

FtsL.   The FtsL (MraR) protein is encoded by *ftsL* (*mraR*), located immediately upstream from *ftsI* in the *mra* region (Ueki *et al.*, 1992; Guzman *et al.*, 1992). It is an essential division gene, first isolated by Ishino *et al.* (1989). Two ts mutants were isolated with different phenotypes. The first,

fts36, was filamentous at 42°C and the second, *lts33*, lysed at 42°C. The construction of three null mutants by Guzman *et al.* (1992) proved that FtsL was a division protein. FtsL is 13.6 kDa protein with a single transmembrane domain. Like the division proteins PBP3, FtsQ and N it is anchored to the cytoplasmic membrane by its N-terminus and has a single membrane spanning helix (Guzman *et al.*, 1992; Dai *et al.*, 1993). The presence of a leucine zipper motif in the periplasmic domain may mean that FtsL has a dimeric form (Guzman *et al.*, 1992). Increased levels of FtsL cause filamentation and aberrant cell shape but only when cells are cultured in minimal media (Guzman *et al*, 1992). There are thought to be 20-40 molecules of FtsL per cell. This is similar to both PBP3 and FtsQ (Spratt, 1975; Carson *et al.*, 1991). Guzman *et al.* (1992) suggest that the three proteins are linked together to form a stochiometric membrane complex which functions in the division process.

EnvA. EnvA is a 33.9 kDa protein which is encoded by *envA*, the terminal gene of the *mra* region (Sullivan and Donachie, 1984; Beall and Lutkenhaus, 1987). The EnvA protein is cytoplasmic and toxic if overproduced (Beall and Lutkenhaus, 1987). The phenotype of the sole *envA* mutant is the formation of chains of cells in which the nascent hemispherical poles of the cells are complete but the cells are still linked together (Normark, 1970; Beall and Lutkenhaus, 1987). The identification of EnvA as UDP-3-O-acyl-N-acetylglucosamine deacetylase indicates that EnvA is directly involved in cell wall metabolism (Young *et al.*, 1994) and not cell division. The pleiotropic phenotype includes hyperpermeablity to antibiotics (Grunstrom *et al.*, 1980).

FtsN. FtsN was isolated as a multicopy suppressor of *ftsA* mutants (Dai *et al.*, 1993) and it was also shown to suppress *ftsI* mutants. FtsN is encoded for by *ftsN* which is located at 88.5 minutes on the chromosome. FtsN is a protein of 36 kDa and is similar in its membrane topology to PBP3, FtsA, L and Q. The phenotype produced by inactivation of FtsN is long aseptate cells similar to those observed for *ftsQ, ftsI* and *ftsL* mutants (Dai *et al*, 1993). The hydropathy profile of FtsN is similar to the FtsQ, FtsA, FtsL and FtsI proteins: that is a short N-terminus in the cytoplasm with a single membrane spanning section. The C-terminus is located in the periplasm. As

these proteins share many features, Lutkenhaus (1993) proposed that they form a stochiometric complex active during division.

FtsY, FtsE and FtsX.   The *ftsY, ftsE* and *ftsX* genes form an operon at 69 minutes on the *E. coli* chromosome (Gill *et al.,* 1986). All three proteins lack a signal sequence and each is associated with the inner cytoplasmic membrane (Gill *et al.,* 1986; Gill and Salmond, 1987). FtsY has similarity with a eukaryotic secretory protein and FtsE has similarity to prokaryotic nucleotide proteins involved in protein transport (Gill and Salmond, 1990; Taschner *et al.,* 1988). The FtsX activity is unknown, it also has no known identity with other proteins. The filamentous phenotypes of *ftsE* and *ftsX* mutants was suggested to be due to inefficient transport of other division proteins across the cytoplasmic membrane (Donachie *et al.,* 1984; Gill and Salmond, 1990).

FtsK.   FtsK is a large protein of 147 kDa encoded by *ftsK* at 20 minutes on the *E. coli* linkage map (Begg et al., 1995). The protein appears to have several transmembrane domains at the N-terminal and contains consensus sequences for an ATP/GTP binding pocket in the cytoplasmic part of the protein. Several parts of the FtsK protein show considerable sequence similarity to a family of proteins from *Coxiella burnetii, Camphylobacter jejuni* and *B. subtilis* (Begg *et al.,* 1995); all of which contain the putative ATP/GTP binding domain. The large central portion of the FtsK protein has some similarity to γ-gliadins in wheat (Begg *et al.,* 1995). The phenotype of the sole *ftsK* mutant is SulA independent and filamentous at 42°C. Division is restored at 42°C by a clone missing the 3' terminus of *ftsK+*. *ftsK+* cannot be cloned in high copy number, indicating that overproduction of FtsK is harmful to the cell (Begg *et al.,* 1995). An *ftsK rodA* double mutant forms filamentous cells with regularly-spaced deep constrictions. This led Begg *et al.* (1995) to propose that FtsK is required at a very late stage in division. The *ftsK* ts phenotype is salt reversible and suppressed by a *dacA* deletion. *dacA* encodes PBP5, a DD-carboxypeptidase (Matsuhashi *et al.,* 1990) and its deletion increases levels of pentapeptide side chains in the murein. Inactivation of *dacB* and *dacC,* two other PBPs with DD-carboxypeptidase activity, did not suppress the *ftsK* ts allele (Begg *et al.,* 1995).

The role of FtsK in cell division has not been determined. The *B. subtilis* homologue to FtsK is SpoIIIE, which transfers DNA from the mother

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cell to the prespore during sporulation (Wu and Errington, 1994; Wu *et al.*, 1995). The chromosomes segregate correctly in the *ftsK* mutant. A second SpoIIIE-like protein may exist in *B. subtilis*, it may be that one is required for spore formation and another for cell division during vegetative growth (W.D. Donachie pers. comm.). Begg *et al.* (1995) hypothesise that FtsK is required for the final stage in division at which the single sacculus is converted into two. The mode of regulation of *ftsK* is unknown. It lies downstream from the *lrp* gene (leucine regulatory protein) and is probably cotranscribed. There is an SOS inducible promoter *dinH* (Lewis *et al.*, 1992) in the 134 bp gap between *lrp* and *ftsK* which may be involved in regulation of expression of *ftsK*.

FtsJ/FtsH. FtsJ and FtsH are 70.7 kDa and 23.3 kDa proteins respectively; encoded by *ftsJ* and *ftsH*, located at 69 minutes on the *E. coli* linkage map (Tomoyasu *et al.*, 1993a). Neither protein is essential for growth but mutants of either produce short filaments (Begg *et al*, 1992; Tomoyasu *et al.*, 1993a, 1993b). The function of FtsJ is unknown and FtsH has been shown to be a membrane bound ATP dependant protease (Tomoyasu *et al.*, 1993a, 1993b; Tomayasu *et al.*, 1995). FtsH has two transmembrane segments with the cytoplasmic segment showing identity to the AAA class of proteins (ATPases Associated with a variety of cellular Activities; Akiyama *et al.*, 1995). The protein is thought to exist as a dimer or tetramer. The thermosensitive *ftsH* mutant H1 causes a decrease in PBP3 with the periplasmic processing of the C-terminal being inhibited (Begg *et al*, 1992). Tomoyasu *et al.* (1995) have shown *in vitro* that for FtsH to have proteolytic activity for the heat shock transcription factor, $^{32}\sigma$. Both *ftsJ* and *ftsH* have consensus sequences for $^{70}\sigma$ transcription factors (Tomoyasu *et al.*, 1993a).

1.2.2. Transcriptional regulation of division.

The study on the regulation of cell division has concentrated on the *mra* cluster which contains seven genes involved in cell division, *ftsL*, *ftsI*, *ftsW*, *ftsQ*, *ftsA*, *ftsZ* and *envA* (Donachie, 1993). In particular, the regulation of expression of *ftsQ*, *ftsA* and *ftsZ*. The *mra* region contains 16 ORFs, some of which have known functions in cell growth or division. A more complete description of this is presented in Chapter 1.5.1. The genes of the *mra* region are transcribed in the same direction and are tightly packed; several of the

genes overlap. There are no transcriptional terminators throughout the *mra* region (the first is located after *envA*.). Therefore it is possible that the genes are transcribed as one massive operon (Beall and Lutkenhaus, 1987; Hara and Park, 1993). Promoters have been identified throughout the *mra* region with evidence for transcriptional coupling of genes (Beall and Lutkenhaus, 1987; Dewar *et al.*, 1989; Mengin-Lecreulx *et al.*, 1989a; Carrión *et al.*, 1995). In addition to control of transcription there is evidence for both translational and functional regulation. Therefore the control of gene expression in the *mra* region appears to be highly complex.
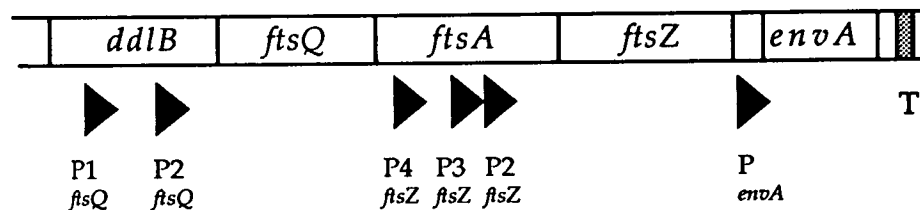
Figure 1.2.2. The recognised promoter sequences in the *ddlB envA* region.

ftsZ is the penultimate gene in the *mra* region and regulation of its expression is the most characterised of the cell division genes. Initial research on the expression of *ftsQ, A* and *Z* revealed that all are transcribed from promoter regions located in the coding regions of upstream genes (Figure 1.2.2.; Robinson *et al.*, 1984, 1986). There have been five promoters identified in the expression of *ftsZ*. Early studies on *ftsZ* revealed that the upstream gene *ftsA*, was required for *ftsZ* expression (Lutkenhaus and Wu, 1980). Four promoter sites within *ftsA*, were subsequently identified and sequencing of the region revealed more putative promoter regions (Sullivan and Donachie, 1984; Robinson *et al.*, 1984). The promoters, P4$_{ftsZ}$, P3$_{ftsZ}$ and P2$_{ftsZ}$ (Figure 1.2.2.) are thought to contribute for 80% of the total *ftsZ* expression (Yi *et al.*, 1985; Garrido *et al.*, 1993). The fourth *ftsZ* promoter in *ftsA*, P1$_{ftsZ}$ has now been shown to be an RNase E cleavage site and therefore it is not a promoter but a region involved in the post-transcriptional control of the other *ftsZ* transcripts (Cam *et al.*, 1994). For complementation of a null strain with a single copy of *ftsZ*, more than 6 kb of upstream DNA is absolutely required (Dai and Lutkenhaus, 1991). This indicates that essential transcripts of *ftsZ* may initiate more than 6 kb upstream.

The presence of so many promoters involved in the expression of *ftsZ* suggests there may be mechanisms by which *ftsZ* expression could be

regulated. However, only one such control is known to exist. This is the *sdiA* product which activates transcription from P2*ftsQ* (Wang *et al.*, 1991). The *sdiA* gene is found at 42 minutes (Wang *et al.*, 1991). Overexpression of *sdiA* increases transcription from P2*ftsQ*. The deletion of *sdiA* results in a 40-50% reduction of transcription into *ftsQ*, *ftsA* and *ftsZ*. The *sdiA* deletion mutant however, showed no division defects and therefore there must be other mechanisms to regulate the levels of *ftsZ* within the cell (Wang *et al.*, 1991). Since P2*ftsQ* is located in *ddlB*, the expression of *ftsQ*, *ftsA* and *ftsZ* may also be regulated by *sdiA* although this has not been shown.

P1*ftsQ* was shown by Aldea *et al.* (1990) to be a gearbox promoter where expression is growth rate sensitive. The expression from P1*ftsQ* is inversely dependant on growth rate. As cell growth rates slow during early stationary phase, expression from P1*ftsQ* increases; the critical mass for cell division decreases and therefore cell division occurs when cells are smaller. It may be that the increased expression from P1*ftsQ* during the later stages of the growth cycle produces the necessary extra FtsZ required for the earlier division events. *ftsA* expression may also be governed by the gearbox promoter as the correct ratio of FtsA to FtsZ is essential for division (Dewar *et al.*, 1992). The expression of the *bolA* gene is also controlled by a gear box consensus promoter.

The presence of putative DnaA binding sites within *ftsA* led Masters *et al.* (1989) to propose that the expression of *ftsZ* may be synchronised with the initiation of chromosome replication. This was however disproven (Garrido *et al.*, 1993; R. Smith, 1995). Other work by Dewar *et al.*, (1989) showed that increasing the levels of a mutant FtsA protein had a regulatory effect on *ftsZ* transcription.

The levels of *ftsZ* transcripts were found to oscillate during the cell cycle. Garrido *et al.* (1993) showed that *ftsZ* transcription increased abruptly when division occurred. In addition, the *ftsZ* mRNA was relatively low in abundance and that it had a half-life of 1.5 minutes. Upon a nutritional shift up, the rate of RNA and protein synthesis rapidly increases (Jensen and Pedersen, 1990). The levels of *ftsZ* mRNA remained low suggesting that *ftsZ* expression is under the cell cycle control (Garrido *et al.*, 1993).

The transcriptional control of other division genes within the two minute region is still poorly understood. The promoter for *ftsW* expression is located at least 1.5 kb upstream from its start and similarly *ftsI* has no promoter region nearby (Ikeda *et al.*, 1989; Hara and Park, 1993). There are

two regions with consensus promoter sequences upstream from ftsL located in *mraZ* and *mraW* respectively. Therefore one or both of these putative promoters may be involved in the expression of *ftsI* and/or *ftsL* respectively. (Guzman *et al.*, 1992; Carrión *et al.*, 1995).

1.2.3. Translational regulation of division proteins.

The transcription of division genes is often from promoters located far upstream and the levels of proteins translated from each ORF in the transcripts can vary dramatically. Examples of this are *ffsQ, ftsA, ftsZ, ftsW* and *ftsI* (Dai and Lutkenhaus, 1991; this work, Chapter 3; Hara and Park, 1993). Mukherjee and Donachie (1990) examined the relationship between the translational efficiencies in *ftsQ, A* and *Z*; thought to be present in a ratio of 50 : 150 : <20000 molecules per cell respectively (Donachie, 1993). In the study they found that the translation of both FtsQ and FtsA was limited, in comparison to FtsZ, partly because of the sequence of the ribosome binding sites (rbs). The rbs of *ftsQ* and *ftsA* did not facilitate efficient binding of ribosomes. The low levels of PBP3 translated from *ftsI* also appear to result from a poor rbs (M. Khattar and G. Roberts pers. comm.). The translation of FtsW mRNA may be regulated by a palindromic sequence which has the potential to form a stem loop structure in which the rbs is sequestrated (Ikeda *et al.*, 1989; Khattar *et al.*, 1994; this work, Chapter 3).

1.2.4. Functional regulation of division proteins.

The regulation of function of division genes has been studied intensively for FtsZ. The inhibition of FtsZ activity by SulA and minCD has already been discussed in 1.2.2.. In addition to these inhibitors, the concentration of FtsZ appears to be critical for FtsZ ring formation *in vitro*, and also the concentration of K^+, again this has been demonstrated *in vitro* (Mukherjee *et al.*, 1992; deBoer *et al.*, 1992). PBP3 also appears to be regulated by other proteins. The MreB protein is thought to be a negative inhibitor of PBP3 activity by repressing expression from *ftsI* (Doi *et al.*, 1988). The deletion of *mreBCD* causes a concomitant increase in the levels of PBP1b and PBP3 (Doi *et al.*, 1988). FtsA has sequence similarity to MreB and may act as a positive regulator of PBP3 activity (Matsuhashi *et al.*, 1990). The activity of FtsH is involved in PBP3 activity. It is a membrane bound ATP

dependant protease which is involved in the post translational processing of the C-terminal of PBP3 in the periplasm (Tomoyasu *et al.*, 1995). Localisation of PBP3 may involve FtsW, which is thought to form part of the functional division complex involving PBP3, FtsA and other proteins (Wientjes and Nanninga, 1989; Matsuhashi *et al.*, 1990)

1.3. Replication of the Chromosome

During each cell cycle of *E. coli* the chromosome must be replicated and an identical copy passed to each daughter cell. The biochemical processes involved in replication of chromosomal DNA are generally understood (Marians, 1992). Replication of the chromosome starts at a fixed point and replication is bi-directional until both replication forks reach a termination point 180° from the origin of replication (Masters *et al.*, 1970; Prescott and Kuempel, 1972; Oka *et al.*, 1980). The rate of DNA synthesis is constant irrespective of the growth rate, being approximately 960 bp/sec for cells growing at 37°C (Cooper and Helmstetter, 1968; Helmstetter and Cooper, 1968). Therefore DNA replication is a constant to which the other cell cycle processes, initiation of replication, chromosome partition and cell division, must accommodate (Donachie, 1993). The cell must contain two chromosomes such that one can be provided for each daughter cell. The DNA concentration in a cell doubles as the cell doubles in mass. The growth rate of the cell determines the frequency of initiation by an unknown mechanism. That is if a cell has a doubling time of 60 minutes, then initiation of replication will occur once every 60 minutes. Alternatively if the doubling time is 40 minutes, then initiation occurs every 40 minutes. With cells which have a doubling time of less than 60 minutes the physical constraints of the time taken for 40 minutes for chromosome replication followed by 20 minutes for division is circumvented by multiple replication forks. These allow multiple rounds of replication where a replicating chromosome will start to replicate again before replication of the template chromosome is complete. The amount of DNA in cells with a doubling time of 20 minutes will be three times greater than that of cells with a doubling time of 60 minutes to accommodate the extra replication occurring in the cell (Cooper, 1990). Therefore initiation is controlled by the cells growth rate. The mass of DNA within a cell will govern the size of a cell (Cooper, 1990; Donachie *et al*, 1995).

1.3.1. Initiation of replication.

The initiation of chromosome replication is a separate process from DNA synthesis, and is controlled by a different group of proteins. The process of initiation involves the opening of the chromosomal DNA duplex at a specific point, *oriC* . *oriC* is the origin of replication and is at 84 minutes on the linkage map (Oka *et al.*, 1980). The separation of DNA strands at *oriC* allows the insertion of the protein complexes required for the bi-directional replication of the chromosome (Prescott and Kuempel, 1972). Under normal conditions initiation always occurs at *oriC*.

For DNA polymerase III holoenzyme to bind at *oriC* the DNA strands must be first separated. The melting reaction involves DnaA, a protein which binds specifically to a 9 bp sequence, the DnaA-box. There are four DnaA-boxes at *oriC*. The binding of 20-40 DnaA molecules to the DnaA-boxes produces the melting of nearby AT rich regions. DnaB and DnaC, other proteins involved in replication, can then bind in the gap, allowing the priming by DnaG and the insertion of DNA polymerase III holoenzyme (Bramhill and Kornberg, 1988). As DnaA initiates the melting of *oriC*, it is proposed to be the controlling element in the timing of initiation. Despite considerable study, a link between the expression of DnaA and the control of initiation has not been entirely established.

Studies on the control of initiation at the origin have shown that replication from *oriC* occurs only once per cell cycle. Initiation of replication requires fully methylated DNA. The existing chromosome is methylated on both strands at the A residue of GATC sequences by *dam* methyltransferase. The newly replicated DNA strands are hemimethylated. *oriC* contains eleven GATC regions which require methylation before initiation can occur (Smith *et al.*, 1985). After replication, most new hemimethylated DNA is methylated after a few minutes; however at *oriC* the GATC sites remain hemimethylated for 30-40% of the cell cycle. This is independent of growth rate. *oriC* is sequestered by the cytoplasmic membrane during this period and cannot be methylated. The delay in methylated *oriC* being available as a substrate for initiation may mean that other factors required for replication are no longer available until the next cell cycle. It has shown that the hemimethylated DNA containing the DnaA promoter is also sequestered,

therefore greatly reducing transcription of DnaA for a period after initiation of replication (Campbell and Kleckner, 1990).

The methylation of *oriC* is not a prerequisite for replication initiation as *dam* mutants are viable with *oriC* probably remaining the site of initiation (Messer and Noyer-Weider, 1988). However methylation of DNA is important in the regulation of function in initiation. The initiation of replication occurs at variable times (not cell cycle dependant) in *dam* mutants (Bakker and Smith, 1989). Both the reduced and increased levels of *dam* methyltransferase affect the number of initiation origins (Boye and Lobner-Olesen, 1990). The sequestering of *oriC* may involve HobH, a peptide which binds to the hemimethylated *oriC*. A *hobH* mutant showed asynchronous initiation (Herrich *et al.*, 1994).

1.3.2. Termination of replication.

The efficient termination of replication is part of the highly co-ordinated processes required during the cell cycle. The sites for termination of the replication forks are approximately 180° from *oriC*, in the 450 kb *Ter* region. This contains two replication fork traps, 5 minutes apart at 28.5 and 33.5 minutes. The T1 (28.5) trap inhibits counter clockwise forks and T2 (33.5) inhibits clockwise forks (deMassy *et al.*, 1987; Hill *et al.*, 1987). The blocks act in a polar fashion; with forks travelling clockwise not affected as they travel through T1 and vice versa for counter clockwise replication forks. Both forks are stopped in the *Ter* region. This is due to action of the *tus* gene product. Tus is a 36 kDa protein which binds to the *Ter* sequences with high affinity (Hill *et al*, 1988). There are six *Ter* sites within the *ter* region. Cells were viable after the deletion of 360 kb from the *Ter* region; although they produced many nonviable and filamentous cells, presumably due to an induced SOS system (Henson and Kuempel, 1985).

1.3.3. Chromosome partition.

The mechanism for the partition of chromosomal DNA after replication has not yet been elucidated. After chromosome replication, nucleoids are separated spatially to the cell quarters (1/4 and 3/4 of the unit cell length, Burdett *et al.*, 1986). The partition event usually occurs 2-3 minutes after the termination of replication. The inhibition of protein

synthesis condenses the chromosomes into compact nucleoids which correspond to the number of chromosomes (Kellenberger, 1988). There is a minimum cell size before division can occur, when the cell length has reached two unit cell lengths in rod-shaped cells (Donachie *et al.*, 1976; Grossman and Ron, 1989). The same phenomenon applies to spherical cells which have a volume approximately 6 times greater than that of rod-shaped cells, but where the ratio of DNA to cell mass is the same as rod-shaped cells and a minimum distance between chromosomes is still required for partition (Donachie and Begg, 1989a). Before partition occurs, there is a period of post replication protein synthesis (Donachie and Begg, 1989b). This may be time required by the cell to increase in size to 2 unit cell lengths, for synthesis of particular proteins required for partition or a combination of both the above.

A model for partition was proposed by Jacob *et al.*, (1963) where each sister chromosome is bound to an attachment site in the cell envelope. As cells grow the daughter chromosomes are mechanically separated as cell length increases. This model is no longer valid as lateral extension of the cell wall is diffuse and not at the midpoint of the cell, a necessary criteria for the hypothesis. If inhibition of protein synthesis is reversed then the daughter chromosomes can migrate to their respective poles. This is independent of the stage in the cells growth.

The phenotype for partition mutants (*par*, Hirota, 1971) is the inability of the chromosomes to divide. Examination of such mutants, after the nucleoids have been condensed and stained, by fluorescence microscopy reveals a large nucleoid mass located at the midpoint of the growing cell. The thermosensitive *par* mutants originally isolated have been shown to be DNA replication mutants and therefore not directly involved in the partition process (Hirota, 1990; Donachie, 1993). Concatermerisation of the chromosomes or inefficient replication are the constraints on partition caused by these mutant alleles.

Hiraga *et al.* (1989) isolated two ts mutant alleles which prevented partition, *mukA* and *mukB*. The *mukA* mutant was mapped to *tolC*, an outer membrane protein involved in the lethal action of Colicin E1. The partitioning of chromosomes in the *mukA* mutant was such that occasionally sister chromosomes moved together to either side of the cell midpoint. Examination of other *tolC* mutants revealed the same phenotype (Hiraga *et al.*, 1989). The second mutant is *mukB*, a cold sensitive non lethal allele, which mapped to 21 minutes on the chromosome. The gene encodes a 177

kDa protein (Niki *et al.*, 1991). The phenotype of the *mukB* mutant was complex. 5% of the total cell population were rod-shaped anucleate cells. Other cells, in pairs, exhibited incomplete partitioning of chromosomal DNA between the daughter cells. Both ts and null alleles were viable at 22°C, but lethal at 42°C (Hiraga *et al.*, 1989; Niki *et al.*, 1991). Cell division and DNA synthesis were unaffected by a *mukB* null allele (Niki *et al.*, 1991).

A hydrophobicity profile of the deduced amino acid sequence of MukB showed it to be hydrophilic. Analysis of the secondary structure shows a putative nucleotide binding sequence in the globular N-terminus and two extended α-helix domains and a large globular domain at the C-terminus (Niki *et al.*, 1991). The α-helix domains have motifs proposed to form coiled coil structures. These are present in eukaryotic filamentous proteins such as the myosin heavy chain (McLachlan and Karn, 1983). Casaregola *et al.* (1990) showed cross reaction of a yeast heavy chain monoclonal antibody and an *E. coli* protein of ~180 kDa. This may be the MukB protein. On the predicted MukB structure, Niki *et al.* (1991) proposed that MukB dimers may form coiled coil structures to pull the daughter chromosomes towards each pole. The globular N-terminus could bind to DNA and the C-terminal region act as a 'motor' domain providing the mechanistic action for partition.

It is tempting to consider that the MukB protein is the cytokinetal element required to effectively partition the daughter chromosomes. The mutant phenotype shows a partition defect and computational protein modelling of amino acid sequence hints at a system where the rapid partitioning of the chromosomes would be possible. However, many of the cells produced by the *mukB* null mutant appear normal with one chromosome per cell (Niki *et al.*, 1991). The segregation of chromosomes without a partition process should be random. If MukB was the only protein involved in partition then a greater proportion of cells with aberrant amounts of chromosomal DNA would be expected. Therefore another, less effective method of partition may exist.

1.3.4. The SOS response with respect to cell division.

E. coli displays a complex response when exposed to conditions that damage DNA (e.g. U.V. light or alkylating agents) or inhibit replication (e.g. nalidixic acid or thymine starvation). Pertinent to this thesis is the effect of the SOS

response on cell division. The transcription of genes linked with the SOS response is repressed by the protein LexA. The LexA repressor binds as a dimer to specific nucleotide sequences, the LexA-boxes (consensus LexA-box taCTGTatata.a.a.CAGta). These are located in or near the promoter regions of SOS dependant genes. The binding of LexA dimers may not completely suppress gene expression, as this depends on the number of LexA-boxes and their position relative to the promoter.

The positive regulator of the response is an activated form of the RecA protein. Single stranded DNA and ATP are required for activation of RecA. Once in the active form RecA acts as a coprotease inducing the LexA repressor to cleave at a specific peptide bond The RecA-mediated cleavage of LexA allows expression of the SOS genes within minutes after DNA damage (Sassanfar and Roberts, 1990). Upon the repair of DNA damage, RecA becomes inactive and levels of LexA increase and again repress expression of the SOS genes. A protein specifically inhibiting cell division is induced by the SOS response. This is SulA (SfiA, Huisman and D'Ari, 1980), which binds to FtsZ, the most plentiful division protein in *E. coli.*. This prevents the formation of the FtsZ ring, thought to be the cytokinetic element in cell division (Bi and Lutkenhaus, 1993). It is thought that the reaction involves GTP (Higashitani *et al.*, 1995).

Upon repair of the DNA damage, RecA becomes inactive and can no longer cleave LexA. The expression of SOS genes is again repressed by LexA binding to their promoter regions. SulA is degraded by Lon protease and FtsZ activity can resume. In *lon* mutants SulA is stabilised, having a half-life of 30 minutes. Therefore in a *lon⁻* mutant division will continue to be inhibited even though DNA damage has been repaired (Gottesman *et al.*, 1981). In addition to FtsZ being affected by the SOS response, the new division gene *ftsK* has an SOS inducible promoter *dinH* located between *lrp* and *ftsK* (Lewis *et al.*, 1992; Begg *et al.*, 1995).

1.4. The maintenance of cell shape.

Mecillinam is an amidino-penicillinam which binds specifically to PBP2 (Lund and Tybring, 1972; Matsuhashi *et al.*, 1979). The resultant morphology of mecillinam treated cells is a change from rod to spherical shape within one generation, with subsequent inhibition of cell division and a decrease in viability (Tybring and Melchior, 1975; Matsuhashi *et al.*, 1979).

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Similarly, the inactivation of the gene encoding PBP2 (*pbpA*) produces a spherical cell phenotype. Selection for cells resistant to mecillinam and other work has identified a range of proteins and genes which give a spherical phenotype; the genes therefore being involved in the maintenance of the cell shape (Adler *et al.*, 1968; Henning *et al.*, 1972; Matsuzawa *et al.*, 1973; Westling-Häggström and Normark, 1975; Wachi *et al.* 1987)). The genes and proteins involved in cell shape can be roughly divided into three classes. The inactivation of PBP2 or RodA produces a spherical morphology which is unaffected by division inhibition. Inactivation of division does not produce a filamentous phenotype in *rodA* or *pbpA* mutants, the cells remain spherical; the division potential of the cells is lost (Donachie *et al.*, 1984). The second class of spherical mutants do filament upon suppression of division and therefore retain a division potential (Donachie *et al.*, 1984). These are *cya*, *crp* and *mreB* mutants. The final group involves *dacA* and *bolA*, the overexpression of either produces spherical cells (Markiewitz *et al.*, 1985; Aldea *et al.*, 1988).

The relationship between PBP2 and the cellular concentration of guanosine tetra phosphate (ppGpp) has been extensively studied by D'Ari and his co-workers. ppGpp is a global regulator in *E. coli*. Levels of ppGpp increase inversely with growth rate. ppGpp is the effector of the stringent response where at a high ppGpp concentration the expression of certain proteins is repressed by the altering the affinity of RNA polymerase for their promoters (Cashel and Rudd, 1987). By this mechanism the growth rate can control translation by altering the levels of ribosomal RNA and aminoacyl-tRNA synthetases. Vinella *et al.* (1992) isolated two spherical mecillinam resistant alleles. The mutant alleles were mapped to the aminoacyl-tRNA synthetases *argS* and *alaS*. High levels of ppGpp, produced by overexpressing *relA*, ppGpp synthase I (Friesen *et al.*, 1976), confer mecillinam resistance (Vinella *et al.*, 1992). High levels of the FtsZ protein was shown to suppress the lethal phenotype of PBP2 inactivation (Vinella *et al.*, 1993). This finding, in conjunction with the suppression of the effects of PBP2 inactivation led Vinella *et al.* (1992, 1993) to propose that ppGpp may control the relationship between division, elongation and growth.

The identification of a second ppGpp synthase allele, *spoT*, led to the construction of a *relA spoT* double mutant (Hernandéz and Bremner, 1991; Xiao *et al.* 1991). There were no detectable levels of ppGpp although cells were viable only when grown in rich media. Cell division was affected

however, with the cells having a short filamentous phenotype, therefore a lack of ppGpp may partially inhibit division (Xiao *et al.*, 1991; Vinella *et al.*, 1992).

RodA is a membrane protein with close similarity to FtsW and can be cross linked to PBP2 (Ikeda *et al.*, 1989; Matsuhashi *et al.*, 1990). The mutual suppression of an *ftsI* ts mutant, TOE23, with a *rodA* amber mutation led *Begg* et al. (1986) to assume there were subtle interactions between the division and elongation processes. The suppression however was caused by elevated levels of PBP2 and PBP5, whose genes are in the same *mrd* operon at 15 minutes on the linkage map (Matsuzawa *et al.*, 1989). The process by which the levels of PBP2, located upstream from *rodA*, and PBP5 are increased due to the *rodA* amber mutation is not known. *rodA* is not an essential gene (K. Begg pers. comm.).

The *crp* and *cya* mutants are involved in the control of a different global regulator, the cAMP-CAP complex. *crp*, located at 85 minutes, encodes the cyclic AMP receptor protein (CAP) and *cya* at 74 minutes, encodes for adenyl cyclase. Both give rise to spherical and mecillinam resistant cells (Aono *et al.*, 1979; Kumar, 1976). The relevance of both mutants to cell shape is doubtful as the growth of both mutant alleles is dependant on the culture conditions. However, mutants of either gene raise the frequency of division, suggesting that perhaps the cAMP-CAP complex exerts a negative regulation on septation (Donachie *et al.*, 1984).

The *mre* operon (*envB.*) was first identified by Westling-Häggström and Normark (1975). They isolated a spherical mutant, *envB*, which was mapped to 71 minutes. The *mre* operon consists of at least six genes *mreBCD*, *orfA*, *orfB* and *cafA* . Mutation of *mreB* or deletion of *mreBCD* produces spherical cells (Wachi *et al.*, 1987; Wachi *et al.*, 1989; Wachi *et al.*, 1991). Overproduction of *mreB* induces filamentation. This is due to a decrease in PBP3; MreB appears to affect *ftsI* (PBP3) expression. The *mreB* mutant and a *mreBCD* deletion have elevated levels of PBP3 and PBP1b (Wachi *et al.*, 1987; Wachi *et al.*, 1989).

An increase of either PBP5 or BolA also causes cells to become spherical. Markiewitz *et al.* (1985) found that overexpression of the *dacA* gene produced spherical cells. It has been proposed that the increase in the tetra-and tripeptide side chains on the murein precursor molecules and murein polymer, produced as a result of the increased DD-carboxypeptidase activity of PBP5, are the preferred substrate for the septum synthesising

enzyme PBP3 and therefore division is favoured over elongation (Begg *et al.*, 1990). *bolA* is located at 9.5 minutes on the genetic map (Aldea *et al.*, 1988). The overexpression of *bolA* also produces a spherical phenotype (Aldea *et al.*, 1988). The deletion of *bolA* has no noticeable effect on growth rate or division. The expression of *bolA* is from a 'gearbox' promoter whereby the expression of *bolA* is inversely proportional to the growth rate of the cell (Aldea *et al.*, 1989). One hypothesis is that BolA increases the number of divisions when growth rate decreases as cells enter the stationary phase.

1.5. The *mra* region.

1.5.1. The organisation of the *mra* region.

As discussed previously, there are many genes involved in the processes of cell growth and division distributed around the chromosome of *E. coli* . Biochemical studies on temperature sensitive mutants produced by chemical mutagenesis by Miyakawa *et al.* (1972), identified a cluster of genes involved in the biosynthesis of peptidoglycan precursors located at 2 minutes on the *E. coli* chromosome. Miyakawa *et al.* (1972) named the cluster *mra* after *murein* synthesis region *a*. Similar studies on growth mutants isolated by Lugtenberg and van Schijndel-van Dam (1972a, 1972b, 1973) identified the genes coding for D-alanyl-D-alanine adding enzyme, L-alanine adding enzyme, D-*m*-diaminopimelic acid adding enzyme and D-alanine: D-alanine ligase. Further genetic analysis on the complementation of these mutant alleles revealed the region in which the genes were located. In addition, further research led to the identification, sequencing and expression of other genes in the 2 minute region (Fletcher *et al.*, 1978; Lutkenhaus and Donachie, 1980; Begg *et al.*, 1980; Lutkenhaus *et al.*, 1980; Salmond *et al;*, 1980; Robinson *et al.*, 1984; Robinson *et al.*, 1986; Maruyama *et al.*, 1988; Ishino *et al.*, 1989; Mengin-Lecreulx *et al.*, 1989; Tao and Ishiguro, 1989; Ikeda *et al.*, 1990).

The gene organisation of the *mra* region is now complete and there are 16 ORFs which have been sequenced (Figure 1.5.1.). The catalytic activity of the products from some of the genes in the *mra* cluster have been confirmed (Table 1.5.1.). The genes within this cluster are closely spaced with seven overlaps between the termini and start codons of adjacent genes (Table 1.5.2.). The genes are contiguous with no transcriptional terminator

sequences until after the last gene in the cluster, *envA* (Beall and Lutkenhaus, 1987). Seven genes from the cluster have been shown to be involved in cell division (Table 1.5.1.; see 1.2.1.). The other genes are involved with the biosynthesis of the murein precursor molecule Lipid II (Table 1.5.1.; see 1.2.1.). The functions of the first two genes of the *mra* cluster, *mraZ* and *mraW*, have not yet been identified. Mutant alleles have been isolated for all of the genes within the cluster with the exception of *mraZ, mraW* and *mraY*.

The division genes *ftsL, ftsI, ftsW, ftsQ, ftsA, ftsZ* and *envA* have already been described in 1.2.1(see Table 1.5.1.). Their relative positions within the *mra* region are shown in Figure 1.5.1. The growth genes encoding the enzymes which make lipid II located in *mra* are shown in Figure 1.5.1. Those proteins involved in the cytoplasmic synthesis of UDP-MurNAc-pentapeptide (Figure 1.1.3.) are encoded by *murE, murF, murD, murC* and *ddlB* respectively. The genes encoding the proteins which catalyse the production of Lipid I and lipid II, *mraY* and *murG*, are also found in the *mra* region (Figure 1.5.1.; Table 1.5.1.).

The similarity between the MurC, MurD, MurE and MurF proteins was examined by Ikeda *et al.* (1990). All of the ligases had two domains of similar identity. The first domain shows considerable similarity with the ATP binding site of other nucleotide-binding proteins (GXXKT/S). The presence of an ATP binding domain has been proposed for MurD by Mengin-Lecreulx *et al.* (1989a) and Parquet *et al.* (1990). The function of the second domain is not known.

The transcriptional organisation of the distal portion of the *mra* region has been intensively studied with 8 promoters identified from *ftsW* until *ftsZ* (Beall and Lutkenhaus, 1987; Dewar *et al.*, 1989; Mengin-Lecreulx *et al.*, 1989; Garrido *et al.*, 1993; see 1.2.2.). The regulation of expression of the distal genes *ftsQ, ftsA* and in particular *ftsZ* appears to be highly complex (see 1.2.2-4.). The identification of promoter regions for expression of the proximal genes of the *mra* cluster is less documented (see Figure 1.5.1.). Promoters have been identified in *ftsI* and *mraY*; these are required for the expression of *murE/murF* and *murD* respectively (Mengin-Lecreulx *et al.*, 1989; see Figure 1.5.1.). The promoter for *ftsI* has been shown to located more than 1 kb upstream (Hara and Park, 1993). Other potential promoter sequences have been identified in *mraW* and *ftsL* (see Figure 1.5.1.), although there is no experimental evidence available to confirm these. The inability to complement the chromosomal disruption of *ftsZ* with a single copy of *ftsZ*

containing 6 kb of upstream DNA suggests that the *in vivo* regulation of the *mra* region is more complex than has already been demonstrated (Dai and Lutkenhaus, 1991). This, in conjunction with the absence of transcriptional terminators throughout the operon, has produced speculation that the entire region may be transcribed as one massive transcript (Dai and Lutkenhaus, 1991; Hara and Park, 1993).

mraZ mraW ftsL ftsI　　murE　　murF　　mraY　murD　　ftsW　　murG　　murC　　ddlB　ftsQ　　ftsA　　ftsZ　　envA

P*_{mraZ}　P*_{ftsL}　　P*_{murE}　　　　　　P*_{murD}　　　P*_{murG}　　　P*_{ddlB}　P1 P2　　　　P4 P3 P2　　P*_{envA}　　T
　　　　　　　　　　　　　　　　　　　　　　　　　　　　　　　　　　　ftsQ ftsQ　　ftsZ ftsZ ftsZ
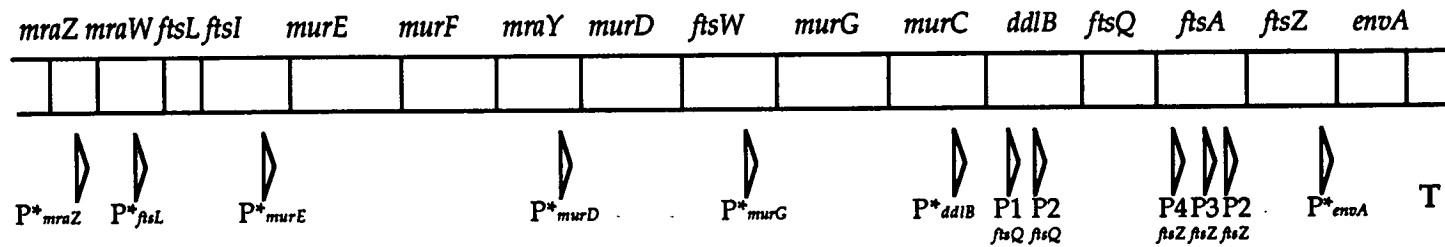
Figure 1.5.1. A schematic diagram of the *mra* region showing the sixteen genes involved in cell growth or division. Also shown are the putative promoters (P*) and the known promoters (P). Promoters are numbered if there are more than one involved in the expression of a particular gene. The terminator (T) is also shown after *envA*, on the right of the diagram.

Table 1.5.1. Shown overleaf are the 16 genes which comprise the *mra* region of *E. coli*. Presented in the table are the sequential order of the genes and the size in bp of each gene. The location of the proteins is based on the hydrophobicity of the peptide sequence or experimental evidence. The isolation of conditional mutants allowed the characterization of 14 of the 16 genes. There are no *mraZ* or *mraW* mutants therefore their function is not known. The proposed functions of the proteins produced by the *mra* genes has been described where possible.

Gene	Size (BP)	Protein	Weight (kDa)	Location	Division/ Growth	Function/Proposed function (*)	Reference
mraZ	456	MraZ	34.8		Unknown	Unknown	
mraW	939	MraW	17.4		Unknown	Unknown	
ftsL	375	FtsL		Membrane	Division	Involved in the localisation of division complex*.	Guzman et al. 1992.
ftsI	1764	PBP3	63.8	Membrane	Division	Penicillin-binding protein involved in the biosynthesis of septal murein	Nakamura et al. 1983.
murE	1845	MurE	53.3	Cytoplasm	Growth	D-m -diaminopimelate adding enzyme	Tao and Ishiguro. 1989.
murF	1359	MurF	47.3	Cytoplasm	Growth	D-alanyl-D-alanine adding enzyme	Parquet et al. 1989.
mraY	1083	MraY	39.8	Cytoplasm	Growth	UDP-MurNAc-pentapeptide : undecaprenyl-phosphate phospho-MurNAc-pentapeptide transferase	Ikeda et al. 1990a.
murD	1317	MurD	46.9	Cytoplasm	Growth	D-glutamate adding enzyme	Ikeda et al. 1990a.
ftsW	1245	FtsW	46	Membrane	Division	A division protein involved in the localisation of PBP3*	Ikeda et al. 1989.
murG	1044	MurG	36.7	Membrane	Growth	UDP-GlucNAc:MurNAc-pentapeptide-pyrophosphoryl-undecaprenol GlucNAc transferase	Ikeda et al. 1990b.
murC	1473	MurC	53.6	Cytoplasm	Growth	L- alanine adding enzyme	Ikeda et al. 1990b.
ddlB	921	DdlB	32.8	Cytoplasm	Growth	D-alanine: D-alanine adding enzyme	Robinson et al. 1984.
ftsQ	828	FtsQ	45.3	Membrane	Division	A division protein, role is unknown	Robinson et al. 1984.
ftsA	1263	FtsA	45.3	Membrane	Division	A division protein, role unknown	Robinson et al. 1984.
ftsZ	1149	ftsZ	40.3	Cytoplasm	Division	Forms a ring at the cell midpoint, thought to contract during septation*	Yi and Lutkenhaus. 1985.
envA	918	EnvA	33.9		Division		Beall and Lutkenhaus. 1987

Table 1.5.1. A list of the relevant properties of the *mra* region. Comments are on the opposite page.

Genes	Separation (bp)	Genes	Separation (bp)
mraZ-mraW	Gap 2	*ftsW-murG*	Gap 21
mraW-ftsL	Overlap 4	*murG-murC*	Gap 54
ftsL-ftsI	Gap 15	*murC-ddlB*	Overlap 7
ftsI-murE	Overlap 14	*ddlB-ftsQ*	Gap 1
murE-murF	Overlap 4	*ftsQ-ftsA*	Overlap 4
murF-mraY	Overlap 7	*ftsA-ftsZ*	Gap 60
mraY-murD	Gap 3	*ftsZ-envA*	Gap 100
murD-ftsW	Overlap 1		

Table 1.5.2. The distance, in bp, between the genes of the *mra* region. There are seven overlapping regions between the genes (this work, Mengin-Lecreulx *et al.*, 1989a; Ikeda *et al.*, 1990a, 1990b; Robinson *et al.*, 1984).

1.5.2. Homologs of the *E. coli mra* region genes with other organisms.

There are now many homologs of genes in the *mra* region which have been identified in other bacteria and higher organisms. A search of the nucleotide database at NCBI identified more than 30 sequences which show similarity to genes of the *mra* cluster. The sequences wee from Gram-positive and Gram negative bacteria, archaebacteria, cyanobacteria, mycobacteria, chloroplasts and mitochondria.

The sequencing of the genomes of *H. influenzae* and *Mycobacterium genitalium* (Fleichmann *et al.*, 1995; Fraser *et al.*, 1995) allows comparisons to be made with the *E. coli mra* region. A region similar to *mra* has been identified in *B. subtilis* at 133°-135° and many of the genes within this region have been mapped and sequenced (Miyao *et al.*, 1992; Daniels and Errington, 1993). In Figure 1.5.2., the *mra* regions of *E. coli*, *B. subtilis*, *H. influenzae* and *M. genitalium* are drawn to show the similarities and differences between the different species.

| E. coli | *mraZ mraW ftsL ftsI murE murF mraY murD ftsW murG murC ddlB ftsQ ftsA ftsZ envA* |

| H. influenzae | *ftsL ftsI murE murF mraY murD ftsW murG murC ddlB ftsQ ftsA ftsZ envA* |

| B. subtilis | *ftsI spoVD murE mraY murD spoVE murG orf2 dds orf4 divIB sbp ftsA ftsZ* |

| M. genitalium | *mraZ mraW orfX ftsZ* |
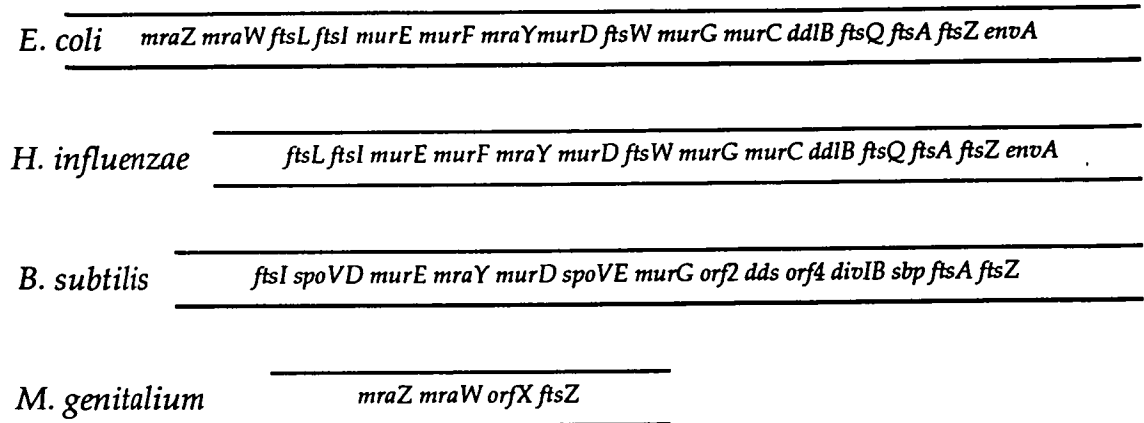
Figure 1.5.2. The order of genes in the *mra* regions of *E. coli, H. influenzae, B. Subtilis* and *M. genitalium*. The diagram is not to scale and intergenic regions are not shown.

The organisation of genes between the *mra* regions of *E. coli* and *H. influenzae* is the same (Fleichmann *et al.*, 1995). The *mra* region of *B. subtilis* also exhibits some similarities. However there are sporulation-specific genes in the *mra* cluster of *B. subtilis*. These include *spoVD*, a sporulation specific protein with similarity to PBP3 of *E. coli* (Daniel *et al.*, 1994). Daniel *et al.* (1994) propose that the gene encoding PBP3 required for vegetative growth lies immediately upstream from *spoVD*. The *spoVE* gene produces a peptide with similarity to the FtsW and RodA proteins of *E. coli* (Ikeda *et al.*, 1989). There are genes encoding MurE, MraY, murG, FtsQ, FtsA and FtsZ in both clusters (Daniel and Errington, 1993; Beall *et al.*, 1988; Gonzy-Treboul *et al.*, 1992; Miyao *et al.*, 1992; Harry *et al.*, 1993; Daniel *et al.*, 1994). Interestingly there is no coding frame which encodes a MurF-like protein in *B. subtilis*. *murF* in *B .subtilis* may be located in a different region of the chromosome. The functions of *orf2* and *orf4* are unknown. The *mra* region of *M. genitalium* has only three homologues of the *E. coli mra* region. These are *mraZ, mraW* and *ftsZ* (Fraser *et al.*, 1995; W. Donachie pers. comm.). Also present in this cluster is *orfX* whose peptide shows no similarity to other peptides (W. Donachie, pers. comm.).

Chapter 2.

Materials and Methods.

2.1 Bacterial, phage strains and plasmids.

Bacterial strains used in this study are listed in table 2.1.1. Bacteria were either maintained on L broth agar plates stored at room temperature or for longer term storage in L broth agar stabs or in frozen storage buffer at -70°C Bacteriophages used in this study are listed in Table 2.1.2. Phage lysates were stored at 4°C as broth suspensions to which a few drops of chloroform had been added to prevent microbial growth. M13 phage lysates were stored at 4°C without chloroform as M13 is chloroform sensitive.

Plasmids constructed in the course of this study are listed in Table 2.1.3.

Table 2.1.1. Bacterial strains used.

Strain	Relevant genotype	Source/reference
BL21(λDE3)	F⁻ *ompT [lon] hsdS*$_B$ (r$_B$⁻ m$_B$⁻); an E. coli B strain) containing λDE3, a λ prophage carrying T7 RNA polymerase.	Studier and Moffat, 1986
BW313	Hfr *lysA dut ung thi-1 recA spoT1*	Raleigh *et al*. 1989
C600	F⁻ e14⁻(McrA⁻) *thr-1 leuB6 thi-1 lacY1 supE44 rfbD1 fhuA21;* the original C600 is *ecoK* r⁺m⁺ McrBC⁺.	Laboratory stocks
C600F	C600 *leuTn::10 murF*^{ts} (from TKL-46)	K. Begg
C600T	C600 *leu::Tn10* Tet^r	This work
D301	(RP4871) Che⁺ *Δ(lacZYA) U169 recD1903 (Tet^r)*	Russel *et al.* 1989
DBHY1	C600 *DftsI mraY::CAT*	This work
DBHY2	DBHY1 *sep*⁺ 82, a λ prophage carrying *leuA-ftsI*	This work
DBHY3	C600 *mraY::CAT.* Cmp^R.	This work
DBHΩ3	DBHΩ2, *recA* Tet^R.	This work
DBWΩ1	DSB1 *ftsW:: Ω.* Tet^R/ Spc^R/Str^R/Kan^R	This work
DBW C1	DSB1 *ftsW::CAT*	This work
DBW C2	DSB5 *ftsW::CAT*	This work
DBYC2	C600 *leu::Tn10 mraY::CAT.* Tet^R/Cmp^R.	This work
DBYΩ1	DSB1 with *mraY::Ω*	This work
DH5αF'IQ	*endA1 hsdR17 (r$_K$⁻m$_K$⁺) supE44 thi-1 l- recA1 gyrA96 Δ80 dlacZ Δ(M15 Δ(lacZYA argF) U169/F' proAB lacIq lacZΔ(M15 Tn5 relA1.* Kan^R.	Laboratory stocks
DL307	F⁻, *recD*	D. Leach
DSB1	SHA6 *recD::minitet*. Tet^R.	This work

Strain	Relevant genotype	Source/reference
DSB2	SHA5 *leu::Tn9*. Cmp^R.	This work
DSB4	DBH Ω3 except *ftsW$^+$*	This work
DSB5	C600T *pcnB::Kan*. Tet^R/Kan^R.	This work
DSB8	C600T *rodA$^{ts.}$* Tet^R.	This work
JC10-240	Hfr:PO45; *lysA>serA ilv--318 thr-300 srlC*300::Tn10 *thi*1 *recA56 relA rpsE*300. Tet^R.	A. J. Clark
JC411	*leuB6 hisG1 argG6 metB1 lacY1 gal-6 xyl-7 mtl-2 malA1 polA^{ts} str104 tonA2 tsx-1* $\lambda^R\lambda^-$ *supE44*. Str^R.	Clark *et al.*, 1969
JC411T	JC411 *leu::Tn10*. Tet^R.	This work
JM101	F' *traD36 lac^q Δ(lacZ)M15 proA$^+$B$^+$/supE thi Δ(lac-proAB)* Str^R.	Laboratory stocks
JM110	F' *traD36 lac^q Δ(lacZ)M15 proA$^+$B$^+$/rpsL (Str^r) thr leu thi lacY galK galT ara fhuA dam dcm supE44 Δ(lac-proAB)* Str^R.	Laboratory stocks
JM263	JM101 *ftsW^{ts}*. Str^R/Tet^R.	This work
KH201	OV2 *leu::Tn10 ftsW^{ts}* Tet^R.	Khattar *et al.* 1994
KH2157	OV2 *leu::Tn10 ftsI^{ts}*Tet^R.	M. Khattar
KH2219	OV2 *leu::Tn10 ftsI^{ts} murE^{ts}* Tet^R.	M. Khattar
KH2690	OV2 with *leu::Tn10 ftsI^{ts}* Tet^R.	M. Khattar
KJB24	W3110 *rodA^{am}* Kan^R.	K. Begg
MM38	*Asn$^+$ argG6 hisG1 leuB6 metB1 pyrE gal-6 lacY1 xyl-7 bgl$^+$ fhuA2 T1R Ph80R gyrA rpsl104 tsx-1 TGR uhp$^-$ l$^-$ ΔpcnB*. Kan^R.	S. MacAteer
MMK129	W3110 *leu::Tn10 ftsI^{ts}*. Tet^R.	M. Khattar
MMK441	W3110 *leu::Tn10 ftsI^{ts}* Tet^R.	M. Khattar
MMK704	W3110 *leu::Tn10 ftsI^{ts}* Tet^R.	M. Khattar
MMK1888	W3110 *leu::Tn10 murD^{ts}* Tet^R.	M. Khattar
MMK2628	W3110 *leu::Tn10 murE^{ts}* Tet^R.	M. Khattar
OV2	*ara$_{am}$ lac125$_{am}$ gala42 K$_{am}$ galE$^-$ trp$_{am}$ leu$^-$ ilv$^-$ his$^-$ thy$^-$ sulIII A81^{ts}*	Laboratory stocks
OV2C	OV2 with *leu::Tn9* Cmp^R.	This work
OV58	OV2 *murG$_{am}$ leu::Tn10* Tet^R.	Salmond *et al.* 1980
OV263	OV2 *leu::Tn10 ftsW^{ts}*Tet^R.	M. Khattar
SHA5	KJB 24 with a chromosomal duplication from ~0 minutes to ~14 minutes *leu$^+$/leu::Tn10* Tet^R/Kan^R.	S. Addinall
SHA6	SHA5 except diploid for *leu$^+$* Kan^R.	S. Addinall

Strain	Relevant genotype	Source/reference
TKL-46	*thr leu trp arg ilvA his thi thyA pyrF tsx lac tonA murF*[ts]	Lugtenberg and van Schijndel-van Dam 1972
TG1	F' *traΔ36 lacI*[q] *Δ(lacZ)M15 proA⁺B⁺/ supE Δ(hsdM-mcrB)5* (r$_K$⁻m$_K$⁻ McrB⁻)*thi Δ(lac proAB)*	Laboratory stocks
TOE23	*arg his pro thr thi thy ftsI*[ts]*leu::Tn10* Tet[R].	Begg *et al.* 1980
TP8503	*Δlac-proB leu⁻ thi supE42 fhuA; T1R, Ph80R ::Tn7* Tmp[R]	Laboratory stocks
Wam6	OV2T with an unidentified TS allele	This work
Wam34	OV2T with an unidentified TS allele	This work
W3110	INV: *rrnΔ-rrnE;1 sup⁻ l⁻*	Laboratory stocks
W3110C	W3110 *leu::Tn9* Cmp[R].	This work
W3110T	W3110 *leu::Tn10* Tet[R.]	This work

Table 2.1.2. Bacteriophages used.

Phage	Description	Source/reference
P1	Wild-type transducing phage	Laboratory stock
λBSD40	λ phage carrying *ftsI murE' lacZSC lacY lacA* Kan[R]	This work
λDE3	λ phage carrying*lac*UV5 T7 *gene1*	Studier and Moffat (1986)
λΔB	λ phage carrying *ftsA ftsZ*	Lutkenhaus *et al.* (1980)
λΔΔ	λ phage carrying *murG*	Begg *et al.* (1980)
λΔE	λ phage carrying *ddlB ftsQ ftsA ftsZ envA*	Lutkenhaus *et al.* (1980)
λddl	λ phage carrying *murG murC ftsQ*	Begg *et al.,* 1980.
λFH16	λ phage carrying *ftsQ ftsA*	Begg *et al.,* 1980.
λGH200	λ phage carrying *envA*	Lutkenhaus *et al.* (1980).
λ16-2	λ phage carrying *murG murC ftsQ ftsA ftsZ*	Lutkenhaus and Donachie (1979).
λ*sep*⁺ 82	λ phage carrying *leuA- ftsI*	Fletcher *et al.* (1978).
λ6CI [109]	EMBL 4 containing stuffer fragment from 1.7 to 2.07 minutes	Kohara *et al.* (1987).
λ6F3 [110]	EMBL 4 containing stuffer fragment from 1.97 to 2.28 minutes	Kohara *et al.* (1987).
λ15B8 [111]	EMBL 4 containing stuffer fragment from 2.18 to 2.54 minutes	Kohara *et al.* (1987).
λ235	EMBL 4 containing stuffer fragment from 24.53 to 24.98 minutes	Kohara *et al.* (1987)
λ540	λ phage carrying *supF*	Borck *et al.* (1976)

Phage	Description	Source/reference
λvir	Virulent	Laboratory stock
M13mp18	M13 based cloning vector	Laboratory stock
M13mp19	M13 based cloning vector	Laboratory stock
MW19	Hin dIII / EcoRI fragment from pDBW4 containing C-terminus of murD ftsW and the N-terminus of murG cloned into M13mp19	This work
MWam	MW19 containing the ftsW^{am} allele	This work
MY18	BglII / Hin dIII fragment containing the gene10-mraY fusion and the N-terminus from pETY3 cloned into BamHI / Hin dIII restricted M13mp19	This work

Table 2.1.3. Plasmids used.

Plasmid	Description	Source/reference
pACEFY1	Hin dIII / BglII fragment from pDEG1 cloned into Hin dIII / BamHI restricted pACYC177. C-terminal region of ftsI, murE, murF, mraY, and the N-terminal region of murD transcribed from Kan^R promoter. 8.3 kb. Amp^R.	This work
pACYC177	p15A replicon. 3.9 kb. Amp^R Kan^R.	Lab stocks
pACYC184	p15A replicon. 4.2 kb. Cmp^R/Tet^R.	Lab stocks
pARα	As pACYC177 with Kan^R removed. 3.1 kb. Amp^R.	M. Templin
pBAD18	ColE1 based replicon. PBAD upstream of polylinker. Amp^R transcribed fron PBAD. 4.4 kb. Amp^R.	Guzman et al. 1995.
pBADG1	EcoRI / HindIII fragment from pDDG1 cloned into pBAD18 restricted with EcoRI/Hin dIII. The C-terminal portion of mraY, murD, ftsW, murG and the N-terminal portion of murC transcribed from PBAD. 8.8 kb. Amp^R.	This work
pBADW1	SmaI fragment removed from pBADG1 and the vector religated. The C-terminal portion of mraY, murD, ftsW and the N-terminal portion of murG transcribed from PBAD. 7.2 kb. Amp^R.	This work
pBAW1	PvuII / SmaI fragment from pDDG1 cloned into pBAD18 with transcription of the C-terminal portion of murD, ftsW and the N-terminal portion of murG from PBAD. 5.8 kb. Amp^R.	This work
pBAW2	As pBAW1 but with the PvuII/smaI fragment orientated against transcription from PBAD. 5.8 kb. Amp^R.	This work
pBBW1	EcoRI / HindIII fragment from pBUBW1 cloned into EcoRI / HindIII restricted pBAD18. The C-terminal portion of murD, ftsW and the N-terminal portion of murG transcribed from PBAD. 6.8 kb. Amp^R.	This work

Plasmid	Description	Source/reference
pBEFY1	*Eco*RI fragment from pDEG1 cloned into *Eco*RI restricted pBAD18. Insert cloned such that the C-terminus of *ftsI*, *murE*, *murF*, *mraY* and the N-terminus of *murD* are transcribed from PBAD. 8.8 kb. Amp^R.	This work
pBPW1	*Eco*RI/*Hin*dIII fragment from pUCW1 cloned into *Eco*RI/*Hin*dIII restricted pBAD18. The C-terminal portion of *murD*, *ftsW* and the N-terminal portion of *murG* transcribed from PBAD. 6.2 kb. Amp^R.	This work
pBSD5	*Sma*I/*Pvu*II fragment from pDBE4 cloned into the *Sma*I restriction site of pUC18. Cloned such that the C-terminus of *ftsI* and the N-terminus of *murE* are transcribed from P*lac*. 3.3 kb. Amp^R.	This work
pBSD6	As pBSD5 with the fragment cloned in the opposite orientation to P*lac*. 3.3 kb. Amp^R.	This work
pBSD30	*Eco*RI/*Bgl*II fragment from pDDG1 cloned into the *Eco*RI/*Bam*HI restriction sites of pRS551. *lacZYA* are transcribed from the C-terminal region of *mraY* and the N-terminal region of *murD*. 12.9 kb. Amp^R Kan^R.	This work
pBSD40	*Eco*RI/*Bam*HI fragment from pBSD5 cloned into pRS551 restricted with *Eco*RI/*Bam*HI. Orientation of the fragment is such that *lacZYA* are transcribed from the insert. 13.1 kb. Amp^R Kan^R.	This work
pBUBW1	*Bgl*II/*Sma*I fragment from pDDG1 cloned into *Bam*HI/*Hinc*II restricted pUC18. The C-terminal portion of *murD*, *ftsW* and the N-terminal portion of *murG* transcribed from P*lac*. 5.1 kb. Amp^R.	This work
pCM4	Cmp^R gene from Tn*9* in pBR322 based plasmid. Cmp^R cassette flanked by *Bam*HI. 4.1 kb. Amp^{R}Cmp^R.	Close *et al.* 1982.
pDBC3	A partial *Sma*I restriction of pTGC3 and religation of the vector fragment. The transcription of the C-terminal portion of *murG*, *murC* and the N-terminal region of *ddlB* is from the φ10 promoter. 5.0 kb. Amp^R.	This work
pDBD3	An *Eco*RI/*Cla*I restriction of pTDG3, filled in and ligated. The transcription of the C-terminal portion of *mraY*, *murD* and the N-terminal region of *ftsW* is from the φ10 promoter. 4.3 kb. Amp^R.	This work
pDBE4	An *Eco*RI/*Eco*RV restriction of pDEFY4 with the *Eco*RV site filled in. The vector was religated where the C-terminal portion of *ftsI*, *murE* and the N-terminal region of *murF* are transcribed from the φ10 promoter. 4.2 kb. Amp^R.	This work
pDBF4	*Pvu*II fragment from pDEG1 cloned into the *Sma*I restriction site of pT7-4. The cloned region was transcribed from the C-terminus of *murE*, *murF* and the N-terminus of *mraY* from the φ10 promoter. 5.3 kb. Amp^R.	This work
pDBG3	An *Eco*RI/*Cla*I restriction of pTDG3 filled in and the plasmid religated. The C-terminal region of *ftsW*, *murG* and the N-terminal portion of *murC* is transcribed from the φ10 promoter. 4.8 kb. Amp^R.	This work

Plasmid	Description	Source/reference
pDBW4	*PvuII/ SmaI* fragment from pDDG1 cloned into pT7-4 such that the C-terminal portion of *murD, ftsW* and the N-terminal portion of *murG* are transcribed from φ10 promoter. 3.7 kb. Amp^R.	This work
pDBY4	*EcoRV* fragment from pDEG1 cloned into the *SmaI* site of pT7-4. The C-terminal portion of *murE, mraY* and the N-terminal region of *murD* are transcribed from the φ10 promoter. 5.1 kb. Amp^R.	This work
pDBY6	As pDBY4 but the *EcoRV* fragment is cloned in pT7-6. 4.9 kb. Amp^R.	This work
pDDC1	*SalI* fragment from λ110 cloned into *SalI* restricted pDDG1. The C-terminal portion of *mraY, murD, ftsW, murG, murC* and the N-terminal region of *ddlB* are transcribed by P*lac*. 8.2 kb. Amp^R.	This work
pDDG1	*EcoRI* restricted pDEG1 religated such that the C-terminus of *mraY, murD, ftsW, murG* and the N-terminus of *murC* are transcribed from P*lac*. 7.1 kb. Amp^R.	This work
pDEF4.	pDEFY4 restricted with *EcoRI* and the vector religated. The cloned region was transcribed from the C-terminus of *ftsI, murE, murF* and the N-terminus of *mraY* from the φ10 promoter. 6.4 kb. Amp^R.	This work
pDEFY4	*HindIII/ BglII* fragment from pDEG2 cloned into *HindIII/ BamHI* restricted pT7-4. The C-terminal portion of *ftsI, murE, mraY* and the N-terminal region of *murD* are transcribed from the φ10 promoter. 6.8 kb. Amp^R.	This work
pDEFY6	As pDEFY4 but the vector is pT7-6. 6.6 kb. Amp^R.	This work
pDEFHY4	*NcoI* and partial *EcoRI* restriction fragment from pEHY16 cloned into pDEFY4 restricted with *NcoI/EcoRI*. The C-terminal portion of *ftsI, murE, murFHY (murF'-gene10'-polyhis-mraY)* and the N-terminal portion of *murD* are transcribed from the φ10 promoter. 7.1 kb. Amp^R.	This work
pDEFHY5	An *EcoRI* restriction of pDEFHY4 with religation of the plasmid. The C-terminal portion of *ftsI, murE, murFHY' (murF'-gene10'-polyhis-mraY')* are transcribed from the φ10 promoter. 6.7 kb. Amp^R.	This work
pDEG1	*KpnI* fragment from λ110 cloned into pUC18. Cloned such that the C-terminal region of *ftsI, murE, murF, mraY, murD, ftsW, murG* and the N-terminal region of *murC* are transcribed from P*lac*. 11.1 kb. Amp^R.	This work
pDEG2	As pDEG1 but with *KpnI* fragment opposing transcription from P*lac*. 11.1 kb. Amp^R.	This work
pDIE4	*EcoRI/ EcoRV* restriction of pDIEFY with the *EcoRI* site filled in. The C-terminus of *ftsL, ftsI, murE* and the N-terminus of *murF* are transcribed from the φ10 promoter. 4.9 kb. Amp^R.	This work
pDIEF4	An *EcoRI* restriction of pDIEFY4 with religation of the vector. The clone contains the C-terminal portion of *ftsL, ftsI, murE, murF* and the N-terminal region of *mraY* are transcribed from the φ10 promoter. 7.0 kb. Amp^R.	This work

Plasmid	Description	Source/reference
pDIEFY4	*Hin*dIII/*Kpn*I fragment cloned into pDEFY4 restricted with *Hin*dIII/*Kpn*I. The clone contains the C-terminal portion of *ftsL, ftsI, murE, mraY* and the N-terminal region of *murD* are transcribed from the φ10 promoter. 7.5 kb. Amp^R.	This work
pDGC1	*Sal*I fragment from pDDC1 cloned into pUC18. The transcription of the C-terminal portion of *ftsW, murG, murC* and the N-terminal region of *ddlB* is from the P*lac* promoter. 5.2 kb. Amp^R.	This work
pDWK1	*Cla*I restricted pDDG1 'filled in ' and the *Hin*cII Kan^R casette cloned into this. Kan^R is transcribed in the same orientation as P*lac*. 8.5 kb. Amp^R Kan^R.	This work
pDWΩ1	*Cla*I restricted pDDG1 with the ends 'filled in'. *Sma*I restricted Ω cassette cloned into the *Cla*I site in *ftsW*. 9.1 kb. Amp^R Spc^R Str^R.	This work
pDYC1	The *Bam*HI Cmp^R fragment was restricted from pCM4 and filled in. This was inserted into the *Xmn*I site in *mraY* by partially restricting pDEG1 with *Xmn*I. The cassette was expressed in the same orientation as *mraY*. 12 kb. Amp^R Cmp^R.	This work
pDYΩ1	Partially restricted pDEG1 with *Xmn*I. The *Sma*I restricted Ω cassette was cloned into this site to disrupt *mraY*. 13.1 kb. Amp^R Spc^R Str^R.	This work
pET3c	pBR322 based replicon. The N-terminal portion of T7 gene 10 is transcribed from the φ10 promoter. 4.4 kb. Amp^R.	Rodriguez *et al.*, 1988.
pETY3c	PCR generated fragment with an *Nde*I site introduced at the start of *mraY*. *Nde*I/*Bgl*III restriction of the PCR fragment cloned into *Nde*I/*Bam*HI restricted pET3c. *gene10' mraY* fusion and the N-terminal portion of *murD* transcribed from φ10 promoter. 6.0 kb. Amp^R.	This work
pE'Y3c	*Sau*3AI fragment from pDBY4 cloned into the *Bam*HI site in pET3c. The *gene10' 'mraY* truncation fusion and the N-terminal region of *murD* are transcribed from φ10 promoter. 5.3kb Amp^R.	This work
pET16b	A pBR322 based replicon. The *gene10* N-terminal region is fused to a polyHis tag. Transcription is from the φ10 promoter. 5.4 kb. Amp^R.	Novagen Corp.
pETHY1	*Nde*I/*Hin*dIII fragment from pETY3c cloned into pET16b restricted with the same enzymes. The *gene10*-polyhis *mraY* fusion and the N-terminal region of *murD* are transcribed from the φ10 promoter. 7.0 kb. Amp^R.	This work
pHP45Ω	Contains the Ω cassette on *Sma*I, *Eco*RI, *Bam*HI and *Hin*dIII fragments in a pBR322 background. 4.4 kb. Amp^R Spc^R Str^R.	Prentki and Kirsch, 1984.
pJF118EH	ColE1 replicon. P*tac* upstream of polylinker. 5.1 kb. Amp^R.	Furste *et al.*, 1988.
pJFY1	*Eco*RV fragment from pDEG1 cloned into *Sma*I restricted pJF118EH. The C-terminal portion of *murF, mraY* and the N-terminal portion of *murD* are transcribed from P*tac*. 7.8 kb. Amp^R.	This work

Plasmid	Description	Source/reference
pJFY2	This is the same as pJFY1 but the *EcoRV* fragment is cloned in the opposite orientation to P*tac*. 7.8 kb. Amp^R.	This work
pJFY3c	*BglII/Hin*dIII fragment from pETY3c cloned into *Bam*HI/*Hin*dIII. Transcription of the *gene10-mraY* fusion and the N-terminal portion of *murD* is from p*tac*. 7.0 kb. Amp^R.	This work
pLysS	T7 *gene1* cloned in pACYC184 Tet^R marker. 5.5 kb. Cmp^R.	Laboratory stocks
pMAK705	A construct with a pSC101 ts replicon. P*lac* with a polylinker downstream. 5.5 kb. Cmp^R.	Hamilton *et al.*, 1986.
pMAKYC1	*EcoRV* fragment from pDYC1 cloned into pMAK705. The insert was transcribed from P*lac*. 8.8 kb. Cmp^R.	This work
pMAKYΩ1	*EcoRV* fragment from pDYΩ1 cloned into pMAK705. The insert was transcribed from P*lac*. 10.2 kb. Cmp^R Spc^R Str^R.	This work
pMDG1	*EcoRI* fragment from pDEG2 cloned into *EcoRI* partially restricted pMAK705. The C-terminal portion of *mraY, ftsW, murG* and the N-terminal portion of *murC* transcribed from P*lac*. 9.9 kb. Cmp^R.	This work
pRS551	A reporter vector containing *lacZ lacY* and *lacA* downstream of *EcoRI/Bam*HI cloning sites. pBR322 replicon. 12.6 kb. Amp^R Kan^R.	Simons *et al.*, 1986.
pT7-3	A ColE1 replicon with polylinker in front of φ10 promoter (Amp^R is transcribed in the same direction as φ10) 2.3 kb. Amp^R.	S. Tabor
pT7-4	As pT7-3 but with polylinker reversed in front of φ10 promoter (Amp^R is transcribed in the same direction as φ10) 2.4 kb. Amp^R.	S. Tabor
pT7-4I	*Bam*HI/*Pvu*II fragment from λ109 cloned into pT7-4. The C-trerminal portion of *ftsL, ftsI* and the N-terminal region of *murE* are transcribed from the φ10 promoter. 5.0 kb. Amp^R.	T. Ogura.
pT7-6	As pT7-4 but with Amp^R reversed relative to φ10. 2.2 kb. Amp^R.	S. Tabor
pTDC3	*Sal*I fragment from λ110 cloned into pTDG3 restricted with *Sal*I. The transcription of the C-terminal portion of *mraY, murD, ftsW, murG, murC* and the N-terminal region of *ddlB* is from the φ10 promoter. 7.9 kb. Amp^R.	This work
pTDG3	*EcoRI/Hin*dIII fragment from pDDG1 cloned into pT7-3 restricted wtih the same enzymes. The transcription of the C-terminal portion of *mraY, murD, ftsW, murG* and the N-terminal region of *murC* is from the φ10 promoter. 6.8 kb. Amp^R.	This work
pT7Wam	*Hin*dIII/*EcoRI* fragment from MWam19. Cloned into *Hin*dIII/*EcoRI* restricted pT7-4. C-terminal region of *murD, ftsWam* and N-terminus of *murG* transcribed from φ10 promoter. 3.8 kb. Amp^R.	This work

Plasmid	Description	Source/reference
pUC4 K	Contains the Kan^R gene from Tn903 on EcoRI, BamHI, SalI, HincII or PstI fragments. pMB1 replicon. 5.3 kb. Amp^R Kan^R.	Laboratory stocks
pUC18	ColE1 derived replicon (pMB1). Plac promoter region and polylinker. 2.7 kb. Amp^R.	Laboratory stocks
pUC19	As pUC18 but with the polylinker reversed.	Laboratory stocks
pUCP1	PstI fragment from pDDG1 cloned into the PstI site of pUC18. Transcription of the C-terminal portion of mraY, murD, ftsW, murG and the N-terminal portion of murC transcribed from Plac. 6 kb. Amp^R.	This work
pUCP2	As pUCP1 but with the cloned insert in the opposite orientation.	This work
pUCW1	SmaI restricted pUCP1. Religated such that the C-terminal portion of mraY, murD, ftsW, murG and the N-terminal portion of murG transcribed from Plac. 4.5 kb. Amp^R.	This work
pUCW18	PvuII/SmaI fragment from pDDG1 cloned into pUC18 such that the C-terminal portion of murD, ftsW and the C-terminal portion of murG transcribed from Plac. 4.1 kb. Amp^R.	This work
pUCWam	HindIII/EcoRI fragment from pT7Wam cloned into HindIII/EcoRI restricted pUC19. C-terminal region of murD, ftsWam and N-terminus of murG transcribed from Plac. 3.8 kb. Amp^R.	This work
pUCY1	EcoRV fragment from pDEG1 cloned into SmaI restricted pUC18. The C-terminal portion of murF, mraY and the N-terminal portion of murD are transcribed from Plac. 5.4 kb. Amp^R.	This work
pUCY2	This is the same as pUCY1 but the EcoRV fragment is cloned in the opposite orientation. 5.4 kb. Amp^R.	This work
pUCF19	This is the 2.7 HindII/EcoRI fragment containing the C-terminus of murE murF and the N-terminus of mraY, subcloned from pDBF4 into pUC19 restricted with HindIII/EcoRI. murF is expressed in the same orientation as Plac. 5.4 kb. Amp^R.	This work
pWAC1	EcoRV/SmaI fragment from pDEG1 cloned into the EcoRV site in pACYC184. The C-terminus of murD, ftsW and the N-terminus of murG are transcribed from the Tet^R promoter. 6.6 kb. Cmp^R.	This work

Growth media and buffers.

Growth media, bacterial/phage buffers and other commonly used buffers
are listed in Tables 2.1.4. and 2.1.5 respectively. L broth and L agar were
used routinely for all bacterial manipulations, except where stated. For work
with phage λ the media were supplemented with 10mM MgSO4 and 0.2%
maltose to maximise the expression of the λ receptor protein; for phage P1
2.5mM CaCl2 was added. VB minimal agar with appropriate carbon

sources, vitamins and amino acids, was used for selection of nutritional markers.

Growth of Bacteria.

Bacteria were routinely grown as liquid cultures at 37°C (or 30°C for temperature sensitive strains). Usually fresh overnight cultures that had been inoculated from a single colony were diluted back the following day and grown as required.

Table 2.1.4. Growth Media.

Luria broth (LB)	Difco bacto tryptone	10 g
	Difco bacto yeast extract	5 g
	NaCl	5 g
Distilled water to 1 litre		
pH to 7.2 with NaOH		

L-agar	L-broth + 15 g Difco agar per litre

LB top agar	L-broth + 6.5 g Difco agar per litre

Nutrient broth	Oxoid No.2 nutrient broth	25 g
	Distilled water to 1 litre	

Nutrient agar	Nutrient broth + 12.5 g Davis NZ agar

MacConkey agar	Peptone	20 g
	Bile salts No. 3	1.5 g
	NaCl	5 g
	Neutral red	0.03 g
	Crystal violet	0.001 g
	Difco agar	15 g
	Distilled water to 1 litre	

Table 2.1.4. Continued.

VB minimal media	20x VB salts	50 ml
	20% carbon source	10 ml
	Thiamine HCl (1mg/ml)	2 ml
	Supplements as required	
	Distilled water to 1 litre	

VB minimal Agar	As VB minimal media + 15 g Difco agar per litre.

20x VB salts	$MgSO_4.7H_2O$	4 g
	Citric acid	40 g
	KH_2PO_4	400 g
	$NaNH_4.HPO_4.4H_2O$	70 g
	Distilled water to 1 litre	

Spizizen's broth	$(NH_4)_2SO_4$	10 g
	K_2HPO_4	70 g
	KH_4PO_4	30 g
	Sodium citrate.$2H_2O$	5 g
	$MgSO_4\,7H_2O$	1 g
	Distilled water to 1 litre.	

Recipe for SOC broth	Bactotryptone	4 g
	Bacto yeast extract	1 g
	5M NaCl	400 µl
	1M $MgCl_2$	2 ml
	1M KCl	0.5 ml
	1M $MgSO_4$	2 ml
	Glucose	0.72 g
	Distilled water to 200 millilitres.	

Table 2.1.5. Commonly used buffers.

Bacterial buffer	$MgSO_4.7H_2O$	2 g
	Na_2HPO_4	7 g
	KH_2PO_4	3 g
	NaCl	4 g
	Distilled water to 1 litre	

Phage buffer	Na_2HPO_4	7 g
	KH_2PO_4	3 g
	NaCl	5 g
	$MgSO_4$ (1.0 M)	1 ml
	$CaCl_2$ (0.1 M)	10 ml
	1% gelatin solution	1 ml
	Distilled water to 1 litre	

TE buffer	10mM Tris-HCl (pH 8.0)
	1mM EDTA (pH 8.0)

TAE buffer   Working solution :
                  40 mM Tris-Acetate
                  2 mM EDTA

	50x conc. stock solution:	
	Tris base	242 g
	Glacial acetic acid	57.1 ml
	0.5 M EDTA (pH 8.0)	100 ml
	Distilled water to 1 litre	

TBE buffer: Working solution:
            89 mM Tris-borate
            89 mM Boric acid

5x conc. stock solution :	Tris base	54 g
	Boric acid	27.5 g
	0.05 M EDTA (pH 8.0)	20 ml

Minimal medium supplements.

Amino acid supplements were stored in stock solutions of pure amino acids at a concentration of between 2-10 mg/ml depending on the solubility of the particular amino acid. Sparingly soluble amino acids such as tyrosine were dissolved in 0.01M NaOH. The final concentration of the amino acids in the media was usually in the order of 20-100 μg/ml. If a rich minimal media was required, vitamin free casamino acids (αCAA) were used. The stock concentration of αCAA was 100mg/ml and the final concentration in the medium was typically 1-5 μg/ml. It should be noticed that αCAA lacks tryptophan and this should therefore be added to αCAA media if the bacterial strain to be used has an auxotrophy for this amino acid.

Selection of antibiotic resistance.

The routine concentrations for the antibiotics used in this work are shown in Table 2.1.6. All antibiotics were used in both complex and minimal media with the exception of trimethoprim, which was only used in minimal medium.

Table 2.1.6. Antibiotic Solutions.

Antibiotic	Solvent	conc, of stock solution (mg/ml)	final conc. in media (µg/ml)
Ampicillin	H_2O	100	50-100
Chloramphenicol	Ethanol	20	25
Kanamycin sulphate	H_2O	25	25-50
Spectinomycin dihydrochloride	H_2O	50	25-50
Streptomycin sulphate	H_2O	100	20
Tetracycline hydrochloride	50% ethanol	10	10
Trimethoprim	Methanol	5	50

2.2. DNA Techniques.

2.2.1. Large-scale preparation of plasmid DNA.

Use a fresh overnight of the plasmid carrying strain (grown in the appropriate antibiotics) to inoculate 500 millilitres of growth medium, containing appropriate antibiotics or other selection, and incubate overnight at 37°C with vigorous shaking. Chill the culture on ice for 15 minutes and pellet the cells by centrifugation at 5000 rpm in a GSA or GS-3 rotor for 15 minutes at 4°C. Discard the supernatant and resuspend the pelleted cells in 200 millilitres of chilled TE buffer, then pellet again as above. Discard the supernatant, allow the cells to drain, and remove any remaining buffer with a Pasteur pipette. Resuspend the cells in 5 millilitres of the Tris-sucrose buffer, then transfer the liquid to a 50 millilitre polypropylene centrifuge tube using a Pasteur pipette to remove any remaining cell suspension from the centrifuge bottle. Add 1 millilitre of 20 mg/ml lysozyme solution in H_2O and leave on ice for 5 minutes, mixing frequently by gentle swirling. Then add 1 millilitre 0.5M EDTA (pH 8.0) followed by 0.8 millilitres of 10 mg/ml RNase solution in H2O. Mix by gentle swirling and leave on ice for a further 5 minutes. Add 6 millilitres of Triton lysis mix, cover the mouth with parafilm, and mix the contents by inverting the tube several times. Leave on ice for about 10 minutes, after which the cells should have lysed well and the solution become viscous and partially cleared. If lysis is slow, then extra

Triton X-100 may be added as a 10% solution in H2O (up to a maximum of 1.5 millilitres). To ensure complete mixing, the contents of the tube can be drawn up into and expelled from a 25 millilitre pipette; do not repeat this more than twice, otherwise chromosomal DNA contamination may become a problem.

Centrifuge the lysate in an SS-34 rotor at 15,000 rpm for 30 minutes at 4°C to pellet the cellular debris and most of the chromosomal DNA. With large plasmids (>15kb) a shorter centrifugation time should be used, 15-20 minutes, since plasmid DNA may also sediment and reduce the yield. Pour off the supernatant into a sterile 25 millilitre measuring cylinder, taking care to leave the pellet of debris. CsCl and ethidium bromide can now be added ready for isopycnic centrifugation to separate the supercoiled plasmid from any remaining chromosomal DNA, RNA and protein. Add 17.1 g of CsCl and mix until fully dissolved. Then add 0.342 millilitres of ethidium bromide (10 mg/ml) and make the final volume up to 23 millilitres with TE buffer and mix thoroughly. Transfer the solution to two Beckman Ti50 Sorvall crimp seal tubes and balance both to within 0.05 g. Spin the tubes in a 50Ti fixed-angle rotor at 38,000 rpm for about 60 hours at 18°C. After centrifugation carefully remove the tubes and place vertically in a rack. Place the tube in a gradient holder and firmly clamp it firmly in place. Under U.V. illumination two bands can be seen; the lower of which is more intense, this is the supercoiled plasmid DNA. Remove this band by inserting a syringe with a 0.9 x 40 mm needle just below the band and carefully withdraw this in 1-2 millilitres. Transfer this to a 25 millilitre sterile plastic universal. Remove the ethidium bromide by extracting 5 times with isopropanol saturated with CsCl and H2O. Transfer to 8/32 inch dialysis tubing, seal and dialyse in 2 litres of TE buffer at 4°C for 30 minutes. Change the TE buffer and leave with stirring for 4 hours and finally repeat leaving the dialysis tubing overnight. Collect the dialysed liquid from the tubing in sterile 10 millilitre beakers. Aliquot 0.5 millilitre amounts into Eppendorf tubes and add 50 microlitres 3M sodium acetate (pH 5.0) to each, vortex and add 0.4 millilitres isopropanol. Vortex and spin at 15,000 rpm in a microcentrifuge at 4°C for 30min. Remove the supernatant and add 1 millilitre of 70% ethanol, vortex the pellet and spin at 15 000 rpm for 5 minutes. Remove the supernatant and vacuum dry the pellet. Dissolve in 110 microlitres TE buffer and measure the absorbance at 260 nm and 280 nm of 10 microlitres DNA solution diluted to 0.4 millilitres in TE buffer. The 260

nm reading can be used to calculate the plasmid DNA concentration and the purity of the DNA can be determined by 260/280. For double stranded plasmid DNA 1.8 represents total purity.

Solutions Used.

1. TE buffer: 10 mM Tris-HCl, pH 8.0, 1 mM EDTA.
2. Tris-sucrose solution : 50 mM Tris-HCl, pH 8.0, 25% (w:v) sucrose
3. Lysozyme solution : 20 mg/ml in H2O.
4. EDTA : 0.5 M EDTA, pH 8.0
5. RNase A solution : 10 mg/ml RNase A (heat treated to inactivate DNase)
6. Triton Lysis Buffer :   50 mM Tris-HCl pH 8.0

    62.5 mM EDTA

    0.1% (w:v) Triton X-100
7. Ethidium bromide solution : 10 mg/ml in T.E

2.2.2 Plasmid minipreparation: Alkaline lysis method.

The routine method of harvesting plasmid DNA was performed using a variation of the method developed by Birnboim and Doly (1979). Inoculate 5 millilitres of medium containing the appropriate selective antibiotics with a single bacterial colony. Incubate with shaking at the appropriate temperature overnight.

Spin down the culture in a bench centrifuge at 5,000 rpm for 5 minutes and discard the supernatant. Resuspend the pellet in 100 microlitres of ice cold buffer containing 1% glucose, 10 mM EDTA and 25 mM Tris-HCl (pH 8.0) and transfer to an Eppendorf tube. Incubate at room temperature for 5 minutes. Add 0.2 microlitres of 0.2 M NaOH/1% SDS solution and mix by gently inverting the tube several times and incubate on ice for 5 minutes. To this add 150 microlitres of 3M Na acetate (pH 5.0), and then briefly vortex and again leave on ice for at least 5 minutes. Spin in a microcentrifuge at 15,000 rpm for 10 minutes and remove the pelleted chromosomal DNA and cell debris with a sterile toothpick. Add 450 microlitres of phenol/chloroform/isoamyl alcohol (25:24:1 v/v) and vortex for 30 seconds. Spin in a microcentrifuge for 5 minutes and transfer the supernatant (~0.5 ml) to a fresh sterile Eppendorf tube.

The DNA can now be pelleted by either absolute alcohol or isopropanol precipitation. There is no requirement for the addition of Na acetate. The pellet can then be washed in 70% ethanol and the pellet drained and dried. Typically the pellet is dissolved in 20-50 microlitres of TE buffer, containing RNase A (20 mg/ml) depending on the plasmid copy number to give a DNA concentration of ~0.1 mg/ml. The DNA can now be used for manipulation.

2.2.3. Preparation of a concentrated phage suspension and extraction of DNA.

Pick a single colony of NM621 and grow in L broth overnight at 37°C. Inoculate 200 millilites L broth supplemented with 0.2M $MgSO_4$ and incubate at 37°C with vigorous agitation. Follow the O.D. till A650 = 0.5. Add phage to give a multiplicity of infection (m.o.i.) of 0.1-1.0 (high m.o.i preferred for cI^+ phage and a low m.o.i. for cI^- phage). Follow the O.D. as it rises and then starts to fall as cells lyse, this may take up to 4 hours. When the O.D reaches a minimum add 0.2 millilitres of chloroform and shake for 10 minutes. Add 8 grammes NaCl to the flask, dissolve and add DNase and RNase to 1 μg/ml each, mix and leave at room temperature for 1 hour. Spin down the cell suspension in a GSA rotor at 10,000 rpm for 10 minutes and decant the supernatant into a 500 millilitre flask. Add 20 grammes PEG6000 to the sample, and dissolve. Leave overnight at 4°C to allow the phage particles and macromolecules to precipitate. Swirl the flask to resuspend the precipitate and transfer to a 250 millilitre centrifuge bottle. Spin at 10,000 rpm for 10 minutes in a GSA rotor at 4°C. Decant the supernatant carefully to avoid losing the precipitate. Remove any remaining supernatant with a Pasteur pipette. Add 5 millilitres of phage buffer and leave shaking gently overnight at 4°C to disaggregate the pellet. To ensure even suspension, draw the liquid up and down in a Pasteur pipette and transfer to a sterile bottle.

To purify the concentrated lysate it is layered on a CsCl density gradient. Prepare CsCl in phage buffer to densities of 1.3, 1.5 and 1.7 g/cc. Using a Beckman polyallomer tube, firstly add 2 millilitres of the 1.3 solution, then 2 millilitres of the 1.5 solution and finally 2 millilitres of the 1.7 solution. Carefully layer the phage suspension on the top of the gradient. Balance to 0.05 grammes with another tube and spin at 35,000 rpm in a MSE 6x14 swinging bucket rotor for 2 hours. After centrifugation, carefully

remove the tube and clamp it firmly in a gradient holder. Remove the opaque band which is near the top of the 1.5 g/cc step using a syringe with a 0.9 x 40 mm needle. A dark background can help to reveal the phage particle band. Dialyse the phage suspension against 5 litres TE buffer overnight at 4°C. Transfer 0.5 millilitre aliquots of the suspension to an equal volume of phenol (Tris equilibrated, pH 8.0) in a microfuge tube. Place the tubes in a blood mixer for 2 minutes and then spin in a microfuge. Remove the aqueous layer and transfer to a fresh microfuge tube and extract with phenol again. Repeat this step once more. Retain the phenol-containing microfuge tubes and add 0.2 millilitres TE buffer to the first and repeat the process sequentially to each tube to back extract any residual DNA at the supernatant/phenol interface.

Dialyse the aqueous layers overnight in 5 litres TE buffer at 4°C. Aliquot 0.5 millilitres volumes into sterile microfuge tubes and add 50 microlitres 3M Na acetate to each and vortex. Add 0.4 millilitres isopropanol, vortex and spin in a microfuge at 15,000 rpm at 4°C. Decant the supernatant, add 1 millilitre 70% ethanol, vortex and spin in a microfuge for 5 minutes at 15,000 rpm. Remove the supernatant and vacuum dry the pellets. Resuspend in 110 microlitres TE buffer and use 10 microlitres in a total volume of 400 microlitres TE buffer to measure the O.D. 260 and 280 to determine phage DNA concentration and purity.

2.2.4. Small-scale preparation of λ phage DNA.

Take 5 millilitres L broth and add 50 microlitres of 100 mM MgSO4. Add 100 microlitres of a freshly grown overnight culture and add 1×10^8 phage/ml. As a control inoculate 5 millilitres L broth with culture only. Incubate at 37°C with shaking for 3-5 hours until lysis occurs, the control can be used to see the cell lysis. Add 200 microlitres chloroform and vortex for 30 seconds. Spin in a bench centrifuge for 10 minutes at 5,000 rpm. Transfer the supernatant to a sterile Bijou bottle. Add 5µg/ml of DNase and RNaseA and incubate with shaking at 37°C for 30 minutes. Transfer liquid to a 15 millilitre sterile Corex tube and add 4 millilitres of PEG solution. Mix and leave at 4°C for at least 1 hour. Pellet the phage particles in an SS34 rotor at 10,000 rpm for 20 minutes at 4°C. Remove the supernatant by aspiration and stand the inverted tube on a paper towel to drain remaining liquid. Add 0.5 millilitres phage buffer and gently resuspend the phage pellet. Add 0.5

millilitres chloroform and briefly vortex three times. Transfer to a sterile microfuge tube and spin at 4,000 rpm for 3 minutes. Transfer the upper layer to a fresh tube. Add 0.5 millilitres phenol (Tris equilibrated, pH 8.0) and 100 microlitres TE buffer and mix on a blood mixer for 5 minutes. Spin for 2 minutes and transfer the upper layer (~500 microlitres) to a fresh tube. Add 500 microlitres phenol/chloroform/isoamyl alcohol (25:24:1 v/v) and mix on a blood mixer for 5 minutes. Spin at 15,000 rpm for 2 minutes and again remove the upper layer and transfer to a fresh tube. Add 500 microlitres of chloroform and mix for 5 minutes. Spin at 15,000 rpm for 2 minutes and transfer 450 microlitres of the upper layer to fresh tube. Add 900 microlitres of 100% ethanol and mix and leave on ice for 10 minutes. Spin at 15 000 rpm for 10 minutes, remove the supernatant and rinse the pellet with 800 microlitres 70% ethanol. Remove the supernatant and resuspend the pellet in 400 microlitres TE buffer. Add 40 microlitres NaAcetate and mix. Add 400 microlitres isopropanol and mix. Spin at 15,000 rpm at 4°C for 15 minutes and remove the supernatant. Add 1 millilitres 70% ethanol, vortex and spin at 15,000 rpm for 5 minutes. Remove the supernatant and vacuum dry the pellet. Resuspend the phage DNA pellet in 50 microlitres TE buffer. Use 5 microlitres (~1 microgramme) per digest.

PEG Solution - 20% PEG 6000, 2M NaCl in phage buffer.

2.2.5. Large scale preparation of chromosomal DNA.

A single colony of the required strain was used to inoculate 5 millilitres of broth, containing the appropriate antibiotics and grown overnight with shaking. This was then used to inoculate 100 millilitres of broth which again was incubated overnight with appropriate selection and incubation temperature with constant agitation. The culture was then chilled on ice for 10 minutes and then transferred to a 250 ml centrifuge bottle and spun at 10,000 rpm for 5 minutes in a Sorval GSA rotor. The supernatant was drained and the pellet resuspended in 20 millilitres of STE (TE buffer with 10 mM NaCl) and 10 millilitre volumes transferred to sterile glass universal bottles. To these were added 0.5 millilitres of 10% SDS solution and 0.5 millilitres of proteinase K solution (4 mg/ml). The solutions were then gently mixed and incubated at 50°C for 6 hours. An equal volume

of phenol/chloroform/isoamyl alcohol (25:24:1 v/v) was added to the solutions and then mixed by gentle inversion of the bottles and left to stand at room temperature for 15 minutes. These were then spun in a bench centrifuge 5,000 rpm for 15 minutes to separate the aqueous and phenolic phases. The aqueous layer was then carefully removed using a 25 ml pipette, leaving the precipitated protein interface and transferred to a sterile 100 ml beaker. The nucleic acids in the solution were then recovered by adjusting the sample to 0.2 M sodium acetate and adding two volumes of ethanol chilled to -20°C. The DNA was spooled from the aqueous ethanol interface using a sterile glass rod. The pellet of DNA was then washed in 70% ethanol, briefly dried and left to dissolve in 10 millilitres TE buffer overnight at 45°C.To the nucleic acid solution was added 0.1 millilitres RNaseA solution (10 mg/ml) and this was incubated at 37°C for 1 hour. SDS solution (10%) in 0.5 millilitre volumes was added followed by 0.25 millilitres proteinase K (4 mg/ml). This solution was gently mixed and incubated at 50°C for 1 hour The solution was again phenol/chloroform/isoamyl alcohol extracted and the DNA spooled as above. The DNA was washed in 70% ethanol, air dried and resuspended in 1 millilitre TE buffer. This took 1-3 days at 37°C. The DNA concentration was determined using spectrophotometry as previously described. A typical yield of chromosomal DNA from 100 millilitre culture is ~500 microgrammes.

2.2.6. Small-scale preparation of chromosomal DNA.

Inoculate 5 millilitres of media containing the appropriate selective antibiotics with a single bacterial colony. Incubate overnight at the permissive temperature. Spin 1.5 millilitres of the culture in a microcentrifuge for 2 minutes, or until a compact pellet is formed and discard the supernatant. Resuspend the pellet in 567 microlitres of TE buffer and add 3 microlitres of proteinase K (20 mg/ml) and 30 microlitres 10% SDS. Mix thoroughly and incubate at 37°C for 1 hour. Add 100 microlitres of 5 M NaCl and mix thoroughly. Add 80 microlitres of hexadecyltrimethyl ammonium bromide (CTAB)/NaCl solution, mix thoroughly and incubate for 10 minutes at 65°C. Add an equal volume of chloroform/isoamyl alcohol (1:1 v/v), mix thoroughly and spin in a microcentrifuge for 5 minutes. Transfer the aqueous upper layer to a new tube and add an equal volume of

phenol/chloroform/isoamyl alcohol (25:24:1 v/v), mix thoroughly and spin in a microcentrifuge. Transfer the supernatant to a fresh tube and add 0.6 volume of isopropanol. Mix the contents and spin in a microcentrifuge for 5 minutes at room temperature. Remove the supernatant and wash the pellet in 1 millilitre 70% ethanol, then spin in a microcentrifuge for 5 minutes at room temperature. Discard the supernatant and briefly dry the pellet in a vacuum dessicator. Resuspend the pellet in 100 microlitres of TE buffer and use 15 microlitres per restriction digest.

CTAB/NaCl solution (10% CTAB in 0.7M NaCl)
Adjust the final volume to 100 millilitres. Dissolve 4.1 grammes NaCl in 80 millilitres H_2O and slowly add 10 grammes CTAB (hexadecyltrimethyl ammonium bromide) while heating and stirring, If necessary, heat to 65°C to dissolve.

2.2.7. DNA precipitation.

Two methods were employed to precipitate DNA from aqueous solution.

1) A 1/10 volume of 3 M sodium acetate (pH 5.0) was added to the DNA solution and then mixed by vortexing. To this add 3 volumes of absolute ethanol, mix thoroughly and leave on ice for 10 minutes. Spin in a microcentrifuge at 15,000 rpm for at least 15 minutes and discard the supernatant. Add 1 millilitre of 70% ethanol and vortex vigorously to wash the pellet. Spin in a microcentrifuge for 5 minutes at 15,000 rpm, remove the supernatant and dry the pellet for 3 minutes in a vacuum dessicator.

2) Alternatively 1 volume of isopropanol could be used instead of 3 volumes of absolute ethanol. An advantage of this was that the final volume was reduced by half. This was the preferred method used for most DNA precipitations.

2.2.8. Determination of DNA concentration.

To determine the concentration of DNA solutions, diluted aliquots (typically 10 microlitres in 390 microlitres of TE buffer) had their adsorption measured at 260 nm. An OD 260 value of 1.0 represents a concentration of

50 μg/ml for double-stranded DNA and 40 mg/ml for single-stranded DNA. The purity of the DNA can be calculated by measuring the adsorption of the DNA solution at 280 nm. The 260/280 ratio should be close to 1.8 to represent protein free double-stranded DNA and 2.0 for single-stranded DNA.

2.2.9. Restriction of DNA.

The digestion of DNA using restriction endonucleases was performed usually in 20-50 microlitre volumes depending on the concentration of the DNA sample, normally 1-10 μg/ml. The digest solution contains 1X concentration of the appropriate buffer and the restriction enzyme was present in two to five fold excess, i.e. 2-5 Units of enzyme per microgram of DNA. The final volume of the digest were made up using 1X TE buffer. For restriction enzymes requiring BSA, this too was added in accordance with the manufacturers instructions, usually 10 μg/ml. For complete restriction of the DNA the digests were incubated for 1-2 hours at the restriction enzymes optimum temperature, 37°C for most enzymes used. The reactions were terminated by the addition of TAE loading buffer in preparation for analysis by agarose gel electrophoresis or phenol extracted, ethanol precipitated and resuspended in a suitable volume of TE buffer for further manipulations.

Partial digestion of DNA. For the partial digestion of DNA then six twofold serial dilutions of restriction enzyme were added to DNA of a fixed concentration. The greatest concentration of enzyme used in such reactions was 0.5 Units of enzyme per microgram of DNA. The digestion reactions were incubated for 1 hour. The reactions were terminated using TAE loading buffer and the samples could now be analysed by agarose gel electrophoresis.

Digestion of DNA using two restriction enzymes. For digestion of DNA using two restriction enzymes then one of two approaches was taken. If the enzymes had a compatible buffer then both enzymes were added in equal concentration to the reaction mixture. If there was no compatible buffer for both, the digestion conditions would be made to suit the restriction enzyme which had a requirement for a low salt buffer. The digest would be

incubated for 1-2 hours and then the digest volume would be doubled using the appropriate amount of the second buffer (high salt) and the second restriction enzyme. The final volume made was up with TE buffer. This would be incubated at the optimum temperature for a further 1-2 hours.

2.2.10. 'Filling in' of recessed 3' termini.

The Klenow enzyme was used to 'fill in ' the 3' recess, formed after digestion of DNA with certain restriction endonucleases, to create blunt ended DNA molecules. The Klenow reactions were performed in 20 μl volumes containing 1 μg DNA, 1X nick translation buffer (Boehringer Mannheim), each dNTP at a final concentration of 20 mM, 2 Units of Klenow enzyme and TE buffer to make up the final volume to 20 microlitres. The samples were incubated at 37°C for 30 minutes and then heat inactivated at 70°C for 15 minutes. TE buffer was added to increase the final volume to 100 microlitres, before phenol extraction to remove protein and unincorporated dNTP's. The DNA was ethanol precipitated and then resuspended in a suitable volume of TE buffer for addition to ligation reactions.

2.2. 11. Agarose gel electrophoresis of DNA.

Agarose gel electrophoresis was used to separate DNA fragments after digestion with restriction endonucleases. The concentration of agarose used depended upon the sizes of DNA fragment being separated. For fragments of 300 bp to 1.5 kb 1.5% agarose was used, between 1.5 kb to 4 kb 1% agarose and above 4 kb 0.8% agarose. The agarose was dissolved in TAE buffer, cooled to ~60°C and then poured into the gel tray, the comb(s) inserted and left to solidify for 20 minutes. Three types of electrophoresis equipment were used, all made by BRL. The minigel (5 x 7.5 cm) used for rapid (1-2 hours) separation of DNA fragments usually for ligation reactions or preparing probes for hybridisation. The midigel (11 x 15 cm) and the maxigel (20 x 25 cm) were used to check plasmid miniprep DNA digests for clones. The midigel apparatus was used also to run chromosomal digests prior to Southern blotting onto nylon filters. The wells of the gels were always loaded dry and then the TAE buffer was carefully added to each reservoir until it overflowed across the surface of the gel joining both together. The minigel was run at 60-80 mA and the larger gels were typically

run overnight at 20-30 mA. Once the gel had run sufficiently (this could be approximately gauged by observing the marker dye migration) the power was terminated and the gel placed in EtBr in TAE, to stain the DNA, and then destained in TAE. After this the DNA fragments could be visualised by exposing the gel to UV illumination.

2.2.12. Extraction of DNA from agarose gel slices.

To extract DNA from agarose gels after electrophoresis the Geneclean kit manufactured by BIO 101 was used. The principle behind the kit is a silica matrix which binds DNA in high salt concentration but not in low salt concentration. Agarose gels were stained with ethidium bromide and the DNA bands could be visualised in the gel when placed on a UV transilluminator. The desired bands were excised using a sterile scalpel blade and each placed in a sterile microcentrifuge tube, weighed and three volumes of sodium iodide added to each. These were incubated at 50°C for 5 minutes until the gel slice dissolves. To each is added five microlitres of Glassmilk then vortexed and placed on ice for five minutes with frequent vortexing. The tubes are spun briefly to pellet the glassmilk and the supernatant discarded. The pellet is resuspended in 0.5 microlitres of New Wash (an ethanol based buffer to remove the sodium iodide) and pelleted again. This process is repeated a further two times and then the pellet is resuspended in 10 microlitres of TE buffer. This is incubated at 50°C for 10 minutes and then spun to pellet the glassmilk. The aqueous solution now contains the DNA previously bound to the Glassmilk. This is transferred to a fresh Eppendorf tube and the pellet is again resuspended in 5 microlitres of TE buffer and incubated for 10 minutes again at 50°C to extract any remaining DNA. The tube is spun once more and the supernatant again transferred to the tube with the first supernatant. The DNA in solution is now available for further manipulation.

2.2.13. Ligation of DNA fragments.

The ligation reactions were performed in 10 microlitres volumes. The total amount of DNA used per reaction was 0.5-1.0 microgrammes with 1x ligation buffer (Boehringer Mannheim), T4 DNA ligase and TE buffer to make up to 10 microlitres when necessary. The reaction mixture for blunt

ended ligations also contained 15% PEG 8000. For sticky ended ligations a threefold excess of fragment to vector was used whilst for blunt ended ligations, a one to one ratio was used. Ligase concentration was 1 Unit per reaction for blunt ended DNA ligations and 0.2 Units per reaction for sticky ended DNA ligations. The ligation mixtures were incubated at 16°C for at least 4 hours although they were usually left overnight.

2.2.14. Preparation and transformation of Competent Cells (Chung Method).

Inoculate 5 millilitres of medium containing the appropriate selective antibiotics with a single colony and incubate with shaking at the appropriate temperature overnight. Dilute 1:50 in 25 millilitres selective media and incubate until the OD540 reaches 0.3-0.4. Place the flask on ice for 10 minutes and transfer the culture to a sterile universal. Spin in a bench centrifuge at 5,000 rpm for 10 minutes and discard the supernatant, invert the universal to drain. Resuspend the cells in 1 millilitre of TSS and place on ice. These cells are now competent and can be used immediately or stored at -70°C for up to 3 months. Plasmid DNA (typically 10-200 ng) was added to 100 microlitres of competent cells, gently mixed and left on ice for 30 minutes. After this time 0.9 millilitres of LBG (L broth supplemented with 2 mM glucose) was added to the transformation mixture. This was then incubated at the permissive temperature for 1-2 hours whilst being mixed on a rotating blood mixer. This is to allow the expression of plasmid based antibiotic markers. One hundred microlitre aliquots were plated onto selective agar and incubated overnight at an appropriate temperature. As a control, an aliquot of cells only was plated on the selective medium.

Transformation of TG1 with double stranded M13 DNA: This method was used to isolate M13 clones after ligation reactions. Competent cells of TG1 were prepared and the Chung transformation reaction performed as described above. However after 30 minutes on ice transformation mixtures were then transferred to sterile test tubes containing 300 microlitres of a fresh overnight culture of TG1. To each was then added 4 millilitres of molten (40°C) LC top agar, containing X-Gal (40 μg/ml) and IPTG (20 μg/ml), and mixed, then poured onto LB plates. These were then incubated at 37°C overnight and colourless colonies were picked stored in 1 millilitre LB.

TSS Recipe:	Difco bactotryptone	10 g
	Difco yeast extract	5 g
	NaCl	10 g
	PEG 3350	100 g
	MgSO4	20 mM
	PIPES (pH6.5)	10 mM
	Distilled water to 1 litre.	

2.1.15. Preparation of cells for electroporation.

DNA ligation mixtures for transforming cells by electroporation have to be in salt free solution to prevent arcing as the DNA solution/cell suspension is exposed to a large electrical charge. The presence of salts result in a premature release of the charge and a greatly reduced transformation efficiency. The ligation mixture was briefly spun in a microcentrifuge and a further 40 microlitres of distilled water added to increase the total volume to 50 microlitres. To this 0.5 millilitres of N-isobutanol was added and the mixture vortexed and then spun for 30 minutes at 15,000 rpm at 4°C in a microcentrifuge. The supernatant was removed and the pellet air dried and resuspended in 10 microlitres of distilled water. For each transformation 1-2 microlitres of the ligation mixture was used.

2.2.16. Preparation of cells for high efficiency electro-transformation.

Inoculate 1 litre of L broth (usually 2 x 500 millilitres in 2 litre conical flasks), containing selective agents if required, with 1 ml of a fresh o/n culture and incubate with vigorous shaking until the OD600 reaches 0.7-1.0. Chill the flasks on ice for 15-30 minutes and then pellet the cells in sterile 250 millilitre GSA centrifuge pots by spinning in a chilled rotor (4°C) at 10,000X Gmax for 5 minutes. Discard the supernatant without disturbing the pellet. Resuspend the cellular pellets in 250 millilitres of chilled sterile d.H2O and repellet as before. Continue this process using decreasing volumes of chilled sterile dH2O (i.e. 125 and 50 millilitres). Then the pellets are pooled by resuspending in 20 millilitres of chilled 50% glycerol in d.H2O, (v/v), transferred to a 36 millilitre corex tube and spun at 10,000X Gmax for 5 minutes in a Sorval SS34 rotor chilled to 4°C. The supernatant is discarded and the pellet resuspended in a final volume of 2 millilitres of sterile chilled

50% glycerol to give a cell concentration of ~2-3 x 10^{10}/ml.. Aliquots of 130 microlitres were placed in sterile Eppendorf tubes and placed on ice. Finally the cells were snap frozen in a dry ice/ethanol bath and stored at -70°C until required for use. Cells could be stored in this manner for 6 months.

2.2.17. Electro-transformation of ligation mixtures.

Thaw the cells at room temperature and place them on ice. Aliquot 2 microlitres of salt free ligation mixture (see 2.2.16.) in a chilled Eppendorf tube and leave on ice. The equipment used was a Biorad Genepulser and Pulse controller. The Genepulser was set at 25 µF and 2.5 kV and the Pulse Controller at 200 Ohms. The pulse at these settings has a time constant of 4.5-5.0 milliseconds (the field strength will be 12.5 kV/cm). Gently mix 40 microlitres of competent cells with the DNA solution by drawing up and down in a Gilson 200 micropipettor. Transfer the mixture of cells and DNA to a chilled electroporation cuvette (1mm width, Biorad) and gently tap the side of the cuvette to ensure that the mixture lies evenly on the bottom. Place the cuvette in the safety chamber slide and insert the slide into the chamber until the cuvette is seated between the two electrical contacts. Pulse the cells and immediately resuspend the cells in 1 millilitre of SOC broth (see Table 2.1.1.) and transfer the suspension to an Eppendorf tube. Leave on a blood mixer for 1-2 hours to allow the transformants to express for antibiotic selection at a suitable incubation temperature. 150 microlitre aliquots of the cell suspension were spread on agar plates with appropriate selection and incubated overnight.

2.2.18. Preparation of bacteriophage M13 DNA.

Infection by M13 phage particles: Single isolated phage plaques were picked and placed in 0.5 millilitres of L broth and vortexed, these were left for 1 hour to allow phage particles to diffuse from the agar into the broth. The strain TG1 was used for the propagation of all M13 stocks. A 5 millilitre aliquot of L broth was inoculated with a single colony of cells and incubated overnight at 37°C. One hundred and fifty microlitres of this culture was used to inoculate 5 millilitres L broth To this was added 100 microlitres of the phage suspension and the culture was incubated at 37°C for 3-5 hours. The culture was then spun for 10 minutes at 5,000 rpm in a bench centrifuge

and 1.3 millilitres of the supernatant transferred to an Eppendorf tube. The bacterial pellet could be used to prepare the double-stranded replicative form of M13 DNA and the supernatant for the single-stranded M13 DNA (or as a fresh bacteriophage suspension).

Preparation of double-stranded M13 DNA: The bacterial pellet was washed once in bacterial buffer and repelleted. The double-stranded DNA was prepared in the same manner as for the small-scale isolation of plasmid DNA.

Preparation of single-stranded M13 DNA: To the 1.3 millilitres of the culture supernatant collected was added 200 microlitres of a solution containing 20% polyethylene glycol (PEG 8000) in 2.5 M sodium chloride and this was then thoroughly mixed by vortexing. The solution was left at room temperature for 15 minutes and then spun in a microcentrifuge to pellet the precipitated phage particles. The supernatant was removed to leave the phage pellet as dry as possible. The pellet was resuspended in 100 microlitres of TE buffer by vortexing and 50 microlitres of phenol (Tris-HCl pH 8.0) was added. The mixture was vortexed for 1 minute. The tube was spun for 2 minutes and the upper aqueous layer was transferred to a fresh Eppendorf tube and the total volume made up to 0.5 microlitres with TE buffer. The single-stranded DNA was phenol-chloroform extracted and ethanol precipitated as previously described. The amount of DNA extracted in this manner was 5-10 microgrammes per millilitre of infected culture. This was of sufficient quality for both DNA sequencing and site-directed mutagenesis.

2.2.19. DNA Sequencing techniques.

Introduction. DNA sequencing was performed using the Pharmacia T7 Sequencing Kit. The kit is based upon the chain-terminating dideoxynucleotide sequencing method developed by Sanger *et al* (1977). In the original procedure, primer extension was catalysed by the Klenow fragment of DNA polymerase I. In the kit, however, the Klenow enzyme has been replaced by T7 DNA polymerase, which has the advantage of creating longer chain terminated fragments with a more even distribution of label between fragments. The major practical difference in using T7 polymerase is

that primer extension reactions are performed in two stages, a labelling reaction and a termination reaction. The two stages are necessary because the enzyme uses dideoxynucleotides very readily, and therefore in order to allow the synthesis of long chain-terminated fragments, dideoxynucleotides are excluded from the first stage of the reaction, being added for the second. Even so, the time required for the reactions using the T7 enzyme is considerably less than those using the Klenow enzyme.

Annealing of primer to single stranded template. The DNA templates used in the sequencing reactions were all single stranded M 13 DNAs and purified as described previously. The concentration of the template was adjusted to 1mg/ml in TE buffer. In most cases the universal primer supplied in the kit was found to be suitable. This primer is 17 bp long and at a concentration of 0.80 mM. If another oligonucleotide is used it should be adjusted to the same concentration.

The following was added to an Eppendorf tube on ice:

Template DNA (1 mg/ml)	2 μl
Primer (0.80 mM)	2 μl
Annealing buffer	2 μl
d.H2O	8 μl
Total	14 μl

The contents of the tube were thoroughly mixed and incubated at 60°C for 10 minutes. The tube was then left at room temperature for at least 10 minutes; if the rest of the sequencing reaction was to be performed at a later time then the tube could be stored at -20°C until required.

Sequencing reaction. For each template to be sequenced, four wells of a microtitre plate were labelled 'A', 'C', 'G' and 'T' respectively and 2.5 μl of the corresponding dideoxynucleotide mix added to each well. To the tube containing the annealed template and primer the labelling mix, (CTP, GTP, TTP and GTP in solution), T7 DNA polymerase and labelled dATP were added as follows:

Annealed template and primer	14 µl
Labelling mix	3 µl
[α-35S] dATP	1 µl (10 µCi)
Diluted T7 DNA polymerase (1.5 Units/µl)	2 µl
Total reaction volume	20 µl

The labelling reaction was incubated at room temperature for 5 minutes. While this was proceeding the previously dispensed sequencing mixes were incubated at 37°C for 1 minute in a water bath. After the 5 minute incubation of the labelling reaction, 4.5 µl was added to each of the prewarmed sequencing mixes and returned to the water bath for a further 5 minutes to allow chain-termination to occur. Finally, 5 µl of Stop solution was added to each reaction, These could then be stored at -20°C until required for electrophoresis. When the samples were needed for loading onto the sequencing gel they were heated to 80°C for 2 minutes to denature the DNA. Immediately after this incubation 2-3 µl of each sample was loaded onto the gel.

DNA sequencing gel electrophoresis. DNA sequencing was performed using a 30 x 40 cm BRL sequencing apparatus. The glass sequencing plates were thoroughly cleaned with ethanol and chloroform. The shorter of the two plates was desiliconized using dimethylsilane to ease separating the plate from the sequencing gel after running the samples. The plates were assembled using 0.2 mm spacers and taped together to prevent leakage.

The gel was prepared by adding together the following:

Acrylamide (filtered, 40% w/v)	15 ml
Urea	43 g
d.H2O	35 ml
10X TBE	10 ml

This was allowed to dissolve with the aid of magnetic stirring. Once dissolved, 1 ml of 10% ammonium persulphate solution was added followed by 35 µl of TEMED. This was then stirred slowly for a few seconds and was then slowly poured between the sequencing plates. The flat edge of a 60 well shark-tooth comb was pushed between the plates to layer the top of the gel. Clingfilm was wrapped around the exposed areas of the plate and the top and each side of the gel was clamped with bulldog clips. The gel was

then set aside for at least 10 minutes for the acrylamide to polymerise. Once set the bulldog clips, cling film, tape and comb were removed and distilled water was squirted along the top of the gel. The shark-toothed comb was then replaced with the teeth pointing downwards until just touching the top of the gel. The gel was then clamped into the sequencing apparatus and 1X TBE buffer poured into the top and bottom reservoirs. The gel was then pre-run at ~66 W (~1500 V) for 1 hour. After this the gel was ready to be loaded with the sequencing reactions. The samples were loaded in the order G, A, T and C immediately after denaturing the DNA (see above). The gel was then electrophoresed at 66 W until the blue dye ran off the end of the gel. Once electrophoresis was complete the glass plates were removed from the apparatus and the shorter glass plate was carefully removed. The plate with the gel attached was placed in a fixing bath containing 10% methanol and 10% acetic acid in water for 20 minutes. The plate and gel were then removed and a sheet of water dampened blotting paper laid over the gel. A dry sheet of blotting paper was then laid over this and gently pressed down. The sheets of blotting paper were then carefully peeled from the glass plate with the gel adhered to the paper. The paper and gel sandwich was then dried in a vacuum gel-drier for 1 hour at 80°C. When dry the gel was placed in an autoradiography cassette and allow to develop at room temperature. In most instances a good signal was achieved after 24 hours.

2.2.20. Southern blotting of DNA onto nylon filters.

This was a method used to detect the presence of null mutations on the chromosome. Genomic DNAs (10 μg) were cut with appropriate restriction enzymes to produce fragments of calculated size. Plasmid DNA controls (50ng) were cut with the same enzymes so that comparisons could be made. The digests were run on a midigel at 20 mA for at least 12 hours and then stained with ethidium bromide and photographed under UV illumination. The gel was then washed in 200 millilitres of 0.25 M HCl for 15 minutes to depurinate the DNA. Next the gel was washed in 200 millilitres of 0.5 M NaOH/1.5 M NaCl solution for 15 minutes to denature the DNA. This step was repeated and the gel rinsed in d.H2O. The gel was then washed in neutralising buffer for 45 minutes and again rinsed in d.H2O. Finally the gel was soaked in 20X SSC buffer (pH 7.2) for 5 minutes.

The gel was now ready to be blotted. Ten sheets of blotting paper, cut to the same size as the gel, were soaked in 6X SSC buffer and laid upon a glass plate in a tray. The gel was then laid upon this with the upper surface laid face down. A sheet of nylon membrane (Boehringer Mannheim), cut to the size of the gel, was soaked in 6X SSC buffer and laid upon the upper surface of the gel. A further six sheets of blotting paper soaked in 6X SSC buffer were laid on top of this followed by 15 sheets of paper towelling. Finally, a glass plate was placed on top and a 1 kilogram weight placed upon this. 6X SSC buffer was then poured into the tray until half the lower blotting paper was submerged. After 2 hours the weight and glass plate were removed and any damp paper towels were removed and replaced with fresh ones and the glass plate and weight placed back. The blot was then left overnight. The nylon filter was removed and a corner cut out as a marker for both sides of the blot. The DNA was then fixed on the filter by exposing to UV using a UV crosslinker (1800 UV Stratalinker, Stratagene). The blot could now be used for hybridisation with the prepared labelled DNA probe.

20X SSC buffer recipe:

NaCl	525.9 g
Sodium citrate	264.6 g
Distilled water to 3 litres	
Adjust to pH 7.2	

2.2.21. Preparation of the labelled DNA probe.

This method is adapted from that described by Feinberg and Vogelstein, 1984. The required probe DNAs were always cloned in plasmids. 1 microgram of the plasmid DNA was cut with appropriate restriction enzymes and the fragment isolated by agarose gel electrophoresis. The gel was stained in EtBr and after destaining the gel was observed on a long wave UV transilluminator and the desired band excised cleanly. The DNA was then purified from the agarose gel slice. The DNA can now be labelled by adding 50 nanograms of the fragment DNA to the following mixture.

OLB	10 µl
BSA (10 mg/ml)	2 µl
α[32]P dCTP (10 µCi/µl)	5 µl
Klenow enzyme (2 Units)	1 µl
d.H2O to 50 µl	

Incubate overnight at room temperature to allow polymerisation to occur in a lead containment vessel. The DNA will now be randomly labelled.

OLB buffer (Solutions A : B : C in the ratios 2 : 5 : 3 [v/v])

Solution A: 1.25 M Tris-HCl pH 8.0

0.125 M MgCl2
0.5 mM of each dATP dGTP and dTTP
0.025 mM β-mercaptoethanol

Solution B: 2 M Hepes-NaOH pH 6.6

Solution C: 4.5 mg/ml pd(N)6 (in TE buffer)
2 ml BSA (10 mg/ml)

2.2.22. Purification of the labelled probe.

Before using the labelled probe for hybridisation it was first purified from the labelling reaction mixture. The Elutip (Schleicher and Schuell) was the preferred method. A silica cartridge in a specially designed plastic tip was charged by eluting 3 millilitres of high salt solution through the column (the flow rate in all stages was ~2 ml/min. The volume of the probe solution was increased to 400 microlitres using TE buffer and this was passed through the column. The labelled probe has now bound to the column. Wash this with 5 millilitres of the low salt solution and elute the bound DNA by passing through 400 microlitres of high salt solution. The eluate containing the labelled probe can now be added to the hybridisation reaction.

2.2.23. Probe hybridisation using phosphate buffer.

Preheat the hybridisation oven (Techne) to 65°C. Place the nylon filter blot in a glass hybridisation tube with the blotted side facing towards the centre of the tube. Add 20 milllilitres of SDS-phosphate buffer and hybridise the filter at 65°C for 1 hour. Add the labelled probe solution and leave to hybridise with the filter overnight incubating at 65°C. Pour the hybridisation mixture into a universal bottle and store at -20°C(*). Remove one of the tube caps and add 30 millilitres of SDS-phosphate wash buffer. Reseal the tube and allow the buffer to wash for 30 minutes at 65°C. Pour the wash solution into a waste bottle and monitor the activity from the filter. If the cps is ~20 the filter may be put down in a autoradiography cassette, if the count is still high (>30 cps) then add a further 30 millilitres of wash buffer and incubate again for 30 minutes at 65°C. This too is then disposed of into the waste bottle. The filter was removed from the tube and placed in a polythene bag. This was then secured to an autorad cassette with a reflector pad. An X-ray film was inserted and the cassette was stored at -70°C. The time of exposure of the film to the labelled filter was dependant on how strong the signal was from the labelled probe.

(*) The frozen buffer/labelled probe mix will retain sufficient activity to be reused for further hybridisations up to one week after labelling the probe. However it must first be placed in boiling water for 5 minutes and then allowed to cool to 50°C before being introduced to the prehybridized filter. This is to prevent the stripping of DNA from the filter.

SDS-phospate buffer solution:

Phosphate buffer (pH 7.2)	25 mls
20% SDS	17.5 mls
d.H$_2$O	7.4 mls
0.5 M EDTA	100 µl

1 M Phosphate buffer recipe:

Na2HPO4	354.92 g
NaH2PO4	156.01 g
Distilled water to 3 litres	

Adjust pH to 7.2

2.2.24. Stripping probes from nylon filters.

Once the Southern blot has been probed, a second different probe may be required and before this can be hybridised to the blot the previous labelled probe must be removed. Incubate the blot at 45°C for 30 minutes in 30 millilitres 0.4 M NaOH and then wash in 40 millilitres 0.1X SSC, 0.1% SDS, 0.2 M Tris-HCl (pH 7.5) solution for 30 minutes at 45°C. The signal from the blot should now be negligible and the blot is ready to be hybridised with the next labelled probe.

2.2.25. Site directed mutagenesis.

The method used for site-directed mutagenesis was described by Kunkel (1987). The oligonucleotide primer, used for the insertion of the base change mutation, was first phosphorylated before use as a primer for the extension reaction. ~250 nanogrammes of oligonucleotide DNA was used in the reaction. One microlitre of 10X kinase buffer and ATP to a final concentration of 2 mM was added to the oligonucleotide in a microfuge tube. To this was added 1 Unit of kinase and the total volume made up to 20 microlitres with sterile distilled water. The reaction was incubated at 37°C for 45 minutes. The reaction was then stored on ice. The phosphorylated primer was then annealed to the uracil rich single stranded M13 DNA. To a volume containing ~1 microgramme of M13 DNA was added 2.5 microlitres of the phosphorylated oligonucleotide solution. This was made up to a total volume of 20 microlitres with sterile distilled water. This annealing reaction was incubated at 65°C for three minutes before incubation at 37°C for 30 minutes for the annealing of the primer to the M13 DNA.

For the primer extension reaction 17 microlitres of the annealed DNAs was used. To this was added MgCl2 (final concentration 0.1 mM), dNTPs (final concentration 2 mM), 3 units Klenow polymerase, three units of T4 DNA ligase, ligation buffer and the final reaction volume made up to 45 microlitres with sterile distilled water. This was subsequently incubated at 16°C overnight. Aliquots of the reaction mixture were then used to transform TG1, using the chung method of transformation.

2.2.26. Amplification of DNA by PCR.

Specific regions of DNA were amplified by using the polymerase chain reaction (PCR). Plasmid DNA was used as a template and specific oligonucleotide primers were obtained commercially (Oswel DNA Service, University of Edinburgh). A typical reaction mix using plasmid DNA as a template was as follows.

10 X Thesit buffer	5 µl
dNTP mix (2.5 mM)	5 µl
Primer 1 (50 µM)	1 µl
Primer 2 (50 µM)	1 µl
Taq polymerase (5 Units)	1 µl
d. H_2O	36 µl
Total volume	50 µl

100 microlitres of sterile mineral oil was layered on top of the reaction mixture and then the reactions were incubated in a Hybaid Thermal Reactor programmed to the length of the desired product and the approximate annealing temperature of the primer/template duplex. Generally the DNA was heated to 94°C for 1 minute and the polymerisation took lace at 72°C for 1 minute/ kb of template.

2.3. Bacteriophage techniques.

2.3.1. Production of bacteriophage P1 lysates.

Inoculate 5 millilitres of L broth containing 2.5 mM CaCl2 with a single colony of the host strain and incubate overnight without shaking. Mix 1 millilitre of the overnight culture with 5×10^5 P1 in a large sterile test tube and incubate for 30 minutes to allow the phage to be absorbed into the cells. Prepare a cells only control. Add 3 millilitres of L broth and 4 millilitres of molten LC top agar, cooled to 45°C, containing 2.5 mM CaCl2 and mix. Immediately pour the mixture onto LC bottom agar plates with 2.5 mM CaCl2 and gently swirl the mixture until the surface has an even covering of the top agar. Leave at room temperature for 15 minutes to allow the agar to set and the incubate overnight. The cells only control should have an even

lawn of cells whilst the infected culture will be partly or completely lysed resulting in cleared top agar. Scrape this off with a sterile scalpel blade into a sterile universal containing 4 millilitres of L broth over 100 microlitres of chloroform. Vortex for 30 seconds and incubate at 37°C with vigorous agitation for 30 minutes. Spin the universals in a bench centrifuge at 5,000 rpm for 15 minutes to pellet the top agar and transfer the supernatant, using a pipette, to a 1/2 ounce Bijou bottle. Add a few drops of chloroform to the lysate and store at 4°C.

2.3.2. Transduction of cells by bacteriophage P1.

Inoculate 5 millilitres of L broth containing 2.5 mM CaCl2 with 1 millilitre of a fresh overnight culture of the recipient strain. Incubate at a permissive temperature until the O.D.600 reaches ~1.0. Aliquot four 1 millilitre volumes into Eppendorf tubes and spin for 15 seconds in a microcentrifuge to pellet the cells. Remove 0.9 millilitres of supernatant from each tube and resuspend the pellet in the remaining 0.1 millilitres of supernatant by vortexing. Take 130 microlitres of P1 lysate and vortex in an Eppendorf tube with a drop of chloroform and then spin in a microcentrifuge for 1 minute to pellet any debris. Lysate aliquots of 50, 10 and 1 microlitre were added to the cell suspension and include a cells only control. A phage only control of 100 microlitres of lysate was also used. Incubate all the tubes for 30 minutes before adding L broth plus sodium citrate (10 mM) and incubate for 1-2 hours. When using auxotrophs, 1 millilitre of phage buffer was added to the cells instead of L broth and then they were plated on minimal selective media without having an incubation period. The cells were concentrated tenfold before plated by pelleting the cells in a microcentrifuge, removing all but 100 microlitres of the supernatant and then resuspending the pellet by vortexing. The plates were then incubated overnight and any colonies growing on the selective agar are transductants provided the cells only and lysate only control plates are clear of growth. When selecting on minimal media at 30°C a 2 day incubation period was sometimes required for the colonies to reach an appreciable size (1 millimetre).

2.3.3. Preparation and selection of λ lysogens.

A lawn of the desired strain to be lysogenized was grown up in L-broth, supplemented with 0.20 mM MgSO4 and 0.2% maltose, to mid-log phase. 0.3 millilitres of the culture was used to inoculate millilitres L-top agar. To this was also added approximately 200 λ phage particles and the mixture poured onto a fresh L agar plate and left to set. This was incubated overnight at 37°C. The aim here was to promote the formation of isolated λ phage plaques. A sterile toothpick was used to touch the centre of a plaque and this was then used to streak onto a fresh L-agar plate which was incubated overnight at 37°C. The resulting single colonies from the plate could now be tested for the presence of λ phage. A lysogenized bacterium will now be immune to lysis by λ phages with the same immunity as the one used to lysogenize the strain but sensitive to λ phages that are virulent, or carrying a different immunity. An L agar plate was streaked with the λ lysate used for the lysogeny and a virulent λ phage (λ*vir*). Sterile toothpicks were used to cross-streak the single colonies over the λ phage and the λ*vir* and incubated overnight at 37°C. The streaks which were immune to the λ phage used for lysogeny and sensitive to λ*vir* were presumed to be λ lysogens.

2.3.4. UV induction of lysogens.

An inoculum from a fresh overnight culture of the lysogenic bacteria was grown in L-broth/ 20 mM MgSO4/0.2% maltose with vigorous agitation until the OD540 reached 0.3. The cells were pelleted by centrifugation and then resuspended in 7 millilitres of 20 mM MgSO4. The cell suspension was transferred to a sterile glass Petri dish and exposed to 600 ergs/mm^2 sec of UV irradiation before being diluted fivefold with L broth + 20 mM MgSO4. This culture was then grown at 37°C with vigorous shaking until lysis occurred. A few drops of chloroform were added and incubation continued for another 15 minutes. The culture was then spun at 5,000 rpm to clear the bacterial debris and the supernatant was retained and titred to determine the λ phage concentration.

2.4. Bacterial techniques and assays.

2.4.1. Hfr mating protocol.

Subculture an overnight culture of the required Hfr strain(s) by diluting 1:25 in LB medium with selection and incubate at 37°C without agitation until the cell density reaches $1\text{-}2 \times 10^8$/ml (~3 hours). Subculture an overnight of the recipient strain by diluting 1:5 and incubate at 37°C on a blood mixer until used. Mix 0.2 millilitres of the donor strain and 0.2 millilitres of the recipient strain in a large test tube and incubate in a waterbath at 37°C for 90 minutes. As controls prepare the recipient and the donor alone. Add 2 millilitres of LB to each tube, and place on a rotor for 2 hours to allow the recombinant colonies to segregate. Plate 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4} dilutions of the cross onto selective agar and incubate overnight at 37°C. Plate a 10^{-1} dilution of the controls onto the same type of media. If the mating has been successful the control plates will be clear of growth whilst the mated cell plates will have colony growth.

2.4.2. Gene replacement using pMAK705.

This is a method for replacing wild type alleles on the *E.coli* chromosome with copies of mutant alleles carried on a plasmid with a temperature sensitive replicon. Hamilton *et al* (1986) constructed such a vector, pMAK705, which has temperature sensitive replicon. At high temperature (43°C) the plasmid cannot replicate and therefore is soon diluted from the culture. The plasmid carries the CAT gene as an antibiotic marker and so at 43°C cells which are not plasmid cointegrants cannot survive in the presence of the chloramphenicol, the selective marker for pMAK. A very small proportion of the cells will survive since the entire mutant clone can integrate onto the chromosome by a single crossover. This is achieved by the cloned null allele having homology with the wild type allele. Upon reducing the incubation temperature to 30°C the plasmids in the cointregrants can now replicate and leave the chromosome. Theoretically 50% of the population of cells will have the wild type allele replaced on the chromosome by the null allele. Cells will remain viable even if the null allele is lethal to the cell as there is the wild type copy on the plasmid, expressed from P*lac*, to complement this.

Transform the strain to be used (it must be *recA*$^+$) with the null allele cloned in pMAK705. All cultures were now grown in or on media containing 15 µg/ml of chloramphenicol. Phenotypic expression and incubation of the plated culture was carried out at 30°C. Spread the cells on LB agar plates and incubate overnight at 30°C. Select a single colony and streak onto a new plate of LB agar and incubate again at 30°C overnight. Inoculate 5 millilitres L broth with a single colony and incubate overnight at 30°C.

Make tenfold dilutions of the culture and plate 200 microlitres of the 10^{-7} and 10^{-5} dilutions onto LB agar plates and incubate the 10^{-7} plate at 30°C and the 10^{-5} plate at 43°C overnight. The 10^{-7} dilution plating is a control for cell viability whilst the 10^{-5} plating will reveal those cells which have become cointegrates, typically there are 20-100 cells/plate for the 43°C cells. Purify 6-8 colonies by streaking on LB agar and incubate overnight at 43°C. Repeat this step. To allow resolution of the cointegrates, inoculate 10 millilitres of LB Cmp with eight colonies and incubate overnight at 30°C. Subculture 10 microlitres of the culture into 10 millilitres of L B Cmp and incubate at 30°C overnight. Repeat this step. Plate 200 microlitres of a 10^{-7} dilution onto LB agar plates and incubate overnight at 30°C. Purify 10-20 single colonies on LB Cmp agar. To identify the resolved cointegrates, plasmid DNA minipreps were performed on 10-20 of the isolates and the DNA cut with restriction enzymes. The digests were examined by agarose gel electrophoresis and UV illumination of the EtBr stained gel. The sizes of the plasmids were compared to the two control plasmid DNAs, the wild type clone and the null mutant clone. If any sample digests were the same size as the wild type pMAK705 clones then this indicates that the null mutation has replaced the wild-type copy on the chromosome. Make bacteriophage P1 lysates of these strains immediately to reduce the chance of the null mutation being replaced on the chromosome by a second cross over with the wild type allele resulting with the null allele back in the vector. As a further check the presence of the null allele being on the chromosome can be detected by a Southern blot.

2.4.3. β-Galactosidase assays.

The method used to determine the β–galactosidase activity from a promoter-*lacZ* clone, was as described by Miller (1972). Take 1 millilitre of

culture and measure the OD600; from this take 0.5 millilitres and add to 0.5 millilitres of Z buffer. If the promoter activity is known to be high then add 0.1 millilitres of culture to 0.9 millilitres Z buffer. Add 50 microlitres of chloroform and vortex for 30 seconds. Store the samples at 4°C until all sampling has been performed. Add 200 microlitres of ONPG (4 mg/ml) to each sample and vortex. Prepare a control of 0.5 millilitres of the culture medium used and 0.5 millilitres of Z buffer. After addition of ONPG place the samples on ice until all have been treated. Place the samples in a 30°C waterbath noting the exact time of the start of incubation. Check the tubes every 10 minutes and when yellowing occurs add 0.5 millilitres of Na2CO3 (1 M), vortex and make a note of the time taken for the colour change and store the sample on ice. When all the tubes have reacted measure the OD420 and OD550 of the samples using the control as a blank. The β-galactosidase activity of the samples can now be calculated and expressed as Miller Units using the equation:

$$\frac{O.D._{420} - 1.75 \times O.D._{550}}{O.D._{600} \times 0.5\ ml \times T} \times 1000$$

Where T = time in minutes for colour change.
0.5 ml is the sample volume taken from the original culture.

Z buffer recipe:

Na2HPO4	4.26 g
NaH2PO4.H2O	3.11 g
KCl	0.375 g
MgSO4.7H2O	0.123 g
β-mercaptoethanol	1.35 ml
SDS (10%)	0.25 ml
Distilled water to 500 ml	

2.4.4. Counting and sizing of bacterial cells.

A Coulter Counter ZB (Coulter Electronics, Herts.) and Coulter Channelyser (model C-1000) were used to count and determine the size of bacterial cells. Cultures were grown in filtered LB (5 micrometre membrane filter, Millipore) and 500 microlitre samples were fixed in filtered fixing solution. Samples were then stored at 4°C until use. When measuring cells, a volume, usually 100-200 microlitres was made up to a total volume of 6

millilitres with sodium azide solution in a glass vial. The sample volume for counting cells was 50 microlitres. The channelyser places cells in different channels depending on their size. From this the distribution of different cell sizes, the mode and median cell size of a culture could be measured.

Fixing solution:

> 80% bacterial buffer
> 20% formaldehyde

Sodium azide solution:

> 0.85% NaCl
> 0.085% sodium azide
> Dissolve in d.H$_2$O

2.4.5. Photography of bacterial cells.

When photographing bacterial cells a Zeiss photo-camera was used. Molten agarose was pipetted onto an ethanol cleaned glass slide using a glass micropipette so that a thin, level layer of agarose covered the surface of the slide. A 10-25 microlitre volume of culture was pipetted onto the agar surface and covered with an ethanol washed coverslip. Cells were photographed through a 100X, phase contrast, oil immersion lens.

2.4.6. Testing UV sensitivity of recA strains.

Single colonies of the strain being tested were streaked across the surface of an LB agar plate using a sterile toothpick. As controls samples of a recA$^+$ and a recA$^-$ strain were also streaked on the plate. Areas of the streaks were then exposed to UV light calibrated to 600 ergs/mm^2. A piece of cardboard was used to protect certain areas from UV light. The areas were exposed for different times. Typically these were 0, 10, 20 and 30 seconds. The plates were then incubated overnight and streaks examined for growth on the UV irradiated regions. Typically the recA mutants could not grow after 10 seconds of exposure to UV light.

2.5. Protein Techniques.

2.5.1. *In vivo* protein labelling using T7 RNA polymerase.

Inoculate 5 millilitres of LB (containing Amp and Cmp) with a single colony of the BL21(λDE3)/pLysS host and plasmid clone. Incubate overnight at 37°C and then spin in a bench centrifuge at 5000 rpm for 5 minutes to pellet the cells. Remove 4 millilitres of the supernatant and resuspend the pellet in the remaining 1 millilitre. Use 0.5 millilitres of this to inoculate 25 millilitres of Spizizen's broth with minimal supplements and Amp/Cmp. Incubate at 37°C with shaking until the OD540 reaches 0.8 and remove four 0.5 millilitre aliquots to sterile Eppendorf tubes which were labelled as follows:

-IPTG/-Rif
+IPTG/-Rif
-IPTG/+Rif
+IPTG/+Rif

The tubes labelled +IPTG had 3 microlitres of IPTG (20 mg/ml) added and all tubes were then incubated at 37°C being mixed on a blood mixer. To the tubes marked +Rif was added 3 microlitres of rifampicin (100 mg/ml) and incubation continued as before for 30 minutes. To each tube was then added 1 microlitre of ^{35}S methionine (Amersham or ICN Flow) at an activity of 5 µCi/ml. The tubes were immediately vortexed and incubated for one minute at room temperature to allow incorporation of the label. Spin the tubes in a microfuge for 15 seconds in to pellet the cells and discard the supernatant. Add 200 microlitres of 1X loading buffer to each tube and vortex the tubes for 2 minutes or until the pellet has been resuspended. Samples were then boiled for 2 minutes or incubated at 37°C for 1 hour. These can now be stored at -70°C until required for analysis by SDS-PAGE.

SDS gel loading buffer 2X solution:
        100 mM Tris-HCl (pH6.8)
        200 mM dithiothreitol
        4% SDS
        0.2% bromophenol blue
        20% glycerol

2.5.2. *In vitro* prokaryotic DNA-directed protein translation of circularized plasmid DNA.

This protocol is based on the bacterial cell-free coupled transcription-translation system first described by De Vries and Zubay (1967) and allows the expression of genes, *in vitro*, contained on a bacterial plasmid or a bacteriophage genome. The system used was supplied as a kit by Amersham (Cat no.380). All of the necessary reagents were supplied with the kit bar the L-[^{35}S] labelled methionine, an additional Amersham product.

Remove the cell extract (solution 1) from storage and thaw it on ice. Centrifuge the extract for 5 minutes in a microcentrifuge, remove the supernatant to a fresh microfuge tube and keep on ice. Prepare the reaction mixtures in plastic microfuge tubes and keep on ice. The supplement solution (solution 2) and the amino acid minus methionine solution (solution 3) should both be vortexed before addition to the reaction mixtures. The precipitate seen in solution 2 should be ignored as will not adversely affect the reaction provided the solution is thoroughly mixed first.

DNA Solution	3.5 µg
Supplement solution (solution 2)	7.5 µl
Amino acids minus methionine (solution 3)	3 µl
L-[^{35}S]methionine (30 µCi)	2 µl
S-30 extract (solution 1)	5 µl

Add dilution buffer (solution 5) to give a total reaction mixture volume of 30 microlitres. Gently mix the contents and incubate at 37°C for 1 hour. Vortex the methionine chase solution (solution 7) and add 5 microlitres to each tube and incubate for a further 30 minutes at 37°C. Terminate the reaction by placing the tubes on ice. An equal volume of 1X SDS PAGE loading buffer was added to each tube then vortexed and subsequently stored at -70°C to await protein analysis by SDS PAGE.

Kit solution used:

Solution 1.   An S-30 extract prepared from *E.coli*, strain MRE 600, RNase I-. A supernatant fraction from a 30,000 X g centrifugation step.

Solution 2..   Supplement solution. This contains sufficient nucleotides for transcription, tRNA for translation, an energy-generating system and inorganic salts.

Solution 3.   Amino acids minus methionine. An equimolar mixture.

2.5.3. *In vitro* prokaryotic DNA-directed protein translation from linear plasmid DNA.

This method is similar to that described in 2.5.2. where peptides are produced from DNA under *in vitro* conditions. In this instance the kit used was supplied by Promega Corp. (Product no. L1030) where the *in vitro* transcription is from a linear DNA template. The mRNA transcript is then translated *in vitro* where the introduction of a label can result in the incorporation into the peptide produced.

The S30 extract used by the kit is prepared from *E. coli* strain SL119 which is deficient in the OmpT and Lon proteases and exonuclease V. Supplied in the kit is an S30 premix without amino acids which is optimised for a given aliquot of S30 with all other requirements. These include an ATP regenerating system, NTPs, tRNAs and appropriate salts. lacking methionine for facilitating the radiolabelling of the translation products.

The reaction labelling was prepared as follows:

DNA template	4 μg
Amino acid mixture lacking methionine	5 μl
S30 premix	20 μl
L [^{35}S] methionine 910 μCi/μl)	1.5 μl
S30 extract	15 μl
d.H2O to a final volume of 50μl	

Gently mix the reaction mixture then centrifuge at 5000 for 5 seconds to bring the reaction mixture to the bottom of the tube. Incubate the reactions at 37°C for 2 hours. Stop the reactions by placing the reactions on ice for 5 minutes. Add 200 μl of acetone to each reaction, vortex and place on ice for 5 minutes. Spin for 5 minutes at 15000 rpm and remove the supernatant. Resuspend the pellet in 200 μl of 1X SDS-PAGE loading buffer and load 20-30 μl per lane for SDS-PAGE analysis.

2.5.4. Preparation of SDS-PAGE gels.

Proteins were routinely separated using SDS polyacrylamide gel electrophoresis (SDS-PAGE) with a discontinuous buffer system. The type of gel apparatus used for all SDS-PAGE work was the Hoefer SE600 dual cooled vertical slab unit which is able to run one or two 16 x 18 cm gels. The gel plates were washed in distilled water and then wiped with absolute alcohol. The gel plates were laid together separated by 0.75 mm spacers and then clamped together. The plates were then clamped into the baseplate of the gel apparatus and were ready to receive the acrylamide mix. The resolving gel was poured first. All the ingredients bar the TEMED and the ammonium sulphate were mixed in 50 ml glass beaker and then the latter two added. The ammonium persulphate was always freshly prepared. The solution was quickly mixed and then drawn up in a 25 ml pipette and poured between the gel plates. The resolving mix was poured into the gap until 4 cm from the top of the plates. This was then layered with isobutanol (saturated with stacking buffer) and left to polymerise at room temperature for 15 minutes. The isobutanol was poured off and the air-gel interface was thoroughly rinsed with distilled water. Excess water was removed from the gel space using a strip of blotting paper. The 4% stacking gel was then poured into the remaining area of the gel and the 0.75 mm 10 well comb inserted. This was left at room temperature for 30 minutes for the stacking gel to polymerise. The comb is then removed and each well rinsed three times with Tris-glycine running buffer to remove any acrylamide which is unpolymerized. The gel was now ready to be used to run protein samples.

Recipe for 10% resolving acrylamide gel:

acrylamide (40% v/v)	10 ml
4X Resolving buffer	10 ml
d.H2O	19.2 ml
10% SDS	400 µl
7.5% ammonium persulphate	400 µl
TEMED	25 µl

Recipe for 4% stacking gel:

acrylamide (40% v/v)	1.3 ml
4X stacking buffer	2.5 ml
d.H2O	6.0 ml
10% SDS	100 μl
7.5% ammonium persulphate	100 μl
TEMED	10 μl

2.5.5. Running SDS-PAGE gels

The samples were first thawed, if frozen, and then boiled for 2 minutes or incubated at 37°C for 60 minutes if a non-boiled sample. The samples are spun at 15,000 rpm in a microfuge for 5 minutes. Remove the comb and rinse each well with 1X running buffer to remove any unpolymerized acrylamide. Remove half the Tris-glycine running buffer from each well to be loaded. Aliquot 30 microlitres of each sample and 8 microlitres of the marker proteins into separate wells. Carefully fill the remaining space in the wells with running buffer and clamp on the upper reservoir. Turn on the water cooling system and fill the lower reservoir with running buffer. Remove the base from the gel plates and place the gel plates and upper reservoir into the tank. Fill the upper reservoir and place the lid with electrodes on the top. Run the gel at 30 mA until the marker dyes have migrated from the stacking gel into the resolving gel, the power can now be increased to 45-50 mA. Run the gel until the marker dyes almost run off the bottom of the gel and then switch off the power and remove the gels. Unscrew the clamps and using a wedge prise open the plates. Place each gel in a polythene sandwich box (25 X 25 X 8 cm) and add 70 millilitres of Coomassie stain to each and incubate at 37°C with gentle shaking for 20 minutes. Pour off the stain and rinse the gel in used in destain to remove traces of the stain from the gel and the box. Add 100 millilitres of destaining solution and foam bungs to absorb the coomassie stain leaching from the gel. Leave this at 37°C with gentle shaking until the marker bands are clearly visible. Remove the destain and add 60 millilitres of fixing solution, this is essential as the gel will shrink and crack during vacuum drying without the presence of glycerol, and incubate at 37°C with gentle shaking for at least 20 minutes. Pour off the fixative and place a sheet of blotting paper, soaked with distilled water, over the gel. Lay a dry sheet of the paper on this and

carefully lift the gel/paper sandwich up. Lay the sandwich on the bed of a vacuum gel dryer gel side up and lay a sheet of Saran wrap over the gel. Dry the gel for 1 hour under vacuum at 60°C. Once dry the gel can be taped in an autoradiogram cassette and an X-ray film added. The gels were usually left overnight to expose the film before developing. The position of the non-labelled marker proteins can be marked on the film by over laying it on the dried gel.

2.5.6. Solutions used in SDS-PAGE.

Stock acrylamide:
The 40% v/v bis-acrylamide was supplied premade by Sigma Chemical Co.

5X Running buffer stock solution:

Tris base	15.1 g
Glycine	94 g
10% (w/v) SDS	50 ml

10X loading buffer:

0.25% bromophenol blue
0.25% xylene cyanol
25% Ficoll (type 400) in H2O
Distilled water to 1 litre

Tris glycine electrophoresis buffer:

Working solution
25 mM Tris.HCl
250 mM glycine (pH 8.0)
0.1% SDS

4X stacking-gel buffer.
15.25 g of Tris base dissolved in two hundred millilitres of distilled water adjusted to pH 6.8 with concentrated HCl and made up to two hundred and fifty millilitres with distilled water, filtered and sterilised.

4X Resolving gel buffer:

45.5 g Tris base dissolved in two hundred millilitres of distilled water adjusted to pH 8.8 with concentrated HCl. Made up to two hundred and fifty millilitres with distilled water, filtered and sterilised.

Staining solution:

Coomassie brilliant blue (type R250)	0.25 g
methanol : H2O (1:1 v/v)	90 ml
glacial acetic acid	10 ml
Distilled water to 1 litre.	

Once prepared, filter through a Whatman No. 1 filter to remove particulates.

Destain solution:

methanol	250 ml
glacial acetic acid	750 ml
Distilled water to 5 litres.	

Fixing solution: As destain solution containing 5% glycerol (v/v).

Chapter 3

Analysis of *ftsW*.

3.1. Introduction.

The isolation of thermosensitive filamentous mutants by Ishino *et al.* (1989) and Ikeda *et al.* (1989) led to the identification of two open reading frames immediately upstream of *murG* in the *mra* region at 2.4 minutes on the *E. coli* chromosome (Salmond *et al.*, 1980; Ikeda *et al.*, 1989). The nucleotide sequence reveals an open reading frame (ORF) which codes for a 414 aa peptide of 46 kDa. However, within this ORF there lies a second ORF which is in frame with the larger sequence (Figure 3.1.1.). The peptide produced from the smaller ORF is 384 aa with a calculated molecular weight of 43 kDa (Figure 3.1.1.). Analysis of the primary structures of these peptides revealed that both had similarities to the RodA protein of *E. coli* and the SpoVE protein of *Bacillus subtilis* (Ikeda *et al.*, 1989). The RodA protein functions in lateral wall extension during cell growth and the maintenance of the rod shape (Stoker *et al.*, 1983; Ishino *et al.*, 1986). The SpoVE protein is involved in spore formation in *B. subtilis* (Bugaichuk and Piggot, 1986; Piggot and Coote, 1976). Subsequent BLASTp2 (basic local alignment search tool, Altschul *et al.*, 1990) searches for FtsW homologues in the Genbank database at the NCBI have revealed three other peptides with significant similarities to FtsW. These are from *Haemophilus influenzae* (Fleichmann *et al.*, 1995), *Enterococcus* and a human sequence OrfX. A homologue of FtsW and SpoVE has been identified in *Cyanophora paradoxa* (Stirewalt *et al.*, 1995). There is an identity of 31.9% with the 320aa overlap between FtsW and RodA and an identity of 39.8% between the FtsW and SpoVE proteins (Ikeda *et al.*, 1989). The predicted *H. influenzae* FtsW peptide has 89.1% identity to the *E. coli* FtsW peptide. The hydrophobicity profiles of FtsW, RodA and SpoVE are very similar, all three being highly hydrophobic and basic, suggesting that they are membrane proteins (Figure 3.1.2.). The RodA protein has already been shown to be located in the membrane and to interact with PBP2 (Matsuzawa *et al.*, 1989). Prediction of the topology of the FtsW protein using the TopPredII program (Claros and von Heijine, 1994) shows a highly hydrophobic peptide with ten membrane spanning sections (Figure 3.1.3.). Matsuzawa *et al.* (1989) have also shown that the RodA peptide has ten membrane spanning regions.

The isolation of another *ftsW*^ts^ mutant further indicated that FtsW is involved in cell division (Khattar *et al.*, 1994). Khattar *et al.* (1994) have provisionally shown that the peptide produced from *ftsW* is the larger 414 aa

peptide. This protein had an unusual mobility in SDS-PAGE gels, migrating as if it had a molecular weight of 32 kDa and not the predicted 46 kDa. This peptide was detected in gels only if the extraction in SDS was carried out at low temperature (37°C) and not at 100°C. Similar properties have also been described for the RodA peptide (Matsuzawa *et al.*, 1989). Unlike RodA, the *in vivo* translation of FtsW is inefficient (Khattar *et al.*, 1994, S. Addinall pers. comm.), perhaps because a palindrome sequence which encompasses the Shine-Dalgarno region of the larger ORF restricts translation (Figure 3.1.1.; Ikeda *et al.*,1989). Excess levels of FtsW are inhibitory to cell growth although cell morphology is unaffected (Khattar *et al.*, 1994).

The smaller 43 kDa peptide has recently been shown to complement an *ftsW*ts allele (J. Nikoliachik, pers. comm.). A comparison of the hydropathy profiles of the *Ecoli* FtsW peptides with RodA and FtsW from *H. influenzae* and SpoVE from *B. subtilis* reveals a greater similarity to the smaller 43kDa peptide (Figure 3.1.2.). Therefore it is unknown which peptide functions as a division protein or perhaps both ORFs code for peptides necessary for cell division.

The close identity and similar hydrophobicity profiles of FtsW and RodA led Ikeda *et al.* (1989) to suggest that they are involved in similar functions, one functioning during elongation of the cell wall and the other in the formation of a septum during division. That is RodA interacts with PBP2 in synthesizing murein for the lateral extension of the cell wall during cell elongation (Ishino *et al.*, 1986; Matsuzawa *et al.*, 1989). PBP3 is solely involved in the assembly of murein during the formation of the septum (Ishino and Matsuhashi, 1981) and is located in the *mra* cluster, 6 kb upstream of *ftsW*. The loci for the PBP2 gene, *pbpA*, and *rodA* are in the same operon, the *mrd* operon (Tamaki *et al.*, 1980). Therefore genes required for cell division constitute one operon whilst the genes involved in elongation are also located together in a different operon. Khattar *et al.* (1994) have recently shown that FtsW is involved at an early stage in division whilst PBP3 is involved later, after the formation of the contractile FtsZ ring (Bi and Lutkenhaus, 1991).

The promoter(s) for *ftsW* has not been identified, studies on the complementation of the *ftsW*ts mutant have shown that it is either very weak or more probably that it lies upstream of the *Eco*RV site in *murD* (Ikeda *et al.* , 1989; Ishino *et al.*, 1989). The aim of this work was to identify the FtsW protein and to construct an *ftsW* null strain to identify the phenotype

produced as the previous *ftsW^{ts}* mutants were dependant on culture density or the composition of the growth medium (Ishino *et al.*, 1989; Khattar *et al.*, 1994). The position of the promoter region for *ftsW* was also searched for by examining which fragments of upstream DNA can complement an *ftsW* null allelle.

Figure 3.1.1. The nucleotide and amino acid sequence of the *ftsW* gene and protein respectively. The start codons for both the 46 kDa and 43kDa proteins are highlighted in bold. Also shown in bold are the methionyl residues that start the 46 kDa and 43 kDa peptides. The underlined regions upstream of the start codons are the putative ribosome binding sites (rbs) for the 46 kDa and the 43 kDa proteins respectively. The arrows indicate the location of the palindromic sequences thought to affect the efficiency of translation of FtsW (Ikeda *et al.*, 1989; Khattar *et al.*, 1994).
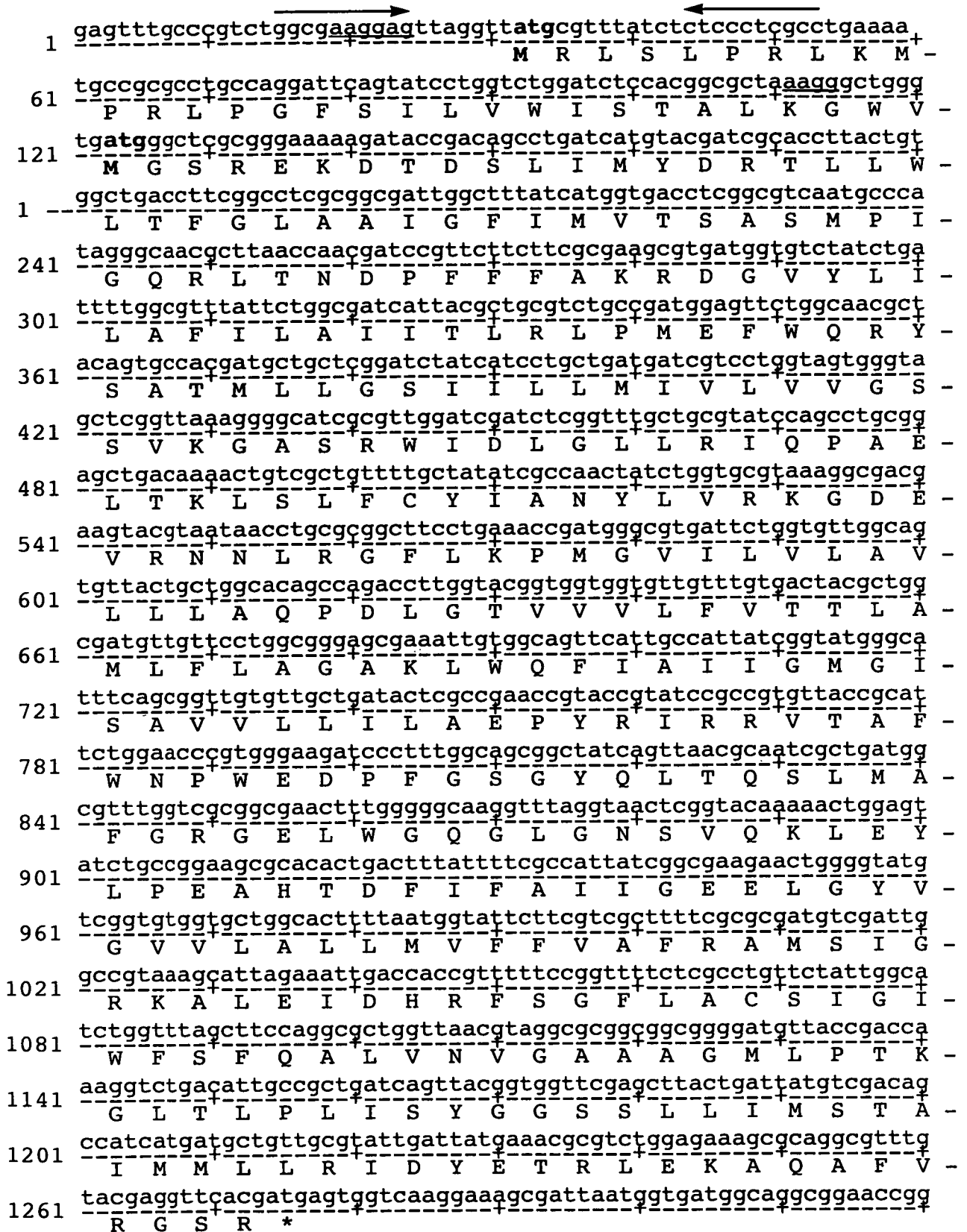
```
        ────────────────────────→                    ←────────────────
   1  gagtttgcccgtctggcgaaggagttaggttatgcgttttatctctccctcgcctgaaaa
                                         M  R  L  S  L  P  R  L  K  M

  61  tgccgcgcctgccaggattcagtatcctggtctggatctccacggcgctaaagggctggg
        P  R  L  P  G  F  S  I  L  V  W  I  S  T  A  L  K  G  W  V

 121  tgatgggctcgcgggaaaaagataccgacagcctgatcatgtacgatcgcaccttactgt
        M  G  S  R  E  K  D  T  D  S  L  I  M  Y  D  R  T  L  L  W

   1  ggctgaccttcggcctcgcggcgattggctttatcatggtgacctcggcgtcaatgccca
        L  T  F  G  L  A  A  I  G  F  I  M  V  T  S  A  S  M  P  I

 241  tagggcaacgcttaaccaacgatccgttcttcttcgcgaagcgtgatggtgtctatctga
        G  Q  R  L  T  N  D  P  F  F  F  A  K  R  D  G  V  Y  L  I

 301  ttttggcgtttattctggcgatcattacgctgcgtctgccgatggagttctggcaacgct
        L  A  F  I  L  A  I  I  T  L  R  L  P  M  E  F  W  Q  R  Y

 361  acagtgccacgatgctgctcggatctatcatcctgctgatgatcgtcctggtagtgggta
        S  A  T  M  L  L  G  S  I  I  L  L  M  I  V  L  V  V  G  S

 421  gctcggttaaaggggcatcgcgttggatcgatctcggtttgctgcgtatccagcctgcgg
        S  V  K  G  A  S  R  W  I  D  L  G  L  L  R  I  Q  P  A  E

 481  agctgacaaaactgtcgctgttttgctatatcgccaactatctggtgcgtaaaggcgacg
        L  T  K  L  S  L  F  C  Y  I  A  N  Y  L  V  R  K  G  D  E

 541  aagtacgtaataacctgcgcggcttcctgaaaccgatgggcgtgattctggtgttggcag
        V  R  N  N  L  R  G  F  L  K  P  M  G  V  I  L  V  L  A  V

 601  tgttactgctggcacagccagaccttggtacggtggtggtgttgtttgtgactacgctgg
        L  L  L  A  Q  P  D  L  G  T  V  V  V  L  F  V  T  T  L  A

 661  cgatgttgttcctggcgggagcgaaattgtggcagttcattgccattatcggtatgggca
        M  L  F  L  A  G  A  K  L  W  Q  F  I  A  I  I  G  M  G  I

 721  tttcagcggttgtgttgctgatactcgccgaaccgtaccgtatccgccgtgttaccgcat
        S  A  V  V  L  L  I  L  A  E  P  Y  R  I  R  R  V  T  A  F

 781  tctggaacccgtgggaagatccctttggcagcggctatcagttaacgcaatcgctgatgg
        W  N  P  W  E  D  P  F  G  S  G  Y  Q  L  T  Q  S  L  M  A

 841  cgtttggtcgcggcgaactttgggggcaaggtttaggtaactcggtacaaaaactggagt
        F  G  R  G  E  L  W  G  Q  G  L  G  N  S  V  Q  K  L  E  Y

 901  atctgccggaagcgcacactgactttattttcgccattatcggcgaagaactggggtatg
        L  P  E  A  H  T  D  F  I  F  A  I  I  G  E  E  L  G  Y  V

 961  tcggtgtggtgctggcacttttaatggtattcttcgtcgcttttcgcgcgatgtcgattg
        G  V  V  L  A  L  L  M  V  F  F  V  A  F  R  A  M  S  I  G

1021  gccgtaaagcattagaaattgaccaccgttttttccggttttctcgcctgttctattggca
        R  K  A  L  E  I  D  H  R  F  S  G  F  L  A  C  S  I  G  I

1081  tctggtttagcttccaggcgctggttaacgtaggcgcggcggcggggatgttaccgacca
        W  F  S  F  Q  A  L  V  N  V  G  A  A  A  G  M  L  P  T  K

1141  aaggtctgacattgccgctgatcagttacggtggttcgagcttactgattatgtcgacag
        G  L  T  L  P  L  I  S  Y  G  G  S  L  L  I  M  S  T  A

1201  ccatcatgatgctgttgcgtattgattatgaaacgcgtctggagaaagcgcaggcgtttg
        I  M  M  L  L  R  I  D  Y  E  T  R  L  E  K  A  Q  A  F  V

1261  tacgaggttcacgatgagtggtcaaggaaagcgattaatggtgatggcaggcggaaccgg
        R  G  S  R  *
```

Figure 3.1.1. The nucleotide and amino acid sequence of *ftsW* as described by Ikeda *et al.* (1989). Comments are on the opposing page.

Figure 3.1.2. A comparison of the hydropathy profiles of the FtsW peptides and their homolgues are shown overleaf. The two potential FtsW peptide profiles from *E. coli* are very similar. The 46 kDa peptide has an extra potential membrane spanning domain at the start of the N-terminus. Otherwise the profiles of both are the same. This is expected as the smaller, 43 kDa, peptide is in frame with the larger peptide and therefore both peptides have an identical amino acid sequence. The *H. influenzae* FtsW peptide has an almost identical profile to that of the 43 kDa peptide from *E. coli*. It has nine potential membrane spanning regions. It does not have the first domain seen with the 46 kDa peptide. With respect to this, the other peptide profiles for RodA in *E. coli* and SpoVE from *B. subtilis*, also lack this first domain whilst the profile of these is also very similar to the 43 kDa peptide. Therefore in contradiction to Khattar *et al.* (1994) the smaller FtsW peptide may be the actual protein involved in division. It has been shown that the smaller peptide can complement the *ftsW* ^{ts} mutant isolated in this laboratory (J. Nikoliachik pers. comm.).
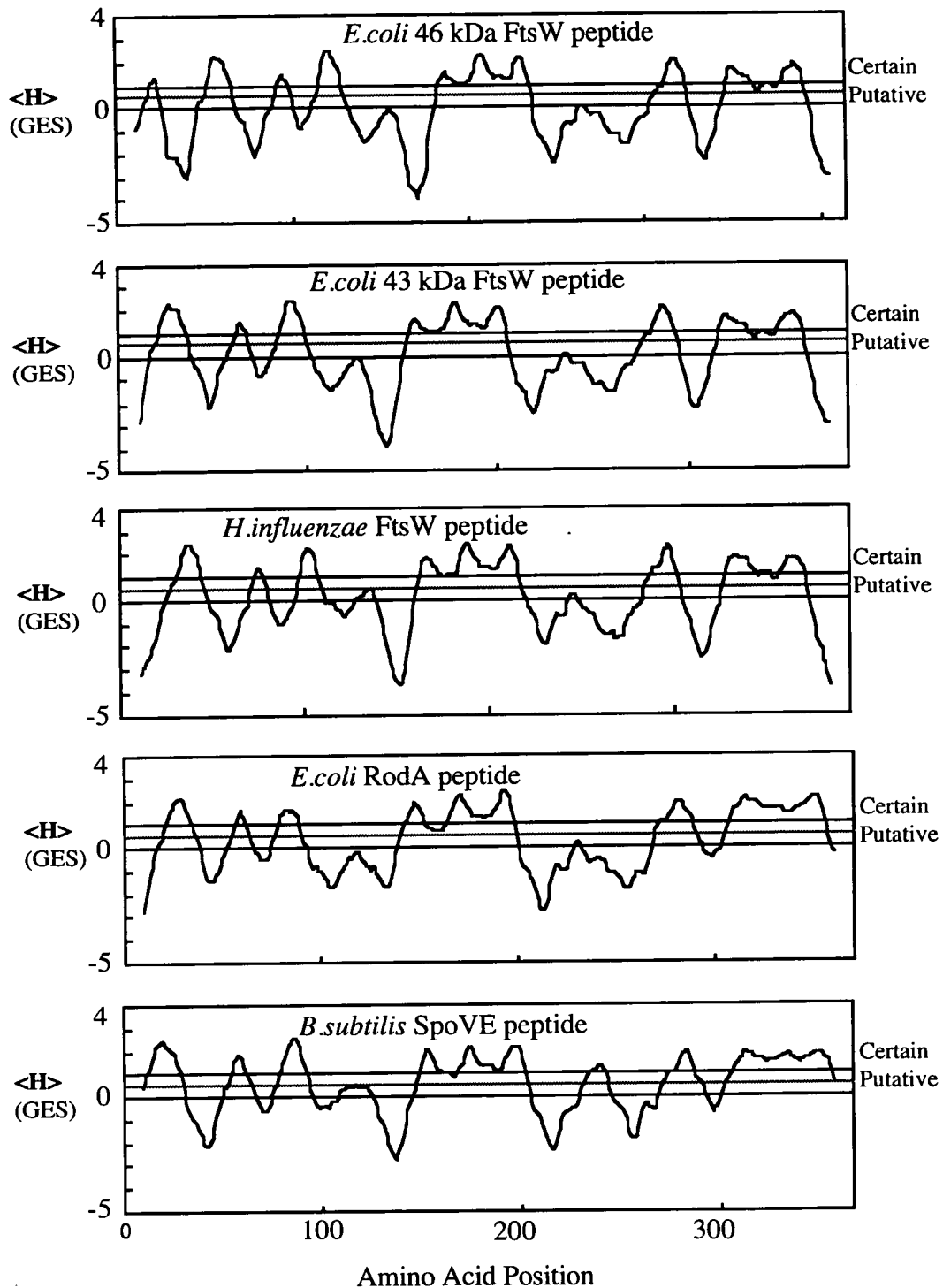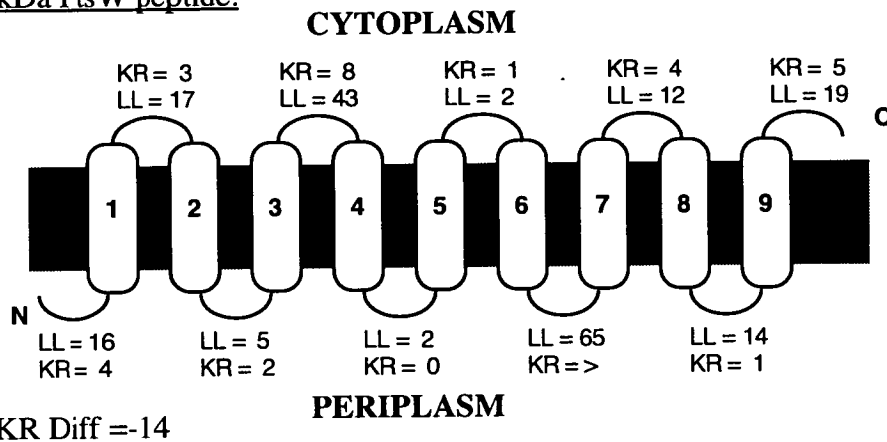
Figure 3.1.2. The hydropathy profiles of the FtsW peptides and their homologues. These are discussed on the opposite page.

46 kDa FtsW peptide.

PERIPLASM

KR = 3   KR = 2   KR = 0   KR = >   KR = 1
LL = 13 LL = 5   LL = 2   LL = 65 LL = 14

1 2 3 4 5 6 7 8 9 10

N

LL = 12 LL = 17 LL = 43 LL = 2   LL = 12 LL = 19
KR = 4   KR = 3   KR = 8   KR = 1   KR = 4   KR = 5

CYTOPLASM

KR Diff = 19

43 kDa FtsW peptide.

CYTOPLASM

KR = 3   KR = 8   KR = 1   KR = 4   KR = 5
LL = 17 LL = 43 LL = 2   LL = 12 LL = 19

C

1 2 3 4 5 6 7 8 9

N

LL = 16 LL = 5   LL = 2   LL = 65 LL = 14
KR = 4   KR = 2   KR = 0   KR = >   KR = 1

PERIPLASM
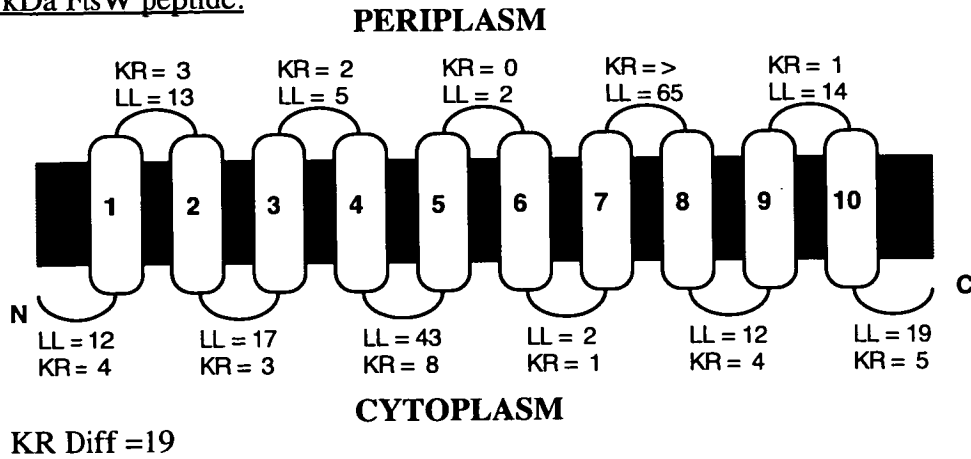
KR Diff = -14

KR Diff : Positive charge difference
> : Too long to be significative   LL : Loop length
< : Too short to be significative   KR : Number of Lys and Arg

---

Figure 3.1.3. A comparison of the predicted topologies of the 46 and 43 kDa FtsW peptides. Both are located in the cytoplasmic membrane although it can be seen that a major difference is the orientation of the hydrophilic domains relative to the cytoplasm and the periplasm. They are in one orientation in the 46 kDa peptide and in the opposite orientation for the smaller peptide. The 46 kDa peptide also has an extra membrane spanning region. The positive charge difference (KR) between the two peptides varies. This is +19 for the 46 kDa peptide and -14 for the smaller 43 kDa peptide.

3.2.1. An attempt to identify the FtsW polypeptide(s).

The ORF for FtsW lies in the centre of the *mra* cluster (Ishino *et al.*, 1989; Ikeda *et al.*, 1989). An 8.4 kb *KpnI* fragment described by Mengin-Lecreulx *et al.* (1989) was shown to contain *ftsW* with large flanking regions of DNA (Figure 3.2.1.).
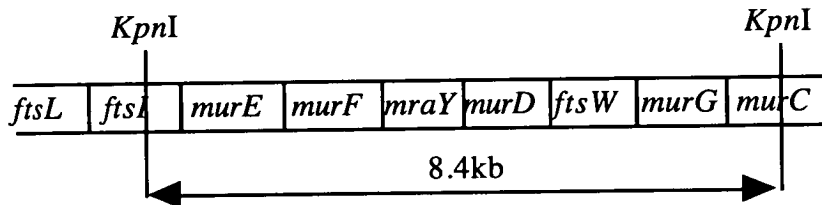

Figure 3.2.1. A schematic diagram showing the 8.4 kb *KpnI* fragment from the *mra* region of *E.coli* and the genes contained within it .

It was decided to clone this fragment since it contained *ftsW* and other genes of interest to this study. The DNA was isolated from the Kohara library, a collection of λ phage clones, which together encompass the entire *E. coli* genome (Kohara *et al.*, 1987). Examination of the Kohara map of the clones revealed three clones spanning the *mra* region, λ6CI(-) [λ109], λ6F3(-) [λ110] and λ15B8(+) [λ111]. The three phages were screened for the presence of the 8.4 kb *KpnI* fragment by screening for their ability to complement temperature sensitive (ts) mutants from the *mra* region and subsequently by DNA analysis.

Recombination between mutations and Kohara λ109, λ110 and λ111. The 8.4 kb *KpnI* fragment can complement *murF, murD* and *murG* ts mutants (Mengin-Lecreulx *et al.*, 1989). It was therefore decided to screen the λ phages for the presence of the 8.4 kb *KpnI* fragment by screening for recombination with the *murF^ts* and *murG^ts* mutants when incubated at 42°C. If any of the phage can recombine with both mutations then it was likely that it contained the desired *KpnI* fragment. The *murF^ts* mutant was originally isolated in strain PC1357 (Lugtenberg and van Schijndel-van Dam, 1973). The *murF^ts* allele from PC1357 was introduced to a C600 background by P1 transduction (C600F). The *murG^am* mutant was OV58, an amber mutant isolated by Salmond *et al.* (1980). The *murG* amber mutation is in a *supF^ts* strain, OV2, in which the amber suppressing tRNA is inactive at 42°C. The *murF^ts* P1 lysate and OV58 were supplied by K.Begg. The ts mutants were

cultured as lawns in BBL top agar supplemented with 0.2 %maltose and 10 mM MgCl$_2$. The lawns were then spotted with 10 µl aliquots of λ109, λ110 and λ111. As a negative control, 10 µl aliquots of Kohara λ235 were used. λ235 contains DNA from the 24 minute region of the *E.coli* genome and therefore should not recombine with either mutation. The plates were incubated at 42°C overnight and the phage spots examined for growth of the recombinants.

λ Phage	C600F	OV58
λ109	+	—
λ110	+	+
λ111	—	+
λ235	—	—

Table 3.2.1. Recombination between *murF*^{ts} (C600F) and *murG*^{ts} (OV58) mutations and Kohara λ phages resulting in growth at 42°C (+). Scoring was for colonies at 42°C.

Of the four phages used in this recombination test, only λ110 recombined with both the *murF* and the *murG* mutations. (Table 3.2.1). λ109 recombined with only the *murF*^{ts} mutant and λ111 with only the *murG*^{ts} mutant. λ235 did not give temperature resistant recombinants with either mutant. Therefore λ110 was chosen for further study and its DNA analysed for the 8.4 kb *Kpn*I fragment.

3.2.2. DNA analysis of λ110 and the cloning of the 8.4 kb *Kpn*I fragment.

Phage DNA was extracted from λ110. The DNA was then restricted with *Kpn*I and analysed by agarose gel electrophoresis. A band corresponding to 8.4 kb was isolated and this result in conjunction with the phage rescue of the ts mutants strongly suggested that λ110 contained the required DNA. The 8.4 kb *Kpn*I fragment isolated from λ110 was cloned into pUC18. C600F, the *murF*^{ts} mutant, was transformed with the ligation mixtures and the cells plated on LB Amp agar with subsequent incubation overnight at 30°C. Amp^R colonies were then patched onto LB Amp agar plates in duplicate. These plates were incubated overnight at 30°C and 42°C respectively. There were only six transformants which grew at 42°C. Three

of these showed confluent growth over the patched area while the other three grew less well.

Restriction analysis of the plasmid DNA isolated from the six transformants which had shown growth at 42°C revealed that all contained the 8.4 kb *Kpn*I from λ110 and pUC18. Surprisingly, the concentration of the plasmid DNA extracted from the three transformants which grew well at 42°C was much less than that obtained from the slower growing transformants. The plasmid DNAs were restricted with *Eco*RI to determine the orientation of the 8.4 kb insert relative to the P*lac* promoter in pUC18 (see Figure 3.2.2.). If the fragment was orientated such that P*lac* could express the cloned genes then an *Eco*RI restriction would produce two fragments of 7.1 kb and 4.0 kb. Alternatively, the opposite orientation to P*lac* would produce two fragments of 6.7 kb and 4.4 kb. After agarose electrophoresis three clones were found to be in the same orientation to P*lac*, and three in the opposite orientation. The former was termed pDEG1 and the latter pDEG2, both were 11.1 kb in size (Figure 3.2.2.). The fact that only isolated colonies of C600F/pDEG2 were formed at 42°C suggests that these were the result of recombination with *murF*^{ts}.

The three C600F transformants which grew well at 42°C contained pDEG1. It is probable that basal expression of *murF* from P*lac* had allowed full complementation of the ts allele. The three C600F transformants which grew poorly at 42°C contained pDEG2. In this instance the expression of *murF* was reliant on an endogenous promoter located in the 8.4 kb *Kpn*I fragment. Mengin-Lecreulx *et al.* (1989) reported the presence of a promoter in a clone which contained the C-terminus of *ftsI* which gave complementation of PC1357, a *murF*^{ts} mutant (Lugtenberg and van Schijndel-van Dam, 1973). This was when '*ftsI murE murF mraY*' was cloned in pUC18 in the opposite orientation to P*lac*. The basal expression from P*lac* may also interfere with the transcription of *murF* by simultaneously transcribing the opposing strand. This may explain why C600F/pDEG2 grew poorly in comparison to C600F/pDEG1. As the amount of plasmid DNA extracted from C600F/pDEG1 was much lower than C600F/pDEG2, it may be that the higher levels of expression of the genes on the fragment are deleterious to the cell and therefore the copy number was affected. More probably the growth rate of C600F/pDEG1 was reduced and therefore fewer cells were present in the samples from which the plasmid DNA was extracted. The effects of pDEG1 expression on cells morphology was

examined by transforming W3110 with both pDEG1 and pDEG2. The morphologies of W3110/pDEG1 and W3110/pDEG2 were very similar upon induction of P*lac* using IPTG.
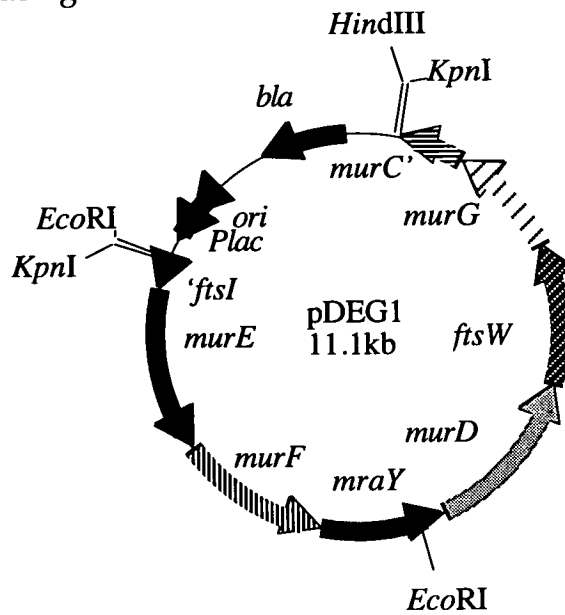

Figure 3.2.2. The 8.4 kb *Kpn*I fragment cloned into pUC18 to form pDEG1.

3.2.3. Cloning and the *in vivo* expression of *fts*W.

Mengin-Lecreulx *et al.* (1989) had previously tried to overexpress *fts*W *in vivo* from a 1.5 kb *Pvu*II/*Sma*I '*mur*D *fts*W *mur*G' clone in pUC18, by employing the maxicell method described previously by Sancar *et al.* (1979). This method failed to produce either of the predicted polypeptides of 46 kDa or 43 kDa. The failure to identify a product by SDS-PAGE analysis may have been due to the method of treating the labelled products (that is by boiling). The close homology to the RodA peptide meant that FtsW may also prove difficult to isolate from the membrane. In this study the choice of vector for *in vivo* expression of *fts*W utilized the bacteriophage T7 RNA polymerase expression system, as described by Studier and Moffat (1986). The vector used was pT7-4 (Figure 3.2.3.), a high copy number plasmid derived from pBR322. The gene of interest is cloned in the polylinker located downstream from the T7 gene10 promoter region. Transcription from the T7 gene 10 promoter (Ø10) requires T7 RNA polymerase. The T7 gene1 encoding T7 RNA polymerase is under the control of P*lac*, cloned in a λ phage. This is

present in the host genome on a lysogenized λ phage (λDE3). Low levels of T7 RNA polymerase are produced by basal expression from P*lac*. A T7 lysozyme, produced from *lysS*, is expressed from a low copy number clone in pACYC184 (pLysS). Since T7 lysozyme binds T7 RNA polymerase, the presence of pLysS reduces basal levels of T7 RNA polymerase. This is important if the overexpression of the cloned gene is toxic to the cell. When the T7 RNA polymerase is induced from P*lac* (by the addition of IPTG), the high levels produced are such that the T7 lysozyme cannot suppress expression by T7 RNA polymerase. The system operates by overexpressing T7 RNA polymerase until a large pool of the enzyme has built up in the cell. Transcription of the host genes by bacterial RNA polymerase is then inhibited by the addition of rifampicin to the culture. T7 RNA polymerase is not inhibited by rifampicin and therefore the only transcription which can occur after the addition of rifampicin is initiated from the Ø10 located in the pT7 vector. After a suitable period of transcription from this promoter (see Chapter 2), the over produced polypeptide is labelled with the addition of ^{35}[S] methionine. At this point the level of bacterial mRNA in the cell is negligible in comparison to the Ø10 mediated mRNA production. Therefore only genes located downstream from and orientated correctly to Ø10 on the pT7 plasmid are expressed and subsequently labelled.
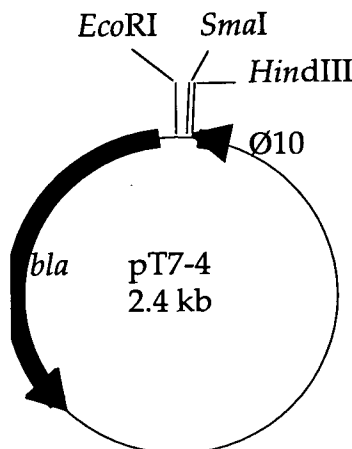

Figure 3.2.3. The *in vivo* expression vector, pT7-4.

To clone the 1.5 kb *Pvu*II / *Sma*I fragment containing *ftsW* into pT7-4, a smaller plasmid, pDDG1 (Figure 3.2.4.), was constructed from pDEG1. pDEG1 was restricted with *Eco*RI and the larger 7.1 kb fragment was isolated and re-ligated; This removes both a 4 kb DNA fragment containing *'ftsI , murE, murF* and *mraY'*.
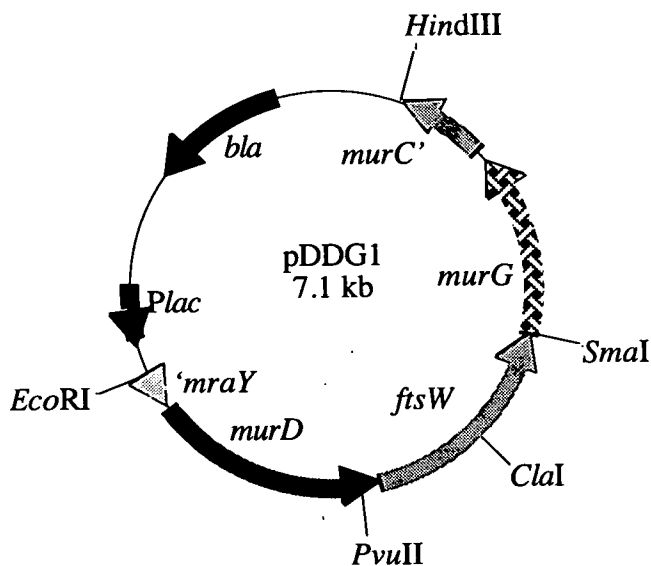
Figure 3.2.4. The clone pDDG1, showing the 1.5 kb *Pvu*II/*Sma*I fragment containing *fts*W.

This construct, pDDG1, contains '*mra*Y *murD fts*W *murG murC*' cloned downstream from P*lac* (Figure 3.2.4.). A 1.5 kb *Sma*I/*Pvu*II fragment was restricted from pDDG1 and cloned into the *Sma*I site in pT7-4. A clone with the *fts*W ORFs in the same orientation as the T7 gene 10 promoter (Ø10, pDBW4, Figure 3.2.5.) was then used to transform BL21 (λDE3)/pLysS. This is the host strain for overexpression of pT7 clones with T7 RNA polymerase.

BL21(λDE3) /pLysS /pDBW4 was then cultured in Spizizen's minimal medium Amp/Cmp at 37°C. The cells were induced and labelled as described in Chapter 2. The labelled samples were treated by boiling for 5 minutes and duplicate samples were prepared by incubation at 37°C for one hour. The samples were then separated by 10% SDS-PAGE electrophoresis. Exposure of the dried labelled gels to X-ray (DuPont) film did not reveal labelled protein bands at either 46 kDa or 43 kDa (Figures 3.2.6. and 7.)

SmaI

bla

pDBW4
4.3 kb   ftsW

Ø10

SmaI / PvuII
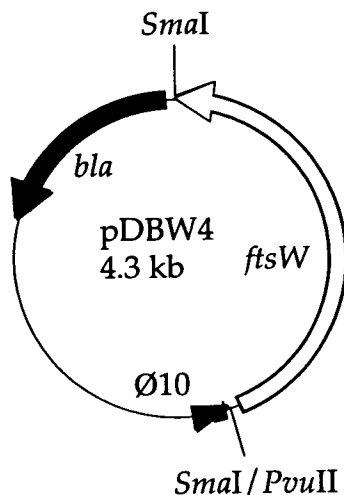
Bands at 31 kDa and 29 kDa were produced from the overexpression and labelling of pDBW4. This was irrespective of the temperature at which the samples were denatured. These are the precursor and mature forms of the β-lactamase protein. The *bla* gene is positioned in the same orientation as the Ø10 in pT7-4 (see Figure 3.2.3.) and is therefore transcribed when T7 RNA polymerase is produced and subsequently translated. The presence of these bands indicates that transcription from Ø10 was successful and that the ^{35}[S] methionine label was incorporated effectively into the proteins during translation.

Therefore the T7 RNA polymerase method was effective for labelling but not for the production of an FtsW polypeptide. The amino acid sequences of the proposed FtsW polypeptides were screened for the number of methionine residues present. If there were no methionine residues in FtsW then the polypeptide would not be labelled. Alternatively if there were few methionine residues then the signal from the labelled peptide would be too weak for detection. The 46 kDa and 43 kDa polypeptides contained 16 and 14 methionine residues respectively. Therefore poor labelling of the polypeptide could be excluded as a reason for the failure to detect a product.

Khattar *et al.* (1994) isolated a 32 kDa polypeptide by expressing the larger coding sequence for *ftsW*. That is the polypeptide with a predicted molecular weight of 46 kDa which had a mobility of 32 kDa when analysed by SDS-PAGE. This unusual mobilty is similar to that reported for the closely related RodA protein. RodA is a 40 kDa peptide which migrates as a

30 kDa peptide on SDS-PAGE gels (Matsuzawa *et al.*, 1989). To produce the 32 kDa protein Khattar *et al.* (1994) made a fusion between the start codon of the larger *ftsW* ORF and the ribosome binding site of T7 gene 10. The expression of the 1.5 kb *SmaI/PvuII* fragment in pT7-6 produced very low levels of FtsW, this also had an unusual migration rate when examined by SDS-PAGE. The pT7-6 vector is similar to pT7-4, the major difference being that β–lactamase is not produced upon the induction of expression (Tabor and Richardson, 1986). The improved expression of FtsW by the fusion may be attributed to the removal of the sequence immediately upstream of the start codon. This sequence forms a palindrome with the sequence immediately after the start codon of the larger ORF (Figure 3.2.8. and 3.1.1.). The mRNA produced from the wild-type DNA could form a stem loop structure which would incorporate the start codon of the larger peptide. This may reduce the efficiency of ribosome binding as the RBS would be unavailable. In light of the work by Khattar *et al.* (1994), pT7-4 was an unsuitable vector as labelled β–lactamase probably masks the very low levels of FtsW produced.
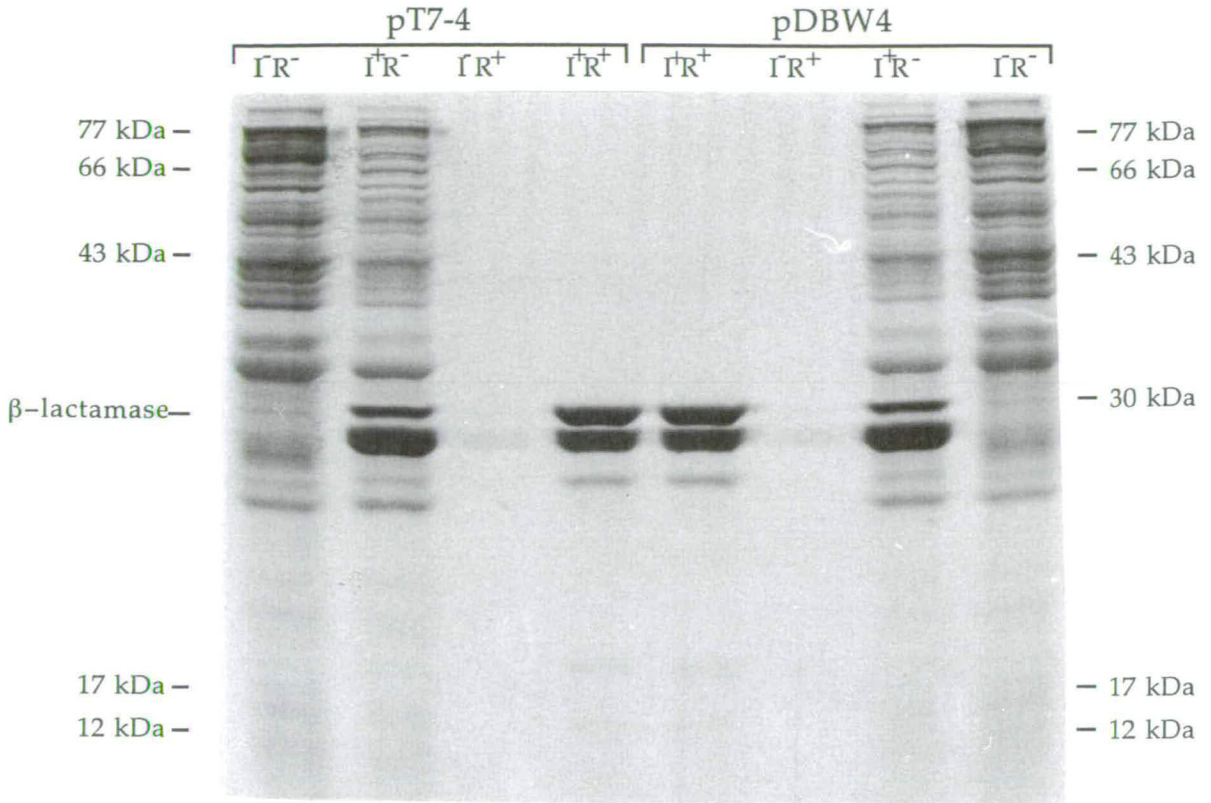
Figure 3.2.6. Over-expression of pT7-4 (lanes 1-4) and pDBW4 (lanes 5-8). The profiles of 37°C treated samples after SDS-PAGE analysis. The SDS-PAGE analysis of the pDBW4 samples prepared at 37°C did not produce peptides of either 46 kDa or 43 kDa. The two labelled peptides visible are the β-lactamase proteins at 31 kDa and 29 kDa. The presence of these in the induced and Rif (I^+R^+) treated samples of pT7-4 and pDBW4 (lanes 4 and 5 respectively) proves that there was expression from the gene 10 promoter upstream of the *ftsW* clone and that the mRNA transcript was translated.
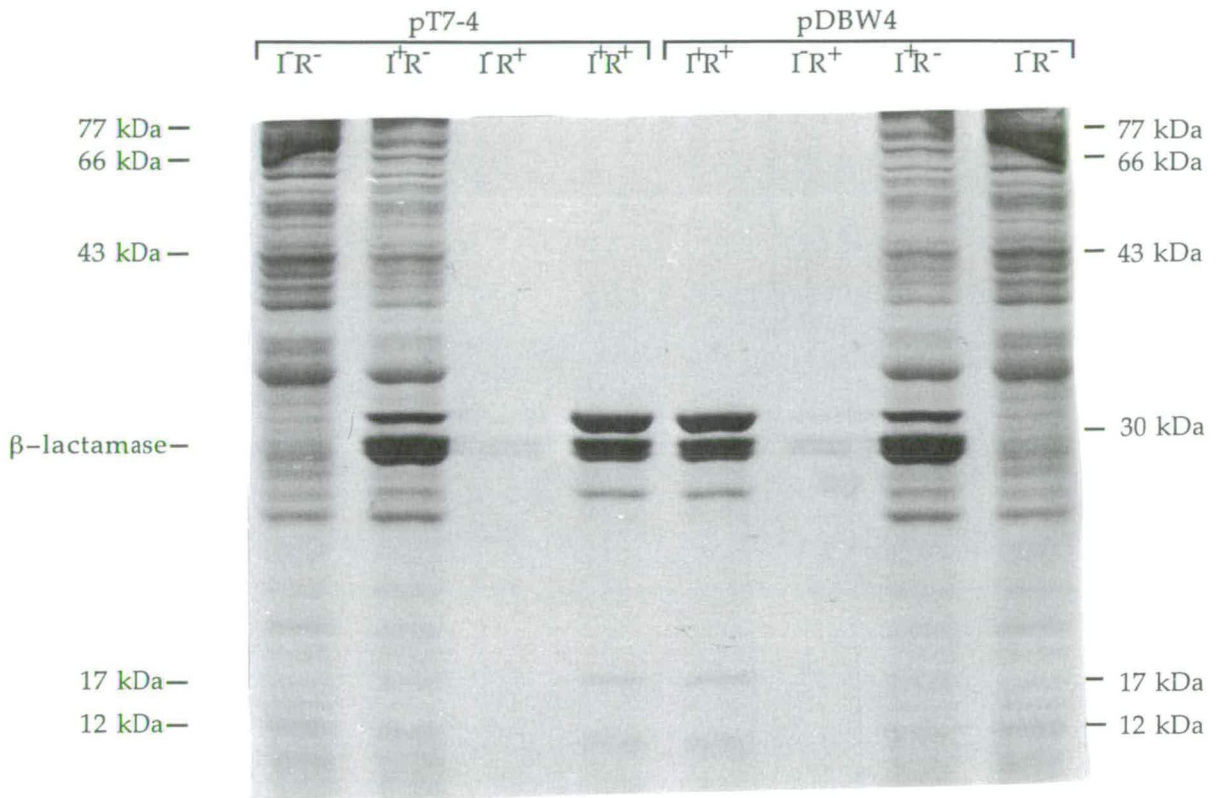
Figure 3.2.7. Overexpression of pDBW4 and pT7-4. The profiles of 100°C samples after SDS-PAGE analysis. The peptides in lanes 4 and 5 are the β-lactamase proteins at 31 kDa and 29 kDa. The presence of β-lactamase proteins in the IPTG induced and Rif (I⁺R⁺) treated samples of pT7-4 and pDBW4 (respectively) proves that there was expression from the gene 10 promoter upstream of the *ftsW* clone and that the mRNA transcript was translated. There was no difference between the 37°C and the 100°C treated samples.

```
      A U
   G       G
  U          C
  U          U
  G          G
  G          U
  A          U
  U          A
                A
     U  G  C U  C
       A  U
       G  C
       G  C
       A  C
       A  U
       G  C
       C  G
       G  C
       G  C
5' GCCCGUCU      UGAAAA  3'
```
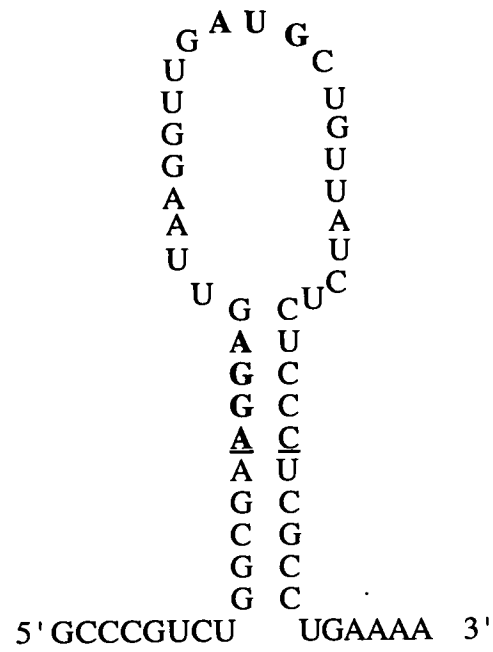
Figure 3.2.8. The potential secondary structure of the *ftsW* mRNA transcript which sequesters the ribosome binding site for the larger peptide (in bold). The start codon is located in the loop (in bold). In each of the palindromic sequences there are single base mismatches for adenine and cytosine (underlined).

3.3.1. Mutagenesis of ftsW.

The previous ftsW[ts] mutant isolated by Ishino et al. (1989) and Ikeda et al. (1989) exhibited a filamentous phenotype at 42°C. This phenotype was sensitive to culture density and the salt concentration of the growth media (Ikeda et al., 1989). The 1.5 kb PvuII/SmaI fragment was shown to complement the ts allele at 42°C. The aim of my work was to construct an ftsW null mutant to confirm that ftsW is an essential cell division gene. Such a mutant is essential to study further the role of ftsW in the E.coli cell cycle. The method chosen was to substitute the thymine residue located at 576 bp after the start of the first ftsW ORF (that is the ORF encoding the putative 46 kDa polypeptide). The thymine residue was replaced with a guanine residue (Figure 3.3.1.) This creates an amber stop codon, UAG, after transcription of either of the two ftsW ORFs.
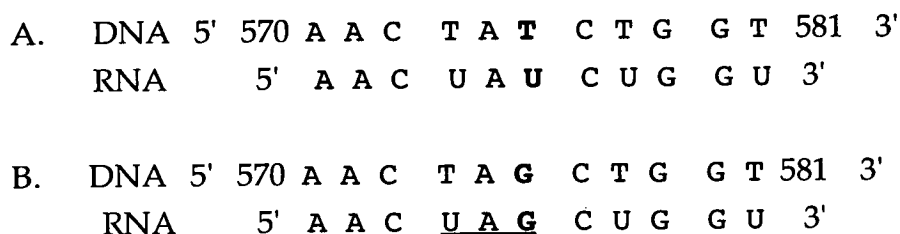
A. DNA 5' 570 A A C   T A **T**   C T G   G T 581 3'
    RNA   5' A A C   U A **U**   C U G   G U 3'

B. DNA 5' 570 A A C   T A **G**   C T G   G T 581 3'
    RNA   5' A A C   <u>U A G</u>   C U G   G U 3'

Figure 3.3.1. Legend A shows the wild-type DNA sequence of ftsW with the thymine residue to be substituted in bold. The RNA transcript shows the tyrosine triplet codon underlined. In legend B the substitution of guanine for thymine is shown in bold. The amber stop codon is shown as underlined.

If the amber suppressor tRNA is inactived then translation from the transcript will be terminated at the UAG codon. It was hoped to replace the chromosomal copy of ftsW with the amber mutant ftsW (ftsW[am]). It was planned that the chromosomal ftsW[am] allele be transferred to OV2, a strain containing a temperature sensitive tyrosine tRNA (supF[ts]), by P1 transduction.

3.3.2. Construction of an amber mutation in ftsW.

The single base change was introduced by site directed mutagenesis using the method described by Kunkel (1985). This method entails the use of

uracil rich, single stranded M13 as a template. The complementary strand to the single stranded DNA is synthesized *in vitro* to produce double stranded M13 DNA. The primer, necessary to initiate the extension of the complementary strand, contains the mutated sequence. Each double stranded molecule of M13 produced is composed of a uracil rich strand and the newly synthesized, uracil free, strand which contains the mutagenized primer. When this DNA is transformed into an *ung*$^+$ strain, the uracil enriched DNA is rendered biologically inactive. *ung*$^+$ encodes uracil transglycosylase which removes uracil from the DNA leaving abasic sites which prevent the ssDNA from replication. The other, *in vitro* synthesized, strand has uracil free DNA and is used as the template for replication. This allows a high proportion of the site directed mutations to be recovered.

The 1.5 kb *ftsW* fragment was subcloned into M13mp19 by restricting both pDBW4 and M13 DNA with *Eco*RI and *Hind*III. The 1.5 kb fragment was isolated, purified and ligated with the linearized M13mp19 DNA. This produced MW19, whose double stranded DNA was 8.8 kb in size. The uracil-enriched ssDNA template was prepared by infecting BW313 with MW19. The relevant genotype of BW313 is *dut ung*. *ung* encodes dUTPase, which degrades dUTP, and therefore the mutant has elevated levels of dUTP. Some dUTP molecules are incorporated, instead of dTTP, into the M13 DNA. The uracil enriched ssDNA is stable because the *ung* genotype produces no uracil transglycosylase in BW313. The single stranded DNA was recovered as described in Chapter 2. A 17-mer oligonucleotide primer with the single base change mutation was supplied by Oswel DNA Services (Figure3.3.2.). The polymerization reaction from the mutagenic primer with the uracil enriched ssDNA was performed as described in Chapter 2.

<div align="center">5' C G C C A A C T A <u>G</u> C T G G T G C 3'</div>

Figure 3.3.2. The mutagenic primer DAV1 with the single guanine substitution underlined in bold.

TG1 was transformed with the reaction mixtures and then grown as a lawn in LC top agar over LC bottom agar. The cells were incubated overnight at 37°C. Fifty isolated plaques were picked and stored at 4°C, each in 0.5ml LB. The introduction of the mutation also introduced a unique restriction site for *Bfa*I into *ftsW*. The successful introduction of the mutation

into MW19 was shown by digestion with *Bfa*I. Double stranded DNA samples were prepared from 10 possible mutants and restricted with *Bfa*I. Analysis of the restriction patterns after agarose gel electrophoresis showed that seven of the ten isolates contained mutated *ftsW*. The mutation was then sequenced from ssDNA collected from one of the potential mutant isolates using the 24-mer primer #1233 (New England Biolabs). The mutation had successfully been introduced to *ftsW* (Figure 3.3.3.).
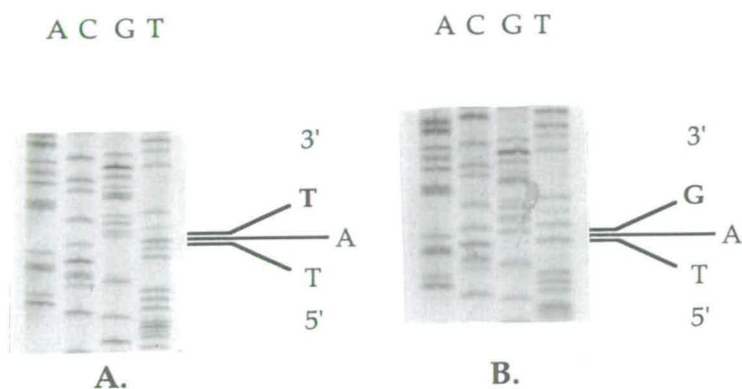

Figure 3.3.3. The sequencing of MW19 (A.) and MWam (B) showing, in bold, the substitution of a thymine residue for a guanine residue.

3.3.3. Attempt to introduce the *ftsW* amber mutation onto the chromosome.

The method chosen to introduce the *ftsW^am^* mutation onto the chromosome utilized a derivative of the *polA^ts^* strain JC411 (Low, 1968; Clark *et al.*, 1969). This method selects for cells in which the plasmid has integrated into the chromosome. *polA* encodes DNA polymerase I, which is essential for ColE1 replication; in a *polA^ts^* strain at 42°C plasmids with a ColE1 origin of replication cannot replicate. As the cell population grows at 42°C, such plasmids are diluted out and eventually lost. If a selection for a plasmid based marker is applied, then cells which no longer contain plasmid DNA will die. Some cells will, however, contain the plasmid as a cointegrant with the chromosome and will therefore survive. The plasmid/genome cointegration occurs by a single cross-over at regions of homology between the plasmid the genome. (Figure 3.3.4.).

The culture is enriched for cointegrants by continual selection for the plasmid marker (*bla*) with incubation at 42°C. Ultimately, a population of cells is produced where all are plasmid cointegrants. By culturing cointegrants at the permissive temperature for DNA polymerase I function, the plasmids may now replicate, following excision from the chromosome, and in doing so may replace part of the genomic DNA with the homologous DNA from the plasmid (see Figure 3.3.4).

The 1.5 kb *Eco*RI/*Hin*dIII fragment containing the *ftsW*^{am} mutation was subcloned from MWam into pT7-4 also cut with *Eco*RI/*Hin*dIII. pT7-4 has a ColE1 origin of replication. The resultant clone, pT7Wam, was 3.9 kb in size. A *polA*^{ts} *leu*::Tn10 strain, JC411T was transformed with pT7Wam with selection for Amp^R colonies on NB Amp agar incubated at 30°C. This strain was cultured in NB Amp/Tet at 30°C overnight with shaking. This culture was used as an inoculum to grow a fresh culture in NB Amp/Tet at 42°C with shaking. Over a period of 3 days the culture was maintained by subculturing daily into fresh NB Amp/Tet at 42°C.

The Figure 3.3.4. represents the integration into the chromosome of a ColE1 based plasmid at a region of homology. **i.** shows the single cross-over reaction between the *ftsW* alleles present in the chromosome and the plasmid, pTWam. The formation of a cointegrant is a rare event. The use of a *polA*^{ts} strain is such that cointegrants can be selected for by selection for Amp^R, as free plasmids cannot replicate without DNA polymerase I and are therefore gradually diluted from the culture when incubated at 42°C. **ii.** The amber mutation can be in either of the *ftsW* alleles depending on the location of the cross-over. **iii.** and **iv.** By shifting the temperature back to 30°C most plasmids excise from the chromosome as (plasmid) excessive replication from the plasmid origin in the chromosome is deleterious to the cell (Yamaguchi and Tomizawa, 1980). These grow much faster than the cointregrants and these are therefore diluted from the culture. The site of recombination for the excision event will determine whether the amber mutation is introduced to the chromosome (**iii.**) or is retained on the plasmid (**iv.**). The *leu*::Tn*10* was used as a selectable marker for the transfer of *ftsW*^{am} into other strains.
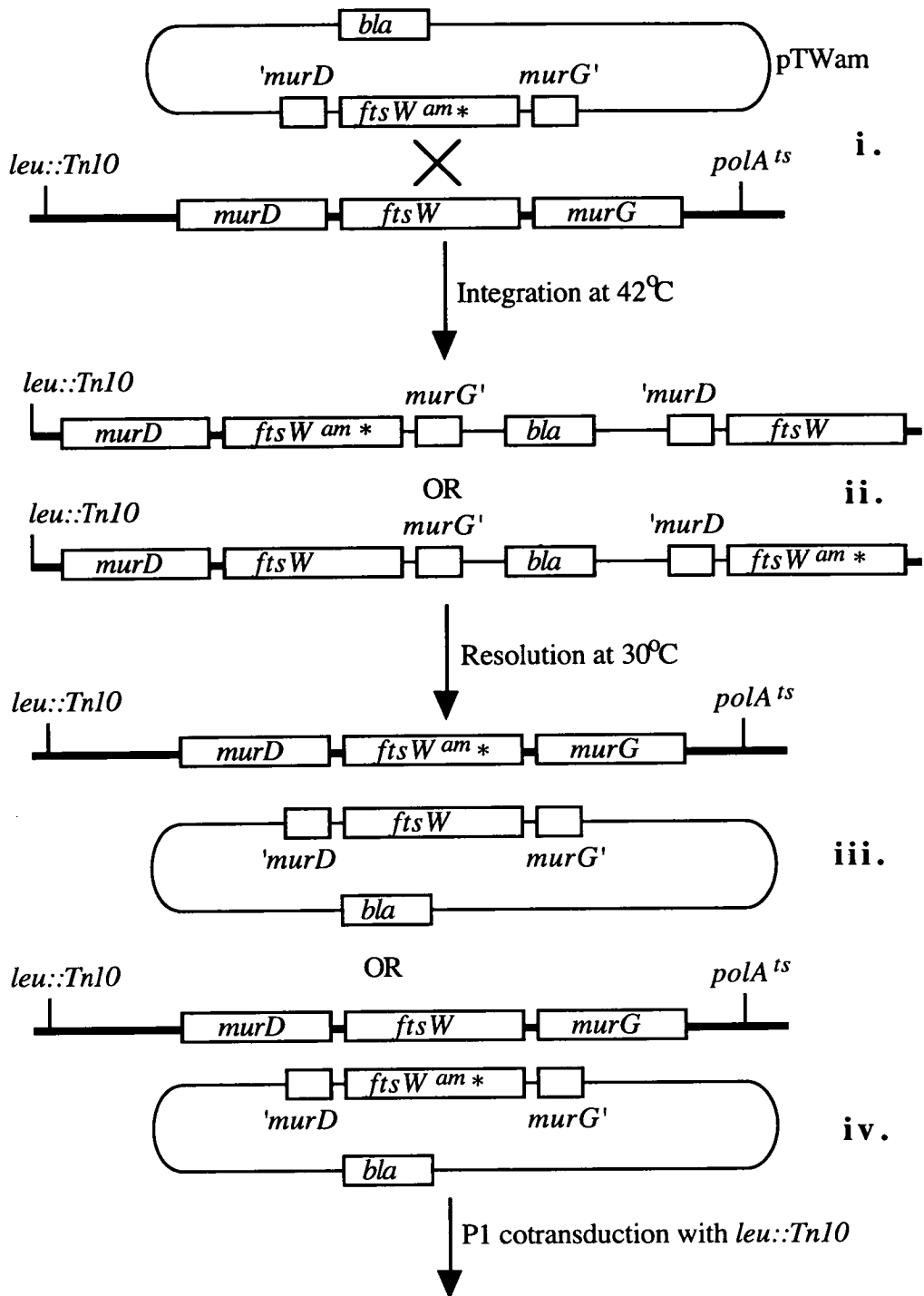
Figure 3.3.4. Insertion of pTWam into the chromomsome of a *polA^{ts}* strain at 42°C and it's subsequent excision at 30°C. The chromosomal mutant allele can be subsequently transferred to other strains by P1 transduction.

5 ml samples of the culture were removed daily and tested for the presence of pT7Wam by restriction analysis of purified DNA. After one day of culturing at 42°C there was no visible sign of pT7Wam, and the culture consisted entirely of cointegrants. Following this treatment, cells were subcultured into fresh medium and incubated at 30°C overnight. Dilutions of the cells were plated on NB Amp/Tet agar and incubated overnight.

Single colonies were collected and their plasmid DNA prepared and restricted with *Bfa*I. The wild-type *ftsW* cloned in pT7-4 contains no recognition sites for *Bfa*I. Therefore if the mutagenized copy of *ftsW* has replaced the wild type *ftsW*, on the chromosome, the plasmid DNA will not be restricted. Thirty colonies were analysed in this manner and on each occasion restriction with *Bfa*I produced a linear band of DNA. This showed that the mutagenized region of *ftsW* has not replaced the wild-type *ftsW* on the chromosome.

P1 lysates were also prepared on JC411T/pTWam at 30°C after the treatment described above. The lysates were used to transduce OV2 with selection for the *leu*::Tn*10* on NB Tet agar at 30°C. Tet^R colonies were then screened for growth at 42°C, the restrictive temperature for *supF*^{ts}. No ts isolates were recovered from screening 1000 Tet^R colonies. OV2 has a *trp*^{am} allele and the presence of *supF*^{ts} was confirmed by culturing ten of the Tet^R transductants on VB minimal agar with complete supplements for the OV2 phenotype except for tryptophan (Trp). These were incubated at 30°C and 42°C. As a control, the isolates were also grown on VB minimal agar, fully supplemented for OV2, at 42°C. The Tet^R isolates were unable to grow on the Trp free media at 42°C. If Trp was supplied in the media then the isolates formed colonies at the restrictive temperature. At 30°C all the isolates grew irrespective of the addition of Trp. Therefore the 10 isolates had all retained the *supF*^{ts} *trp*^{am} phenotype associated with OV2.

The choice of pT7-4 as the vector may have been responsible for the failure to isolate any *ftsW*^{ts} mutants. Ikeda *et al.* (1989) found that the *Pvu*II/*Sma*I *ftsW* fragment could not complement the *ftsW*^{ts} mutation at 42°C unless an exogenous promoter was present upstream of the *Pvu*II site. pUC118 was used in their research, which expresses cloned DNA from the P*lac* promoter. Therefore the *ftsW*^{am} fragment in pTWam was transferred to pUC19 by restricting both plasmids with *Eco*RI/*Hin*dIII. pUC19 also has a ColEI based origin of replication and therefore is suitable for the selection of cointegrants in a *polA*^{ts} strain at 42°C. The 1.5 kb *ftsW*^{am} fragment was

ligated into pUC19 to create pUCWam (4.2 kb). This was transformed into JC411T and the temperature shift again repeated, as described for JC411T/pTWam.

A small scale plasmid preparation was performed on a 5 ml sample of JC411T/pUCWam which had been subcultured daily into fresh NB Tet at 42°C for 3 days. An aliquot from this was restricted with HindIII and the presence of linear pUCWam screened for by agarose electrophoresis. An additional measure was employed to verify that JC411T/pUCWam grown at 42°C contained only cointegrants. C600 cells were transformed with part of the plasmid minipreparation sample. Amp^R transformants were screened for on LB/Amp and incubated at 37°C. The results from both the restriction digest and the transformation of C600 revealed no free plasmid DNA in the cells at 42°C. Aliquots of the 42°C culture were infected with P1 bacteriophage and grown in LC top agar at 30°C. P1 lysates were prepared from these and were used to transduce OV2 to Tet^R, by selection on NB Tet agar at 30°C. To screen for Tet^R ts isolates, the colonies were patched in duplicate on NB Tet and incubated at 30°C and 42°C.

From 500 colonies incubated at 42°C, two appeared temperature sensitive Microscopic examination revealed the colonies were composed of rods with some filamenting cells. At 30°C, all the patches grew. The two potential ts mutants (Wam6 and Wam34) were then patched on NB Tet/Str and incubated at 30°C. This was to ensure that JC411T/pUCWam was not a contaminant carried over in the P1 transduction of OV2. Wam6 and Wam34 were Str^S and Amp^S. The supF^{ts} genotype of the OV2 host strain for the transductions was checked by sceening for the trp^{am} genotype. OV2 used as the host for the P1 transductions was trp$^-$ at 42°C, therefore the supF^{ts} allele was present

The location of the lethal ts alleles in Wam6 and Wam34 was investigated using P1 transduction. The leuA allele is 17 kb upstream from ftsW. The cotransduction frequency between these alleles is ~70%. The leu::Tn10 marker in Wam6 and Wam34 was replaced with a leu::Tn9 marker by P1 transduction with P1[leu::Tn9 ftsW$^+$]. That is cells became Cmp^R and Tet^S. Transductants were plated in duplicate on NB Cmp agar and incubated at 30°C and 42°C. At 30°C there was confluent growth on all patches. At 42°C, there was no growth. The transduction of Wam6 and Wam34 with a P1[leu::Tn9 ftsW$^+$] lysate was repeated and another 100 Cmp^R/Tet^S isolates grown at 30°C and 42°C. Again no cells grew at 42°C.

These results indicate that the lethal ts mutation is not linked to *leu.*. Therefore *ftsW* does not contain the amber mutation.

This was confirmed by an attempt to transfer the temperature sensitive phenotype by P1 transduction to OV2C (OV2 *leu*::Tn9). P1 lysates were made from Wam6 and Wam34. These were used to transduce OV2C to OV2T with selection for Tet^R transductants at 30°C on NB/Tet. In this instance the *ftsW^{am}* would again be cotransduced with *leu*::Tn10 at a frequency of ~70%. Transductants were grown at 30°C and 42°C and the transductants grew at both temperatures. Therefore the temperature sensitive growth phenotypes of Wam6 and Wam34 were not linked to the *mra* region.

3.3.4. Selection of *ftsW* amber mutants on minimal and LB agar.

The media used for the isolation of an *ftsW* amber mutant had been nutrient broth (NB). This is a nutrient rich medium which contains 0.5% NaCl. The phenotype of the ts mutant isolated by Ikeda *et al.* (1989) was dependant on the salt concentration of the media. An attempt was made to isolate the *ftsW^{am}* mutant by screening Tet^R transductants of OV2 using a P1 lysate from JC411T/pUCWam for a lethal phenotype on NB, minimal media (+Trp) and LB agars, all of which contained Tet.

Tet^R transductants were isolated on all three types of solid medium. The Tet^R colonies were then patched onto fresh plates in duplicate and incubated at 30°C and 42°C overnight. All patches grew at both temperatures. Therefore the *ftsW^{am}* allele had not replaced the wild-type allele on the chromosome.

Therefore the composition of the selective media had no effect on the isolation of an *ftsW^{am}* allele. The possibility remained that the wild-type copy of *ftsW* could not complement the *ftsW^{am}* allele on the chromosome of a non-suppressor (*sup^o*) host, even when expressed from P*lac*. The isolation of JC411T/pUCWam cointegrants was again performed at 42°C. However, a 0.5 ml aliquot from the 42°C culture was used to inoculate 25 ml NB Amp/IPTG which was then incubated overnight at 30°C, with shaking. The addition of IPTG will induce expression from P*lac* increasing the expression of *ftsW* to levels anticipated to be sufficient to complement the *ftsW^{am}* on the chromosome. Tenfold serial dilutions of 10^{-5}, 10^{-6} and 10^{-7} were made using the 30°C culture and were plated on NB Amp/Tet/IPTG agar at 30°C. Single

colonies were propagated in NB Amp/IPTG and the plasmid DNA from each was extracted after incubating the cells overnight at 30°C.

pUCWam contains a single recognition site for the enzyme *Bfa*I at the amber stop codon in *ftsW^{am}* not present in wild-type. Therefore plasmid DNA from isolates which have acquired the *ftsW^{am}* onto the chromosome in exchange for the wild-type would not be restricted by *Bfa*I. The plasmid DNA from twenty isolates was tested by restriction with *Bfa*I, and each plasmid DNA sample was restricted proving that the wild-type *ftsW* had been retained on the chromosome. This was repeated with a further twenty isolates. The plasmid DNA recovered from these cells also retained the *ftsW^{am}* allele in pUC19.

3.3.5. An enrichment technique to isolate *ftsW^{am}* mutants.

As no *ftsW^{am}* mutants had yet been isolated, another approach was attempted where OV2 was transduced with a P1 lysate cultured on JC411T/pUCWam made in the previous experiment. The transduced culture was then enriched for thermosensitive division mutants. The method used was based on that employed by Begg *et al.* (1980) in the isolation of the Thermosensitive Oscillatory Enrichment (TOE) mutants. The formation of filamentous cells was selected for by "oscillating" the incubation temperature between the permissive and restrictive temperatures for ts alleles.

In the experiment, 1 ml of a P1 lysate grown on JC411T/pUCWam was used to transduce 10 mls of OV2. Phage adsorption was for twenty minutes with incubation at 30°C before the transduction mixture was pelleted and resuspended in 3 ml phage buffer. This was used to inoculate 100 ml NB Tet which was incubated at 30°C for a culture mass doubling and the culture was then incubated at 42°C for five minutes before shifting it back to 30°C. When the culture mass doubled, the temperature shift was again repeated for the same period of time. The cycle was repeated once more. The cells were then drawn through a 15 μm membrane filter (Millipore), taking care not to draw air through the trapped cells. The aim of this was to entrap cells which were filamentous due to inhibition of division during the shift ups to the restrictive temperature. The filter was placed in 100 ml NB Tet, prewarmed to 30°C and the oscillatory amplification technique was performed again. This culture was then drawn through another sterile 15

µm filter to further remove smaller cells which were trapped in the first filtration step. Another 100 ml of prewarmed NB was drawn through the filter to wash the trapped cells. The filter was placed in 10 ml NB and incubated at 30°C for five minutes to dislodge the trapped cells from the filter membrane. Tenfold serial dilutions of the culture were then plated on NB Tet agar and incubated overnight at 30°C. Single Tet^R colonies were then patched in duplicate on NB Tet agar and incubated overnight at 30°c and 42°C. Of 1500 Tet^R isolates screened in this manner, none were temperature sensitive.

3.3.6. Complementation of an *ftsW*^{ts} allele at 42°C with pUCWam.

This experiment was designed to ensure that *ftsW*$^{a\,m}$ could complement *ftsW*^{ts} in the presence of an amber suppressor, *supF*. The results from this may help explain why *ftsW*^{am} did not replace the wild-type *ftsW* on the chromosome. A P1 lysate, made from OV263, an *ftsW*^{ts}, strain was supplied by M. Khattar. OV263 also carried a *leu*::Tn9 marker. This was used to transduce OV2T (*leu*::Tn10, *ftsW* $^+$) to OV263 (*leu*::Tn9, *ftsW*^{ts}) by initially selecting for Cmp^R at 30°C. Cmp^R transductants were then screened for ts at 42°C. To test for complementation of an *ftsW*^{ts} at 42°C by amber suppression of pUCWam, a λ prophage carrying *supF*, λ540, was supplied by K. Begg. This allele codes for *su*III$^+$, the tyrosine inserting amber suppressor tRNA, which is not temperature sensitive (Borck *et al.*, 1976). OV2 was infected with λ540 and lysogens were selected for by growth on fully supplemented minus tryptophan minimal agar at 42°C. OV2 has a *trp*^{am} allele, therefore growth of OV2 on minimal agar lacking Trp at 42°C can only occur if amber suppressor tRNA is present, that is if the cells are λ540 lysogens. The *ftsW 263* allele was then cotransduced with *leu*::Tn9 into OV2 (λ540) with selection for Cmp^R at 30°C on LB Cmp agar. Cmp^R transductants were screened for ts behaviour at 30°C and 42°C. The strains used and their genotypes are listed in Table.3.3.1.

Strain	Relevant genotype
OV263	*supF*^{ts} *leu*::Tn9 *ftsW*^{ts}
OV263 (λ540)	(λ540 *supF*) *supF*^{ts} *leu*::Tn9 *ftsW*^{ts}
JM263	*supE leu*::Tn9 *ftsW*^{ts}

Table 3.3.1. The genotypes of the strains used to test complementation of *ftsW 263* with pUCWam.

A *supE* strain, JM101, was also used. *supE* strains carry a glutamine inserting amber suppressor tRNA. This was used to find whether a glutamine insertion instead of the original tyrosine residue would produce a functional FtsW protein, capable of complementing *ftsW 263* at 42°C. Therefore JM101 was also transduced to *leu*::Tn9, *ftsW 263* (JM263). There were three different strains, carrying *leu*::Tn9, *ftsW 263*. These were OV2 (*supF*^{ts}), OV2 (λ540 [*supF*]) and JM101 (*supE*) which are listed in Table 3.3.1. A pUC18 clone containing the 1.5 kb *Pvu*II / *Sma*I fragment was made by cloning the fragment, restricted and purified from pDDG1, into the *Sma*I site of pUC18 to make pUCW18, 4.2 kb in size. The orientation of *ftsW* was such that it was expressed from P*lac*. Each *ftsW 263* mutant was transformed with pUC19, pUCWam and pUCW18. Transformants were selected for Amp^R on NB Amp at 30°C. Amp^R colonies were then streaked at 30°C and 42°C. The results of the plasmid complementation are shown in Table 3.3.2.

	pUC18		pUCWam		pUCW18	
Strain	30°C	42°C	30°C	42°C	30°C	42°C
OV263	+++	−	+++	−	+++	+++
OV263 (540)	+++	−	+++	+	+++	+++
JM263	+++	−	+++	−	+++	+++

Table 3.3.2. The complementation of the *ftsW* ^{ts} 263 allele in different *sup* backgrounds.

The phenotype that the *ftsW 263* allele produced in these strains was filamentous, even at 30°C. When the strains were transformed with pUCW18 the cell morphology became more uniform with rod shaped cells being predominant at 30°C. At 42°C pUCW18 complented all three mutants for growth on NB Amp. However, the morphology of these cells was

filamentous, suggesting the incomplete complementation of the *ftsW 263* allele with pUCW18. pUCWam complemented strain OV263 (λ540) for growth at 42°C. This therefore shows that the *ftsW^{am}* allele produced by site directed mutagenesis can complement *ftsW 263* if present in a *supF* strain, in a high copy number expressed from an exogenous promoter. The morphology of the cells at 42°C was also filamentous. The *supE* transformant, JM263 was not complemented by pUCwam at 42°C. However at 30°C the presence of filaments was reduced and most cells had a rod shaped morphology. This suggests a partial suppression of the *ftsW 263* with the FtsW protein containing a glutamine residue instead of tyrosine.

3.3.7. Comments on the failure to insert *ftsW^{am}*.

The attempts to replace the chromosomal copy of *ftsW* with *ftsW^{am}* had failed. The *ftsW^{am}* allele could complement the ts mutant when full suppression is afforded by *supF*. The *supF^{ts}* allele differs from the temperature insensitive gene in the efficiency of the suppressor tRNA. The efficiency of suppressor tRNA produced by the *supF^{ts}* allele at 30°C is only 10% of that of *supF^+* . Therefore the translation of *ftsW* mRNA from *ftsW^{am}* is greatly reduced. The poor translation of *ftsW* mRNA (M. Khattar, pers. comm.) in conjunction with the inefficiency of suppressor tRNA may mean that insufficient levels of FtsW are produced in a *supF^{ts}* background at the permissive temperature resulting in a non conditional lethal mutant. 50% of the suppressor tRNAs carry serine and not tyrosine, therefore it may be that the substitution of a serine for a tyrosine residue results in an inactive form of FtsW. This would explain why OV2 was unable to harbour *ftsW^{am}* successfully as the sole *ftsW* allele in the cell.

However the screening of plasmids, by restriction, for the presence of the wild-type allele on pUC19 proved fruitless, even in the presence of the P*lac* inducer IPTG. The complementation of the *ftsW 263* with pUCW18 was incomplete suggesting that the *PvuII/ SmaI* fragment cannot confer complete complementation of an *ftsW* mutant. If the chromosomal *ftsW* allele was replaced with the amber mutation, complementation with pUCW18 would be incomplete in a *sup^o* strain. The cells with the wild-type allele on the chromosome would have a faster growth rate and would out compete the *ftsW^{am}* mutants from the mixed culture. Later work presented in this thesis shows that *ftsW* clones with longer upstream sequences produce more

effective complementation of the *ftsW 263* allele. It may be that the amber mutation is lethal when on the chromosome because it is polar and effects the expression of downstream genes.

3.3.8. Attempts to construct an *ftsW* null strain by linear transformation.

An attempt was made to replace the chromosomal copy of *ftsW* with a null copy of *ftsW*. The copy of *ftsW* in pDDG1 was disrupted by the insertion of a selectable marker. The *Cla*I site in *ftsW* was restricted and the 3' ends blunted by using the Klenow fragment. A 1.4 kb Kan cassette was excised from pUC4K (Vierra and Messing, 1982) using *Hinc*II. This was ligated into the blunted *Cla*I site in pDDG1. The Kan cassette was cloned in both orientations relative to the surrounding genes and P*lac* promoter in pUC18. The clone where Kan was expressed in the same orientation as P*lac* was used for further work. This was pDWK1, 8.5 kb in size (Figure 3.3.5.).
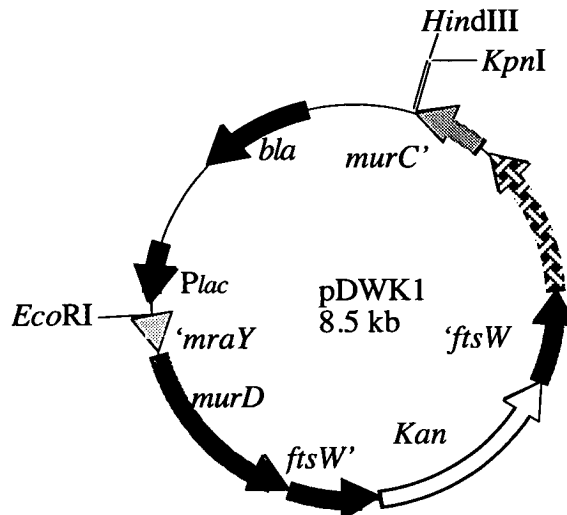
Figure 3.3.5. Construct pDWK1 used to supply an *ftsW* null allele for linear transformation.

This clone was used to provide a 5.8 kb *Eco*RI/ *Kpn*I, fragment containing '*mraY murD ftsW::Kan murG murC*', which was to be used for linear transformation of *recD* strains. Two *recD* strains were transformed with this fragment. In a *recD* strain the exonuclease activity of Exonuclease V is abolished, but not recombination activity (Biek and Cohen, 1986). Therefore linear DNA is more slowly degraded in the cell and homologous

recombination with the chromosome may occur by a double cross-over event between the transformed linear DNA and the chromosome (Figure 3.3.6.).

The *recD* strains used were DL307 (a gift from D. Leach) and D301 (Russel *et al.*, 1989). As the *ftsW*::Kan allele was expected to be lethal, a complementary copy of *ftsW* was present in a low copy number vector, pWAC1. This was constructed by cloning a 2.3 kb *Eco*RV/*Sma*I fragment, from pDDG1, into the *Eco*RV site in the Tet gene of pACYC184 (Chang and Cohen, 1978) to create pWAC1 (6.6 kb in size) in which *ftsW* is expressed from the promoter of the disrupted Tet gene (Figure 3.3.7.). DL307 and D301 were both transformed with pWAC1.
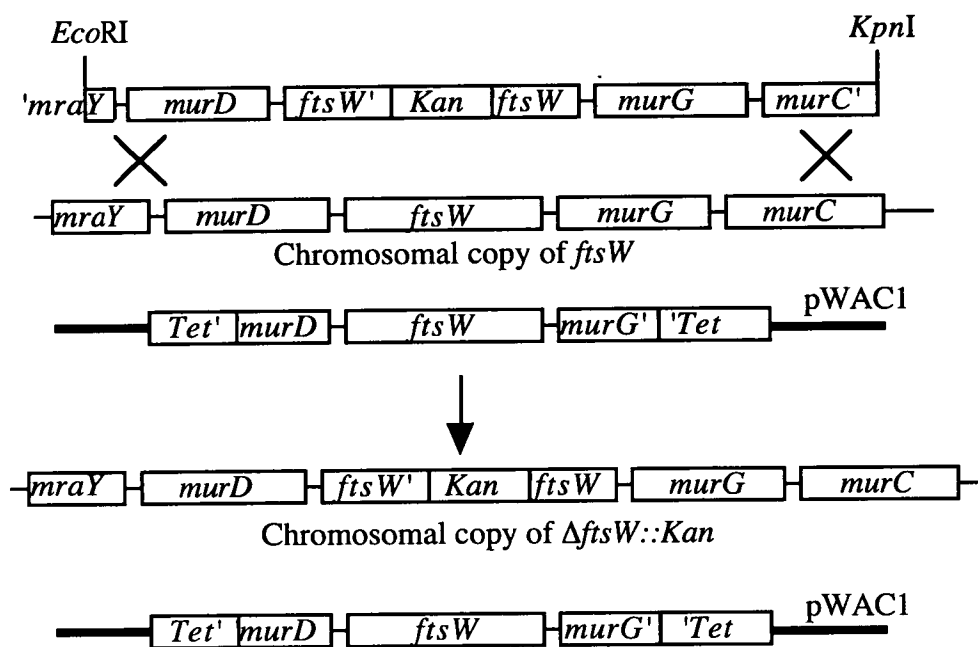
Figure 3.3.6. The insertion of an *ftsW* null allele into the chromosome by linear recombination. pWAC1 is also represented as the complementary copy.
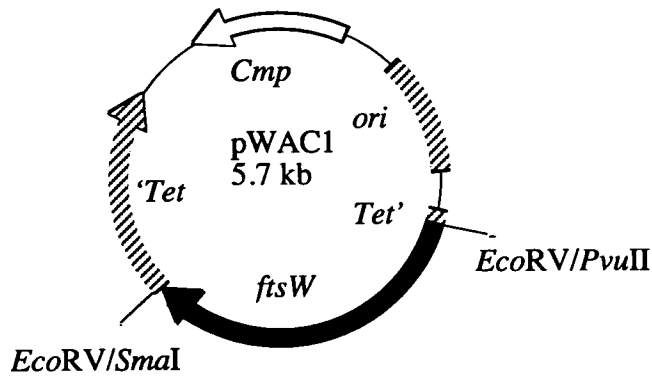
Figure 3.3.7. The plasmid pWAC1 used to complement linear transformants.

The 5.8 kb *Eco*RI/*Kpn*I fragment was excised from pDWK1. The method of transformation was electroporation. Transformants were selected for on LB Kan/Cmp agar with overnight incubation at 37°C. For a cell to become Kan^R there are three events which may occur. (1). The cell could be transformed with unrestricted pDWK1 as a contaminant, (2), there could be recombination between the complementing plasmid and the linear DNA or finally (3), recombination could take place between the chromomsome and the linear DNA (Figure 3.3.6.). To determine whether the Kan^R cells had been transformed with unrestricted pDWK1, the isolates were patched on LB Amp agar. Linear transformants are Amp^S, therefore Amp^R cells contain pDWK1. Plasmid DNA was extracted from the Kan^R/Amp^S isolates and examined by restriction analysis. This determines whether the linear DNA has replaced the plasmid based copy of *ftsW* or the chromosomal copy. The plasmid DNAs were restricted with *Eco*RI which would restrict pWAC1 at a unique site and has no recognition sites within the Kan^R cassette cloned in *ftsW*. Therefore if pWAC1 remains the same size, the Kan^R cassette must be located on the chromosome, presumably in *ftsW*. If the restriction of pWAC1 with *Eco*RI produces a linear band of 8 kb, then the *ftsW*::Kan disruption has replaced *ftsW* on pWAC1 by homologous recombination.

All of the Kan^R isolates were shown to either contain pDWK1 or have the Kan cassette inserted in pWAC1 (data not shown). There was no evidence of a chromosomal replacement. The high frequency of insertion of *ftsW*::Kan into pWAC1 probably was a consequence of the higher copy number of the plasmid based *ftsW* compared to the single chromosomal copy

of *ftsW*. To circumvent the problem of the complementing plasmid being transformed with the linear DNA, another approach was applied. This was to linear transform the *ftsW* interuption into a strain that was diploid for *ftsW*.

3.3.9. SHA5, a "partial diploid" strain.

While studying chromosome segregation and partition in spherical cells, K. Begg isolated a strain which appeared to be diploid for the *leu* region (Donachie *et al.*, 1995). That is the cells were *leu$^+$/leu*::Tn10. SHA5 is derived from KJB24, which is a derivative of W3110 carrying a *rodA* amber mutation linked to a Kan marker. W3110 is *sup^0* and therefore cannot suppress the *rodA* amber mutation, and hence KJB24, SHA5 and their derivatives grow as spherical shaped cells. SHA5 was shown to be Tet^R, due to *leu*::Tn10, whilst remaining *leu$^+$*. S. Addinall made a P1 lysate on SHA5 and transduced a haploid strain (W3110) to *leu$^-$/Tet^R* with P1[SHA5]. W3110 *leu*::Tn9 was also transduced with P1[SHA5] with selection for *leu$^+$/Cmp^S* cells. This also was achieved (S. Addinall, 1994). The *leu*::Tn9 marker was then transduced into SHA5 with selection for Tet^R/Cmp^R cells. Further characterisation of the Cmp^R/Tet^R isolates revealed that they were *leu$^-$*. Therefore SHA5 had two copies of the *leu* region. Other selectable phenotypic markers were then transduced into SHA5 to determine the size of the chromosomal duplication (Table 3.3.3.).

Gene	*thr*	*leu*	*murF*	*purE*	*nadA*	*recA*	*argA*	*argG*	*ilv*	*argE*	*rpoB*
Haploid (H) or Diploid (D)	D	D	D	D	H	H	H	H	H	H	D/H
Position (min)	0	2	2	12	14	58	60.5	69	85	89.5	90

Table 3.3.3. The ploidy of SHA5 showing presenence of diploid or haploid regions throughout the chromosome (Adapted from S. Addinall, 1994).

SHA5 was diploid for at least 14 minutes and therefore termed a "partial diploid". The chromosomal repeat in SHA5 appeared to be stable. Lin *et al.* (1984) reported the isolation of an *E. coli* strain which contained a

large chromosomal duplication encompassing *rrnB*. The phenomenon has also been recorded in S. *typhimurium* (Anderson and Roth, 1981).

As SHA5 was shown to be diploid for the two minute region, it was assumed that an *mra* region could be mutated without detriment to the cell as there is another wild-type allele of the gene present to complement. Therefore the "partial diploid" strain would be ideal for introducing null mutations into the two minute region. A Tet^S derivative of SHA5, SHA6, was a gift from S. Addinall. This was diploid for *leu*$^+$. To be a host for linear transformations SHA6 was transduced to *recD* with a P1 lysate made from D301 (Russel *et al.*, 1989). Selection was for Tet^R as the *recD* allele in D301 is disrupted with a minitet marker. The Tet^R *recD* partial diploid was DSB1.

3.3.10. Introduction of a disrupted *ftsW* allele into DSB1.

The introduction of the Kan^R disruption of *ftsW* into DSB1 was unsuitable as DSB1 already contains a Kan^R marker linked to the *rodA* amber allele. Therefore a different selectable marker was inserted into *ftsW* in pDDG1. The marker chosen was the Ω fragment (Prentki and Kirsch, 1984). This is a 2 kb cassette which confers Spc^R/Str^R. The fragment also contains two T4 transcriptional terminator sequence and translational stop codons in all three reading frames at each end of the cassette (Figure 3.3.8.; Prentki and Kirsch, 1984).

```
                A
         T        G
         C        A
           C G
           C G
           A T
           G C
           G C
           G C
```
5'. *Sma*I   Spc^R/Str^R N$_{44}$ ACTAATTAATTG CTTTTTT N$_{66}$ *Sma*I
3'
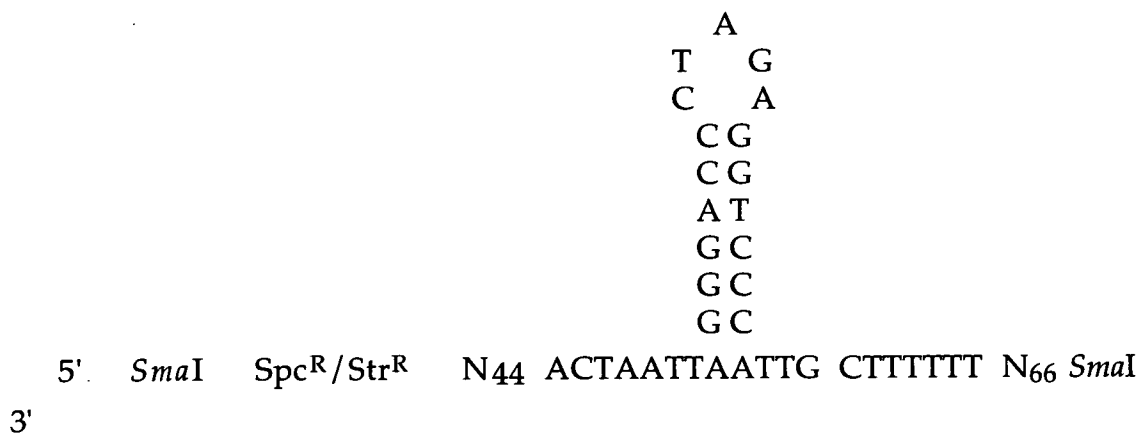
Figure 3.3.8. The T4 transcriptional terminator in the Ω cassette.

pDDG1 was restricted with *Cla*I and the 3' ends filled in using the Klenow fragment. The 2 kb Ω cassette was excised from pHP45Ω (Prentki

and Kirsch, 1984) with *Sma*I and cloned into the blunt ended *Cla*I site of pDDG1. This made pDWΩ1, 9.1 kb in size (Figure 3.3.9.).

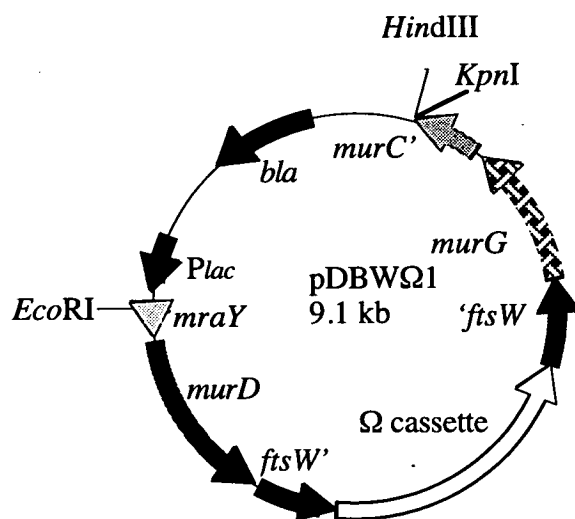**Figure 3.3.9.** pDWΩ1. A plasmid based disruption of *ftsW*.

3.3.11. The linear transformation of DSB1.

As DSB1 contains two wild-type *ftsW* alleles, it seemed possible to mutate one without affecting the cell. The second, wild-type copy complementing the null allele. The DNA for the linear transformation of DSB1 was excised from pDWΩ1, again restricting with *Eco*RI/*Kpn*I, producing a 6.4 kb fragment. DSB1 was transformed with the 6.4 kb fragment by electroporation. Linear transformants were selected for on LB Kan/Tet/Spc agar with incubation at 37°C for two days. Spc^R isolates were then cultured on LB Amp to ensure that Spc^R was not due to the transformation of unrestricted pDWΩ1. Five Spc^R/Amp^S isolates were recovered. This suggested that the *ftsW*::Ω allele had been integrated successfully into the chromosome. These were then screened by P1 transduction before one was chosen for further work and was named DDWΩ1.

The location of the *ftsW*::Ω allele in DDWΩ1 was determined by a series of P1 transductions. DDWΩ1 cells were transduced with P1[*leu*::Tn9 *ftsW$^+$*] and Cmp^R transductants were recovered on LB Cmp agar. Cmp^R transductants were then screened for Spc^S on LB Cmp/Spc. From screening 100 Cmp^R transductants, 67 were Cmp^R/Spc^R. That is, 33% of the

transductants had *ftsW+* cotransduced with *leu*::Tn9 and therefore lost the *ftsW*::Ω allele. The presence of the chromosomal repeat means that the *leu*::Tn9 introduced by P1[*leu*::Tn9 *ftsW+*] can insert in either of the duplicated regions (Figure 3.3.10.). In a haploid strain, *ftsW* is cotransduced with *leu* with a frequency of ~70%. In the diploid strain, DDWΩ1, the cotransduction frequency was 33%. Correcting this for haploidy would give a frequency of 66%. The reciprocal transduction was carried out. A P1 lysate prepared on DDWΩ1 was then used to transduce DSB1 (*leu*::Tn9) to Spc^R. The Spc^R isolates were then screened for Spc^R/Cmp^R on LB Spc/Cmp agar. From a sample of 100 Spc^R transductants, 32 were Cmp^S. Therefore the *leu*::Tn9 *ftsW+* region of DSB1 (*leu*::Tn9) was replaced by *leu+ ftsW*::Ω with a cotransduction frequency of 32%. Again this is half the expected frequency for a haploid due to the diploidy of the 2 minute region. It does show however that the *ftsW*: Ω allele is closely linked to *leu* in DDWΩ1.

Figure 3.3.10. The schematic diagrams **i.** to **v.** represent the chromosome of the partial diploid strain DSB1 showing the duplicated regions as thicker lines. Both contain *leu* and *ftsW*. **i.** The linear recombination event where *ftsW*::Ω is introduced into the chromosome at the expense of one *ftsW* allele. The *ftsW/ftsW*::Ω strain DDWΩ1 is depicted in **ii.** **iii.** to **v.** show the events which may occur if DDWΩ1 is transduced with P1[*leu*::Tn9 *ftsW*]. In **iii.** the *ftsW*::Ω allele is replaced by DNA linked to *leu*::Tn9. The cotransduction frequency of *leu*::Tn9 with *ftsW*+ is ~35% in the diploid strain. This gives a Cmp^R/Spc^S phenotype. In **iv.** the insertion of *leu*::Tn9 does not remove the *ftsW*::Ω allele although it is inserted in the same *mra* region, this occurs with a frequency of ~15% in the partial diploid, with the cells retaining a Cmp^R/Spc^R phenotype. The alternative to **iii.** and **iv.** is that the *leu*::Tn9 inserts in the other duplicated region, **v.** This will not remove *ftsW*::Ω as it is located in the other duplication. The frequency of *leu*::Tn9 transducing into the other duplication is 50% in a partial diploid strain.
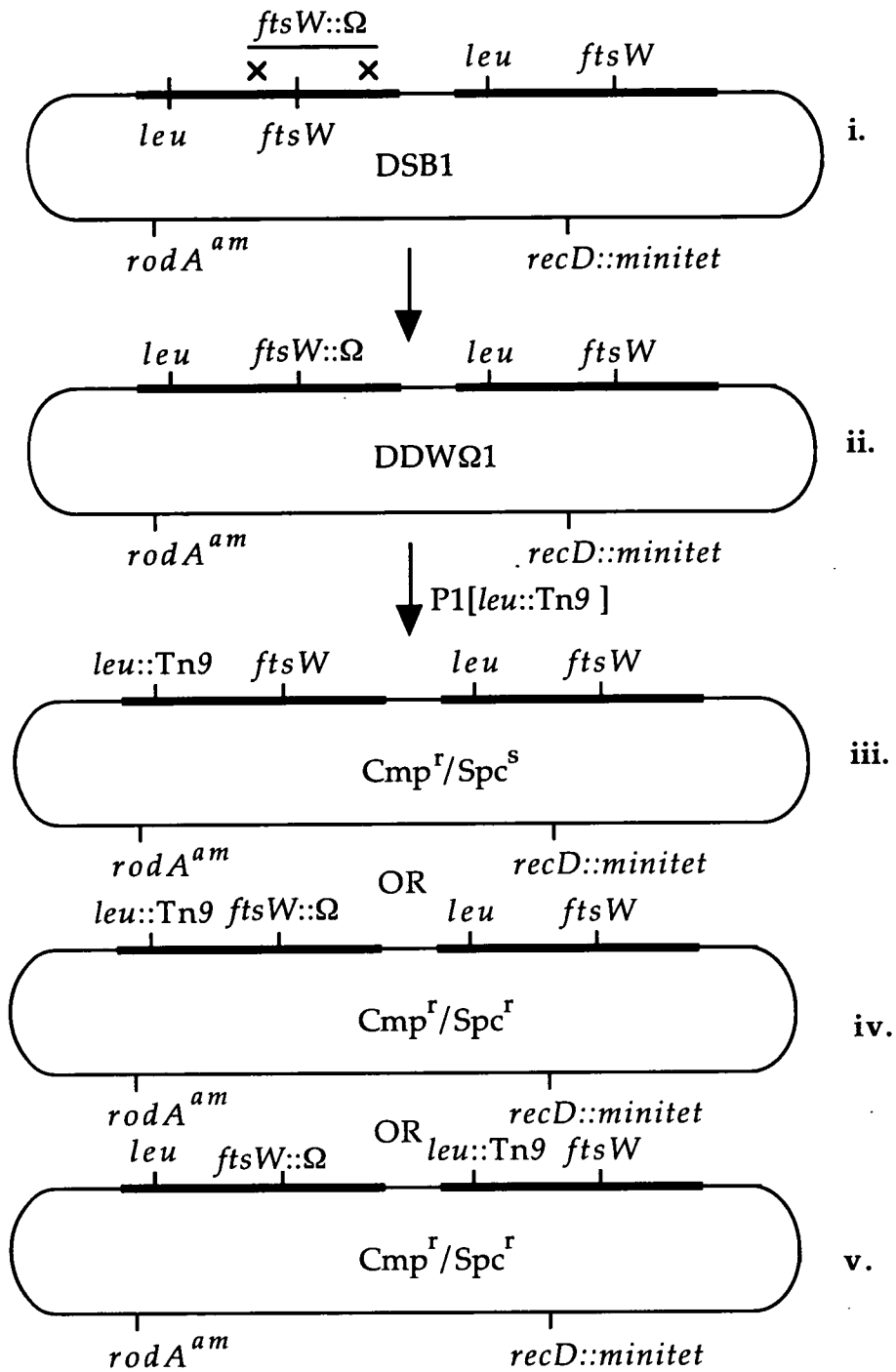
Figure 3.3.10. The linear transformation of DSB1 with *fts*W::Ω.

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3.3.12. Southern blot analysis of DDWΩ1.

The previous section showed that *ftsW*::Ω was linked to *leu*. In this experiment the presence of the *ftsW*Ω fragment in the diploid strain DDWΩ1 was shown by Southern analysis of the genomic DNA. Samples of genomic DNA from DSB1 and DDWΩ1 were prepared as described in Chapter 2. Both DNAs were restricted with *Mlu*I. This produces a fragment of 2.4 kb which contains *ftsW*. By restricting DDWΩ1 chromosomal DNA with *Mlu*I, two fragments containing *ftsW* will be produced. These are 2.4 kb, where the *ftsW* wild-type allele is located, and a larger fragment of 4.4 kb which contains the mutated *ftsW*::Ω allele. The wild-type strain DSB1 should only produce the 2.4 kb *Mlu*I fragment. The digested DNAs were electrophoresed through agarose and blotted onto a nylon membrane (Boehringer Mannheim). The filter was first probed for *ftsW* using a [32P]dCTP labelled *Pvu*II/*Sma*I *ftsW* probe (Figure 3.3.11.). Exposure of the filter revealed a single band of 2.4 kb in the lane containing *Mlu*I restricted DSB1 DNA. The *Mlu*I cut DNA from DDWΩ1 produced two bands of 2.4 kb and 4.4 kb. These are the *ftsW* wild-type and the *ftsW*::Ω alleles (Figure 3.3.11.). The control DNAs used were pDEG1 and pDWΩ1. When restricted with *Mlu*I, pDEG1 produces a band of 2.4 kb containing the *ftsW* wild-type DNA. The restricted pDWΩ1 DNA produces a fragment corresponding to 4.4 kb in size. These can be seen in Figure 3.3.11. for pDEG1 and in Figures 3.3.11. and 12. for pDWΩ1. This data indicates that the Ω fragment is located within *ftsW* and in the chromosome. To show that the Ω fragment was located in the heavier 4.4 kb band, a labelled probe was made using the 2 kb Ω cassette. The filter was stripped of the *ftsW* probe and hybridized with the Ω probe. This only revealed the 4.4 kb band (Figure 3.3.12.). As expected the smaller 2.4 kb band corresponding to the *ftsW* wild-type fragment in DSB1, pDEG1 and DDWΩ1 was not visible. Therefore DDWΩ1 does contain a mutated *ftsW* allele and a wild-type *ftsW* allele. To study the effects of this on cell growth, the mutant allele was transferred to a haploid strain.
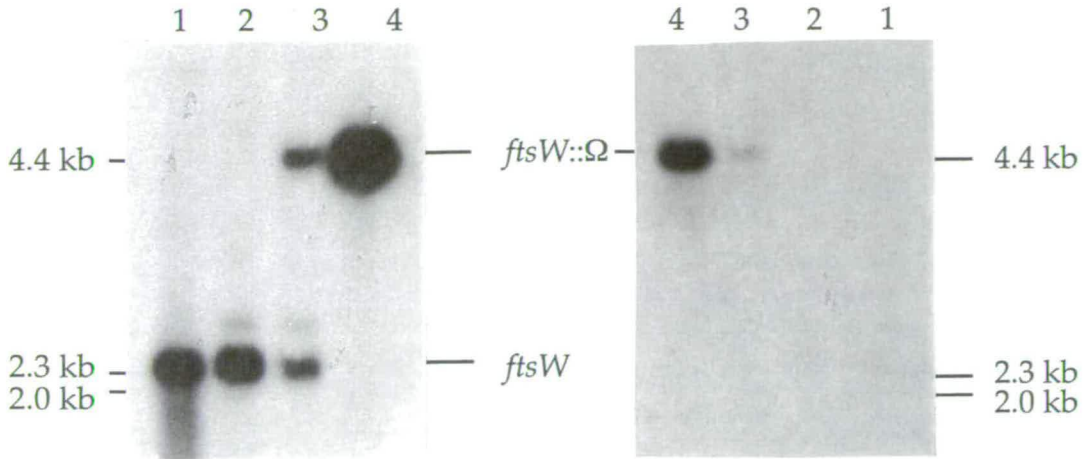
Figure 3.3.11.   Figure 3.3.12.

Figures 3.3.11. Key: 1. pDDG1, 2. DSB1, 3. DDWΩ and 4. pDWΩ1.
All the DNAs were restricted with *Mlu*I. Figure 3.3.11. on the left shows the
filter probed with *fts*W. The probing of genomic DNA, from both the partial
diploid strains, with *fts*W revealed that for DSB1 (the *fts*W wild-type strain,
lane 2) there was only one band lit which was at 2.4 kb. This corresponds to
exactly the same size as *fts*W control DNA from pDDG1also restricted with
*Mlu*I (lane1). In lane 3 the *fts*W mutant strain produces two bands when
probed with *fts*W. One band is 2.4 kb in size which is the wild-type *fts*W
*Mlu*I fragment. There is another at 4.4 kb. This is the *fts*W::Ω allele. This is
the same size as the *Mlu*I fragment produced from the *fts*WΩ control DNA,
pDWΩ1 (lane 4).

Figure 3.3.12. Key: 1. pDDG1, 2. DSB1, 3. DDWΩ and 4. pDWΩ1. The
probing of the same filter with the Ω fragment shows that both the wild-type
plasmid and DSB1 DNA do not hybridize with the Ω cassette. Both the
mutant control, pDDWΩ1 (4) and the diploid DDWΩ1 (3) contain a larger
band at 4.4 kb. This is the 2.4 kb *fts*W *Mlu*I fragment also containing the 2 kb
Ω cassette. The 2.4 kb bands representing *fts*W wild-type DNA highlighted
in Figure 3.3.11 are absent. Therefore the partial diploid mutant strain
contains both the wild-type and mutant alleles of *fts*W.

3.3.13. Introduction of *ftsW*::Ω into a haploid strain.

C600T was transduced with a P1 lysate grown on DDWΩ1 and *leu*[+] transductants selected for on VB minimal agar lacking Leucine. *leu*[+] isolates were patched on LB Tet agar and LB Spc agar. All the *leu*[+] transductants were Tet[S] indicating the *leu*::Tn*10* marker had been replaced. None of the *leu*[+] isolates were Spc[R] suggesting that *ftsW* is an essential gene. In order to introduce *ftsW*Ω into a haploid strain, the following strategy was adopted to monitor the effects of FtsW depletion in a haploid *ftsW*::Ω strain. A plasmid with a temperature sensitive replicon was used complement the null mutation once it had been introduced onto the chromosome. Later curing of the complementing plasmid would allow the effects of depletion of FtsW in growing cells to be monitored. The plasmid used was pMAK705 (Hamilton *et al.*, 1986). The 4.4 kb *Eco*RI fragment from pDEG2 was cloned into pMAK705 partially restricted with *Eco*RI. The clone was pMDG1 (Figure 3.3.13.).
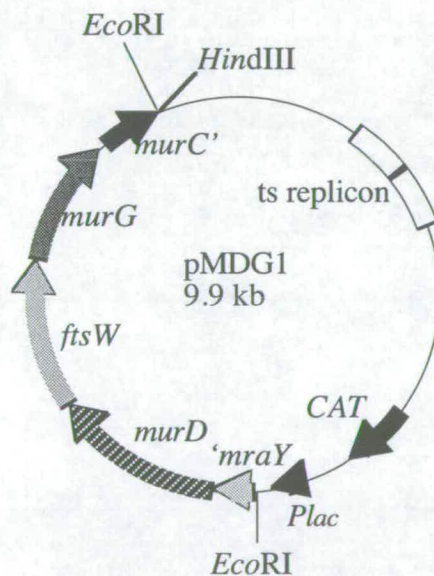
Figure 3.3.13. The vector pMDG1 with a ts origin of replication. This was used to complement *ftsW*::Ω at 30˚C.

The cloned insert is such that *ftsW* is expressed from P*lac*, located upstream of the polylinker in the vector. At 30°C the plasmid is stable. However, at 42°C replication of the plasmid is inhibited and the plasmid is lost by dilution from the culture. Therefore complementation of *ftsW* will be

lost from the null strain when incubated at 42°C and the effects of FtsW depletion from the cells can be monitored. C600T was transformed with pMDG1 and transformants isolated on LB Cmp incubated at 30°C overnight.

A culture of C600T/pMDG1 was transduced with a P1 lysate made from DDWΩ1. *leu*$^+$ cells were isolated on fully supplemented leucine-free VB Cmp agar incubated at 30°C. The isolates were then screened for Spc^R on LB Cmp/Spc agar incubated at 30°C. The cotransduction frequency of *leu* with *ftsW*::Ω was 35%, this was as expected since a P1 lysate from the diploid strain will have phage particles carrying either of the two *mra* loci in the partial diploid. That is *leu* $^+$*ftsW* and *leu* $^+$ *ftsW*::Ω. It was presumed that *ftsW*::Ω had replaced *ftsW* on the chromosome and was therefore complemented by pMDG1. The Spc^R strain was called DBHΩ1.

An overnight culture of DBHΩ1/pMDG1 grown in LB Spc/Cmp was used to inoculate LB Spc and this culture was incubated at 30°C with the OD$_{540}$ measured at intervals until the cells reached a steady growth rate (Figure 3.3.14.). This culture was then used for a shift in temperature to 42°C. As a control, cells were also cultured at 30°C. Culture density measurements were taken regularly and dilutions made when appropriate. The OD$_{540}$ of the two cultures was plotted against the sampling times and compared (Figure 3.3.14.).
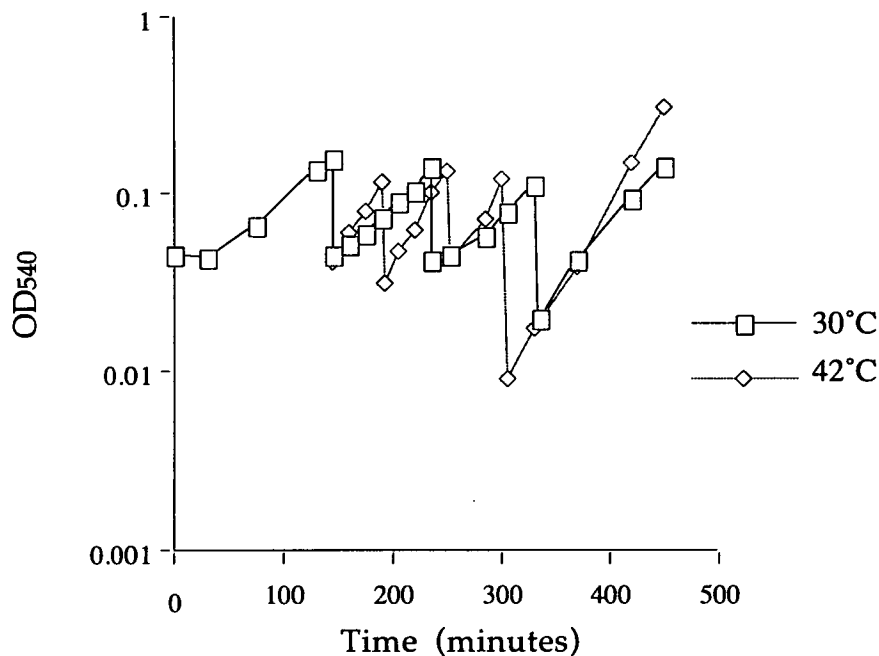
Figure 3.3.14. The effects of a temperature shift on the complementation of *ftsW*::Ω with a temperature sensitive plasmid, pMDG1.

The data reveal that the temperature shift to 42°C did not affect the cells in the expected manner and instead of the growth rate of DBHΩ1 slowing with the loss of the complementing plasmid, it increased. This would be expected if the strain was wild-type as growth of a culture would be faster at 42°C than at 30°C. This result could mean either that the plasmid was no longer ts or that the *ftsW*::Ω allele had not replaced the chromosomal copy of *ftsW*. The cotransduction frequency of *ftsW*::Ω with *leu*+ suggested that *ftsW* had been replaced and therefore the plasmid was thought to be no longer ts for replication. 10^{-7} dilutions of DBHΩ1/pMDG1 were plated onto LB Cmp and incubated at 30°C and 42°C. If the plasmid was ts then the number of Cmp^R colonies isolated at 42°C would be much less than at 30°C. There will be a proportion of cells in the overnight culture which are chromosome/plasmid cointegrants and therefore will grow on Cmp media at 42°C. The results from the testing of temperature sensitivity of pMDG1 showed that pMDG1 was ts with the dilutions incubated at 42°C having only a few colonies, while at 30°C ~700 colonies were isolated.

pMDG1 was isolated from DBHΩ1/pMDG1 and examined by restriction analysis with *Hind*III. The size of pMDG1 linearized with *Hind*III is 9.9 kb. The size of pMDG1 isolated from DBHΩ1 was not 9.9 kb when

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restricted with *Hin*dIII. Instead of a linear band of 9.9 kb, there were three fragments of sizes 2 kb, 4.4 kb and 7.5 kb. This meant that the *ftsW*::Ω allele had been inserted in pMDG1 as there are *Hin*dIII sites in the Ω fragment cloned into *ftsW*. This was also shown by transforming C600 with part of the DNA sample isolated from DBHΩ1/pMDG. Transformants were selected on LB Cmp and LB Spc agars at 30°C. Transformants were isolated on both agars; significantly the number of transformants per plate was similar. Therefore pMDG1 now contained *ftsW*::Ω. C600 was transformed with other plasmids containing *ftsW*. These were pDEG1, pDDG1 and pUCW18. The transformants were then transduced with the P1 lysate made from DDWΩ1 and Spc^R transductants isolated on LB Amp/Spc agar. Spc^R isolates were recovered from each transformant species transduced. Plasmid DNA was isolated from ten Spc^R colonies from each type of transformant and restricted with *Hin*dIII. Digestion of pDEG1, pDDG1 and pUCW18 with *Hin*dIII will produce linear bands of 11.1 kb, 7.1 kb and 4.1 kb respectively. After agarose electrophoresis of the *Hin*dIII digested plasmid DNAs, bands of the predicted sizes for pDEG1, pDDG1 and pUCW18 were observed. However in every digest there were other bands which corresponded in size to fragments that would be produced if the Ω fragment was inserted in *ftsW*. A band at 2 kb was seen in every lane, corresponding to *Hin*dIII restricted Ω fragment. Therefore upon P1 transduction with a lysate containing *ftsW*::Ω, the only cells which can survive on LB Spc are those which have replaced the null allele on the chromosome with the wild-type copy of *ftsW* based on the plasmid. This can occur by integration of the plasmid into the chromosome via a single cross-over event and the subsequent resolution of the plasmid from the chromosome in which *ftsW*::Ω is replaced with the wild-type plasmid copy of *ftsW*.

Dai and Lutkenhaus (1991) made an *ftsZ* deletion in *E.coli* Complementation studies using λ phages revealed that *ftsZ* null phenotype cannot be complemented by *ftsZ*$^+$ containing phages which only contain upstream DNA as far as *murG*. They predicted that 30-40% of the total FtsZ protein is made from a large transcript which begins upstream of *murG*. The Ω fragment used to disrupt *ftsW* has a T4 transcriptional terminator (Figure 3.3.8.). This may prevent the formation of the *ftsZ* transcript. The effect of the Ω fragment on the levels of FtsZ being produced from cells may be why *ftsW*::Ω could not be isolated on the chromosome, even in the presence of complementary DNA. Alternatively the expression of other downstream

genes may be affected. To investigate this a series of λ lysogens were constructed to determine if *ftsW*::Ω could remain stable on the chromosome. The λ phages used to make the lysogens were a gift from K. Begg (Table 3.3.4.).

λPhage	Genes encoded
λΔD	*murG*
λ*ddl*	*mur G murC ddlB ftsQ*
λΔE	*ddlB ftsQ ftsA ftsZ envA*
λΔB	*ftsZ envA*
λFH16	*ftsQ ftsA*
λGH200	*envA*
λ16-2	*murG murC ddlB ftsQ ftsA ftsZ*

Table 3.3.4. λ Phage clones and the genes located on the cloned inserts.

These contained a selection of genes downstream from *ftsW*. The host strain for infection with the λ phages was C600/pWAC1. Each of the C600/pWAC1 lysogens was then transduced with a P1 lysate grown on DDWΩ1. Transductants were selected for on LB Spc/Cmp agar at 37°C. Only two of the seven lysogens produced Spc^R transductants. These were the λ16-2 and λΔE lysogens. Significantly these contain *ftsZ* and large portions of the upstream DNA (Table 3.3.4.). The other λ phage containing *ftsZ*, λ ΔB, did not complement, presumably due to a lower level of expression of *ftsZ* as it does not contain a large region of upstream DNA from the start of *ftsZ*. Plasmid DNA was extracted from Spc^R isolates and digested with *Hind*III. This restricts pWAC1 only once. If *ftsW*::Ω has been inserted then the plasmid will be cut at three sites. Analysis of plasmid digests revealed that pWAC1 was cut once with *Hind*III. This indicated that *ftsW*::Ω was on the chromosome.

Therefore λ16-2 and λΔE allow the insertion of *ftsW*::Ω to remain stable in a haploid strain. A P1 lysate was made from C600 *ftsW*::Ω (λΔE)/pWAC1. C600 (λΔE)/pDDG1 (see Figure 3.2.4.) was then transduced with P1[*ftsW*::Ω] with selection for Spc^R transductants on LB Spc. Spc^R colonies were isolated and the plasmid DNAs checked for the presence of *ftsW*::Ω by restriction analysis with *Hind*III. The plasmid DNAs were cut

three times with *Hind*III and therefore *ftsW*::Ω had once more replaced the plasmid-based wild-type copy. Therefore only λ16-2 and λΔE lysogens transformed with pWAC1 could maintain a stable copy of *ftsW*::Ω on the chromosome. This may be due to the copy number of the plasmid clones. The pUC18 based clones are in high copy number. pWAC1 is constructed from pACYC184 which has a much lower copy number. It contains a different origin of replication from the pUC18 ColE1 and the pMAK705 pSC101 replicons.

3.3.14. Construction of *ftsW* clones in a vector with an inducible promoter, pBAD18.

As *ftsW*::Ω was stable in the chromosome in C600 (λΔE)/pWAC1 it was decided to introduce *ftsW* clones in a vector where the expression of *ftsW* could be regulated. By introducing a *recA* mutation, plasmid cointegration with the chromosome could be prevented and therefore *ftsW*::Ω would remain stable in the chromosome. The new *ftsW* clones could be introduced by transformation and pWAC1 cured from cells. The vector pBAD18 constructed by Guzman *et al.* (1995) was chosen for the inducible promoter P_{BAD} (Figure 3.3.15.).
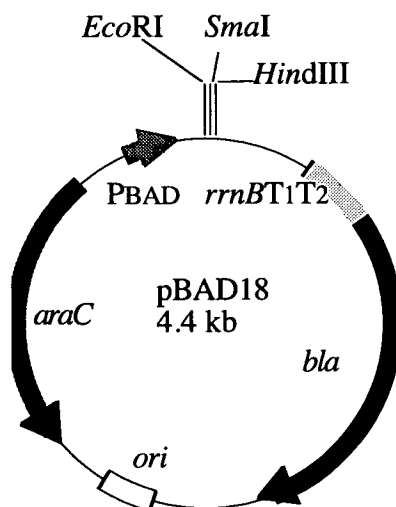
Figure 3.3.15. The plasmid pBAD18.

The plasmid replicon originates from ColE1 and it contains the P_{BAD} promoter from the *ara*BAD operon. The vector also encodes *araC* which is

involved in negative and positive regulation of P$_{BAD}$. In the presence of arabinose, transcription from P$_{BAD}$ is induced and without arabinose transcription is repressed. The addition of glucose further represses expression from P$_{BAD}$. P$_{BAD}$ promoter based vectors have already been used to study null mutations (eg *ftsQ*, Carson *et al.*, 1991; *ftsL*, Guzman *et al.*, 1992). Therefore a series of pBAD18 constructs were made containing *ftsW* with varying amounts of the upstream sequence and tested for their ability to complement the *ftsW*::Ω allele.

Four clones of *ftsW* were made in pBAD18. These are shown in Figure 3.3.16. The 1.5 kb *PvuII* / *SmaI* fragment was cut from pDDG1 and cloned in both orientations in *SmaI* cut pBAD18. This made the clones pBAW1 and 2, both 5.9 kb in size. A second *ftsW* clone was made by cloning the *BglII* / *SmaI* fragment from pDDG1 into the *Bam*HI/*HincII* of the pUC18 polylinker to make pBUBW1, 5.1 kb in size. This was then cut with *HindIII* / *EcoRI* and the 2.4 kb fragment containing *ftsW* was subcloned into pBAD18, also restricted with *HindIII* / *EcoRI* to create pBBW1, 6.8 kb in size (Figure 3.3.16.). The final pBAD18 clone was constructed by cloning the 4.4 kb *EcoRI*/*HindIII* fragment from pDDG1 into pBAD18 to make pBADG1, this was 8.8 kb. By restricting pBADG1 with *SmaI*, the 3' end of *murG* was removed. The 7.2 kb fragment, containing '*mraY murD ftsW murG*' and pBAD18, was re-ligated to make pBADW1 (Figure 3.3.16.)
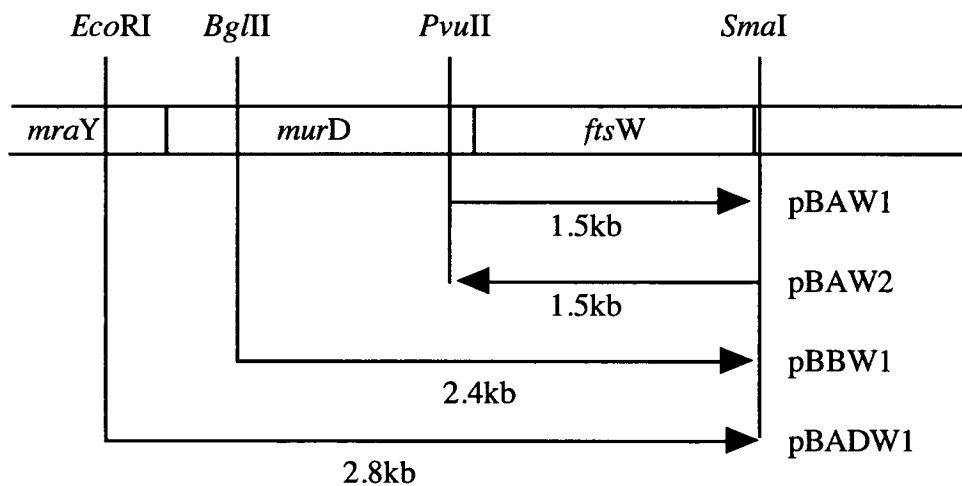

Figure 3.3.16. The various *ftsW* clones in pBAD18. Arrows indicate direction of transcription from P$_{BAD}$.

Before attempting to cure pWAC1 from C600 ($\lambda\Delta$E) *ftsW::*Ω (DBHΩ2) by using the pBAD18 clones, the *recA* mutation was introduced to DBHΩ2/pWAC1 by Hfr mating with the *recA56* mutant JC10-240. This will inhibit the replacement of the chromosomal copy of *ftsW::*Ω with the wild-type alleles of *ftsW* present in the pBAD18 constructs. The selective marker was Tet[R] and the conjugated cells were plated on LB Tet/Spc to select for potential *recA56* mutants. Tet[R] isolates were then streaked on LB Tet/Spc and exposed to varying times of UV irradiation (see Chapter 2). The *recA56* mutants are unable to repair UV damaged DNA and therefore a UV sensitive isolate was chosen, this was known as DBHΩ3 [*ftsW::*Ω *recA* ($\lambda\Delta$E)]. DBHΩ3/pWAC1 was then transformed with the pBAD18 *ftsW* plasmids, with selection for Amp[R] on LB Amp agar.

To cure pWAC1 from DBHΩ3, pARα was transformed into the DBHΩ3/pWAC1/pBAD18 clone transformants. pARα has the same origin of replication as pWAC1 and a selective marker for Kan[R]. If selection was for Kan[R] and Amp[R] then both pARα and the pBAD18 clones would be retained by DBHΩ3. If the pBAD18 *ftsW* clones could complement *ftsW::*Ω then pWAC1 would be cured from the cells.

The DBHΩ3/pARα/pWAC1/pBAD18 *ftsW* plasmids were streaked on LB Amp/Kan/Spc agar supplemented with 1% arabinose and incubated at 37°C. Single colonies from each type of pBAD18 *ftsW* transformant were streaked onto fresh LB Amp/Kan/Spc/ara agar daily. DBHΩ3/pARα transformants of pBAW2, pBBW1 and pBADW1 grew normally. However the pBAW1 transformants grew poorly on this medium (Figures 3.3.17.). If grown on LB Amp/Kan/Spc 1% glucose the pBAW1 transformants grew normally (Figure 3.3.18.). C600 ($\lambda\Delta$E)/pBAW1 grew on both arabinose and glucose based media and therefore inhibition of growth was not solely caused by the expression of FtsW from P$_{BAD}$. After 4 days of subculturing the cells on LB Spc/ Tet/ Amp 1% arabinose agar, single colonies were patched on LB Amp/Cmp/Spc/1% ara arabinose agar. The selection for Cmp would reveal if pWAC1 had been cured. DBHΩ3/pARα transformed with pBBW1 or pBADW1 were now Cmp[S]. Therefore pWAC1 had been cured from these cells. Transformants containing pBAW1 and pBAW2 remained Cmp[R], thus retaining pWAC1.
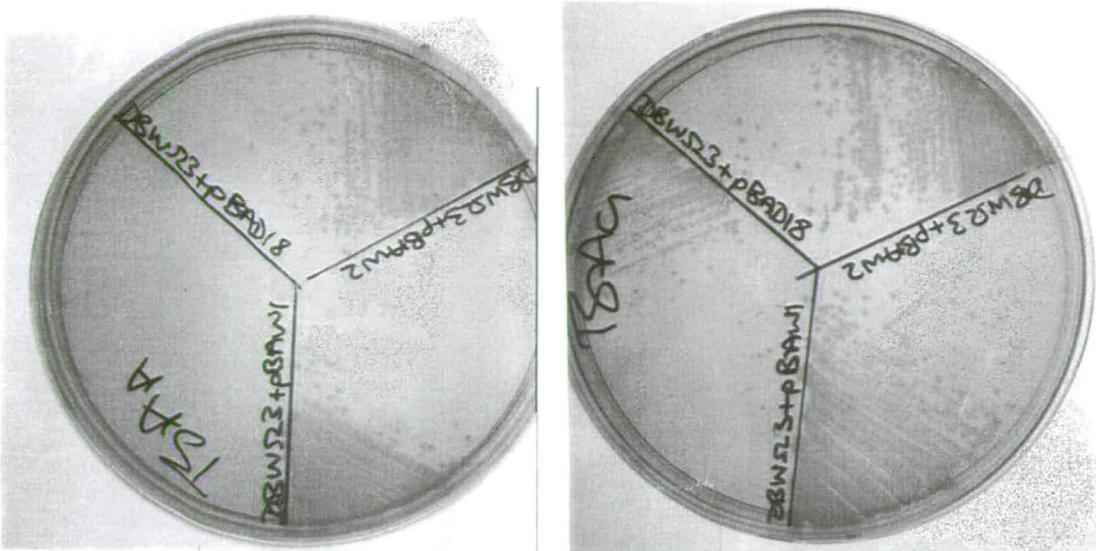
Figures 3.3.17. and 18. The effects of arabinose induction of pBAW1 in DBHΩ3/pWAC1/pARα/pBAW1 when plated on LB Spc/Amp/Tet agar supplemented with 1% arabinose (left) or 1% glucose (right). Also streaked on the same plates was DBHΩ3/pWAC1/pARα containing either pBAW2 and pBAD18. On the plate containing arabinose , the pBAW1 transformants were restricted in growth. The transformants of the other two plasmid species grew unhindered. On the glucose containing agar all three types of transformant grew.

As DBHΩ3/pARα transformants of pBBW1 and pBADG1 had lost pWAC1 they could therefore complement the *ftsW*::Ω allele when the medium was supplemented with 1% arabinose. The strains were then cured of pARα by daily subculturing on LB Amp/Spc/Tet supplemented with 1% arabinose. To determine if the clones could complement *ftsW*::Ω without expression from P$_{BAD}$. Both DBHΩ3/pBBW1 and DBHΩ3/pBADW1 were streaked on LB Amp/Spc supplemented with 1% glucose with incubation overnight at 37°C. Transformants containing pBADW1 grew on the glucose based medium. Cells containing pBBW1 did not grow on the glucose based media. Microscopic examination of the cells from the glucose plate revealed them to be long smooth filaments, many of which had lysed. This was similar to the phenotypes described previously for *ftsW*[ts] mutants (Ishino *et al.*,1989; Ikeda *et al.*; 1989; Khattar *et al.*, 1994). By comparison, DBHΩ3/pBBW1 grown on arabinose showed a mixed phenotype of long and short rods. The morphology of DBHΩ3/pBADW1 grown in the presence of either arabinose or glucose was the same, being a mixture of short and long rods.

The ability of pBADW1 to complement *ftsW*::Ω when expression from P$_{BAD}$ is repressed with glucose indicates that the upstream sequence from *ftsW* in this clone contain promoter activity which can express FtsW at levels sufficient to complement *ftsW*::Ω. The clone pBBW1 cannot complement *ftsW*::Ω when P$_{BAD}$ is repressed by glucose. The difference between pBBW1 and pBADW1 is the amount of DNA upstream from *ftsW* present in the clones (Figure 3.3.16.). pBADW1 contains an extra 424 bp of DNA from the *Bgl*II site in *murD* to the *Eco*RI site in *mraY*. Therefore this region contains a promoter region which can express *ftsW* in the absence of expression from P$_{BAD}$.

3.3.15. Morphological effects of the FtsW depletion.

Only one pBAD18 clone, pBBW1 (Figure 3.3.16.) was dependent on arabinose for the expression of *ftsW*. DBHΩ3/pBBW1 was therefore chosen to study the effects of FtsW depletion in a growing culture of cells. An overnight culture of cells grown in LB Tet/Spc/Amp/1% arabinose was used as an inoculum for LB Tet/Spc containing either 1% arabinose or 1% glucose and incubated at 37°C with shaking. The culture density was measured regularly at 540 nm. The cultures were diluted when appropriate. The optical densities were plotted against time and compared with each other, Figure 3.3.19.

The effects of glucose repression on P$_{BAD}$ did not effect the growth rate of the culture until 200 minutes. During the sampling cells were observed microscopically. The cells growing in the LB glucose culture formed long filaments whilst the LB arabinose culture was formed mainly of rod shaped cells with occasional filaments. At this point the growth rates were still the same. As the growth rate of the LB glucose culture started to lag the morphology of the cells was still filamentous although many had lysed. The smooth and aseptate filamentous phenotype exhibited by the *ftsW*::Ω allele indicates that *ftsW* may play an early role in division. Other cell division mutants from the two minute region form slight invaginations at potential division sites. Both *ftsL* and *ftsZ* are implicated in an early role in division as they form aseptate filaments (Guzman *et al.*, 1992; Dai and Lutkenhaus, 1991). It appears from this observation that *ftsW* may also be involved at an early stage in division. The filamentation of the LB glucose culture suggests that FtsW is not recycled by the cells or that there may be a

minimum concentration below which the cells may not divide. Therefore *ftsW* may be required to be made in every cell cycle (as for FtsA; Donachie *et al.*, 1976).
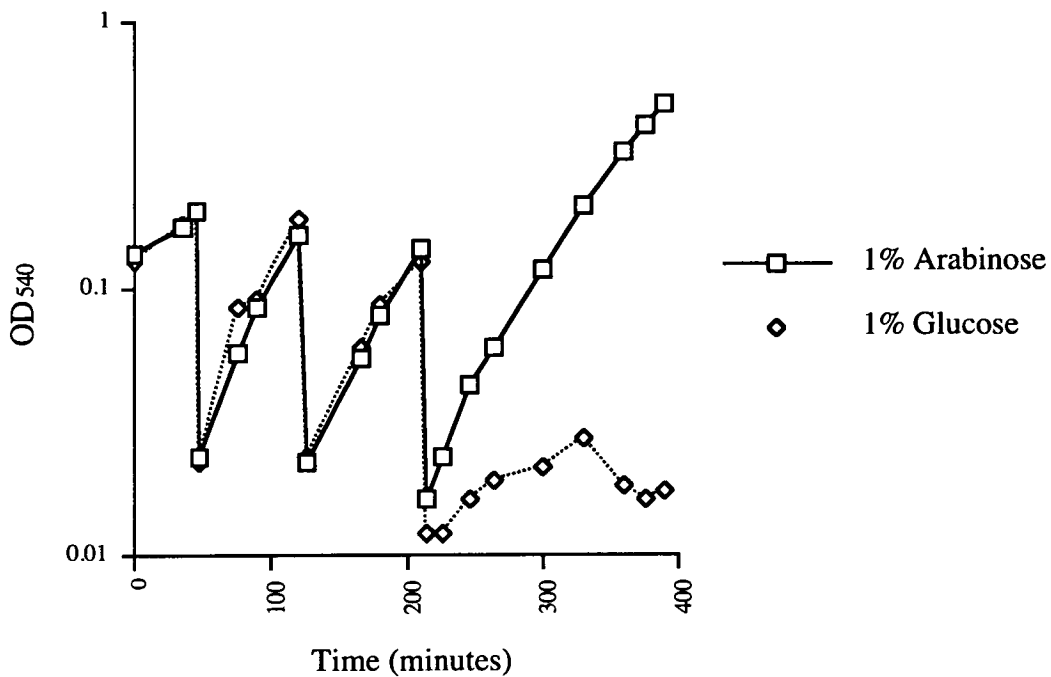

Figure 3.3.19. Growth of DBHΩ3/pBBW1 in LB media with either 1% arabinose or 1% glucose. After 180 minutes of growth, the growth rate of the LB glucose grown cells decreased rapidly for a further 120 minutes before the cells in the culture lysed and the culture density dropped. The culture grown in LB containing arabinose was not affected and grew at a constant rate when in log phase growth.

3.3.16. The effect of FtsW depletion on growth rate and cell size.

The previous experiment had shown that cells ceased to divide after being cultured in glucose. By monitoring the cell size of the mutants grown in LB supplemented with either arabinose or glucose, a more accurate measurement of the effects of FtsW depletion on growing cells could be made. This was achieved by counting and measuring the cell sizes in a Coulter counter and Channelyser. As a control, C600 [*recA* 56 *ftsW* $^+$ (λΔE)]/pBBW1 (DSB4) was grown under the same conditions. Overnight cultures of the mutant and the control strains were used to inoculate filtered LB Tet/Spc containing 1% arabinose. The cultures were incubated at 37°C

with shaking. The OD of the cultures were read at 540 nm. At each sampling for the OD_{540} measurements, a 0.5 millilitre aliquot of cells was fixed in 0.5 millilitres of fixing solution and stored at 4°C. Once the cells had reached a steady growth rate in arabinose based LB, cells from 1 millilitre aliquots of culture were pelletted. The pellets were then washed in 1 millilitre of LB arabinose or LB glucose, prewarmed to 37°C. The aim of this step was to remove arabinose from the inoculum to be used for the glucose grown cells. Filtered LB Tet/Spc/1% glucose, prewarmed to 37°C, was inoculated with the LB glucose washed cells. LB arabinose Tet/Spc was also inoculated. The cultures were then incubated at 37°C with shaking and the culture densities measured at 540 nm with samples fixed at each sampling for further analysis using the Coulter counter and Channelyser. The culture densities were plotted against time and the data compared, Figures 3.3.20 and 3.3.21. Samples of cells were photographed 180 minutes after the switch of cells from arabinose to glucose (Figures 22 and 23.).

Figure 3.3.20. The graph shows the optical density of the *fts*W::Ω strain, DBHΩ3/pBBW1, cultured in LB Tet/Spc with either 1% arabinose (square) or 1% glucose (diamonds and circles) at 37°C. After the inoculation of the glucose culture the growth of the cells in this media slows in comparison to the arabinose grown culture. For 120 minutes both cultures grew at an exponential rate. After this the effect of FtsW depletion in the cells was marked by a sharp decrease in the growth rate. After this point the culture density for the undiluted culture remained static, the diluted culture shows slight fluctuations in cell density. When DBHΩ3/pBBW1 was grown in the presence of 1% arabinose the culture density increased at a steady rate compared to the glucose grown culture.

Figure 3.3.21. In this diagram the control DSB4/pBBW1 was cultured in LB Tet/Spc with either 1% arabinose (square) or 1% glucose (diamond). The growth rates of the two cultures remained identical throughout. The suppression of P$_{BAD}$ with glucose did not impede the growth of the culture. After 2 hours of growth there was no decrease in the growth rate of the glucose culture, as seen for the mutant DBHΩ3/pBBW1. This remained unchanged for the remainder of the experiment.
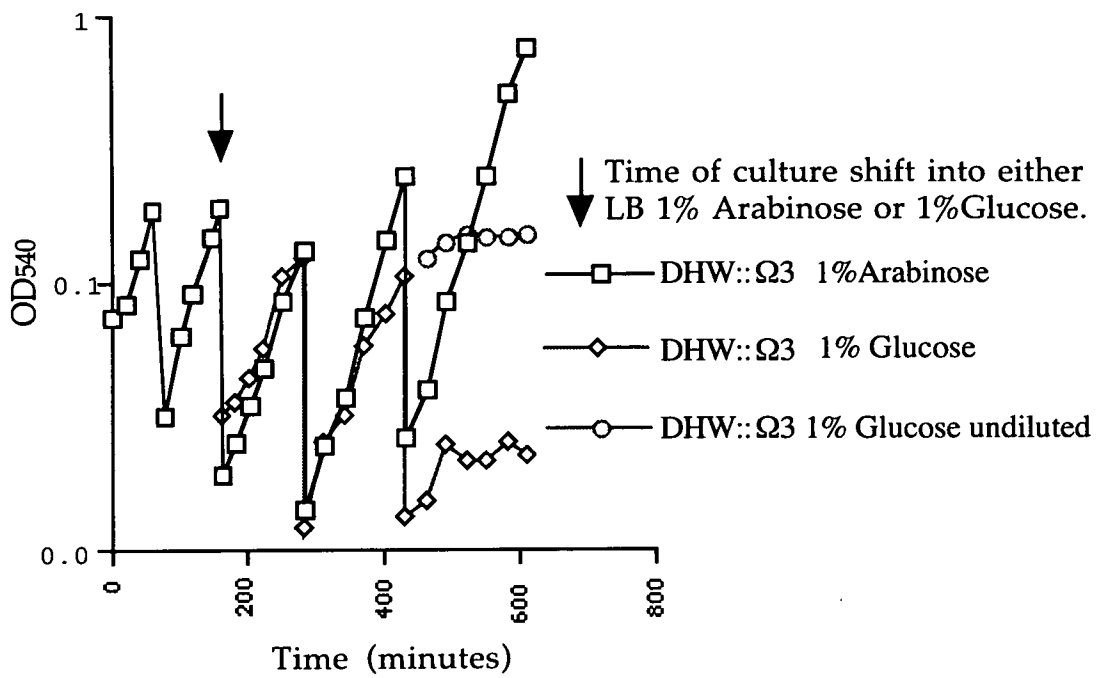
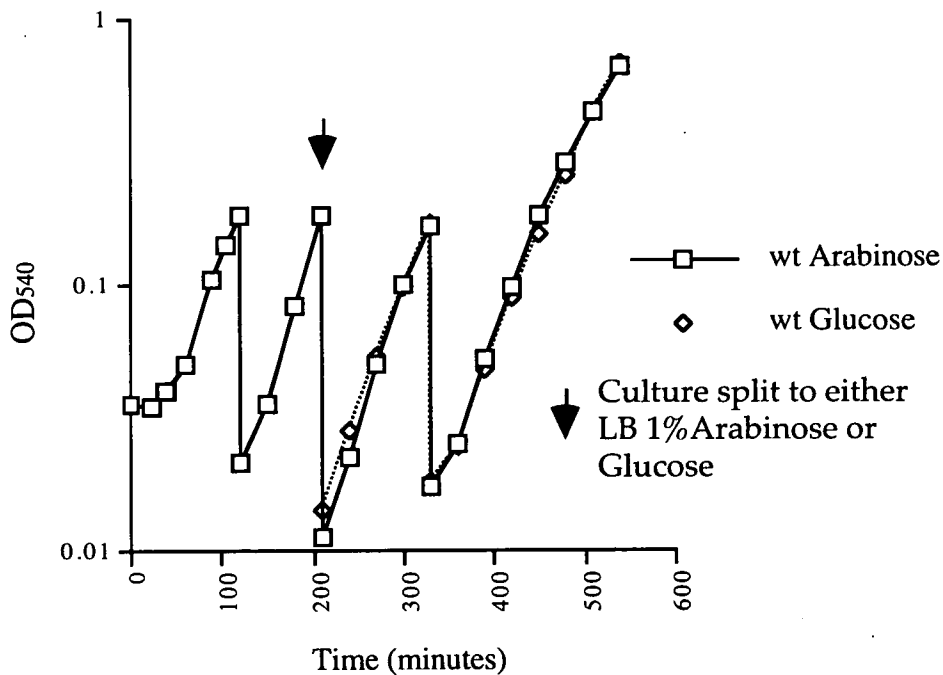**Figure 3.3.20.** Growth of DBHΩ3/pBBW1 in 1% arabinose and glucose.

Figure 3.3.21. Growth of the DSB4/pBBW1 strain in 1% arabinose or glucose.

Figure 3.3.22. DBHΩ3/pBBW1 cells after 180 minutes of culturing in LB Tet/Spc/1% gluc at 37°C with shaking. Bar is equal to 10 μm.

Figure 3.3.23. DBHΩ3/pBBW1 cells after 180 minutes of culturing in LB Tet/Spc/1% ara at 37°C with shaking. Bar is equal to 10 μm.

Figure 3.3.24. This depicts the culture densities of the *ftsW*::Ω mutant (DBHΩ3/pBBW1) and the *ftsW* wild-type strain DSB4/pBBW1 grown in LB 1% arabinose at 37°C. The growth rates of the two cultures were very similar with DSB4/pBBW1 growing at a slightly faster growth rate. Therefore the complementation of the *ftsW*::Ω allele by pBBW1 was sufficient that the growth rate of the mutant was almost indistinguishable from the wild-type control.

Figure 3.3.25. A comparison between the mutant (diamond legend) and the wild-type (square legend) grown in LB Tet/Spc with 1% glucose at 37°C. The growth of the mutant was immediately affected upon the addition of glucose. The wild-type had a slight lag before increasing it's growth rate. The growth of the wild-type strain after this continued exponentially. The mutant however maintained a slower growth rate and after 300 minutes ceased to grow. At this point the cells remained unchanged for the next three hours. Microscopic examination of the mutant cell at this point revealed a culture of lysed filamentous cells. The wild-type strain grew predominantly as short rods.
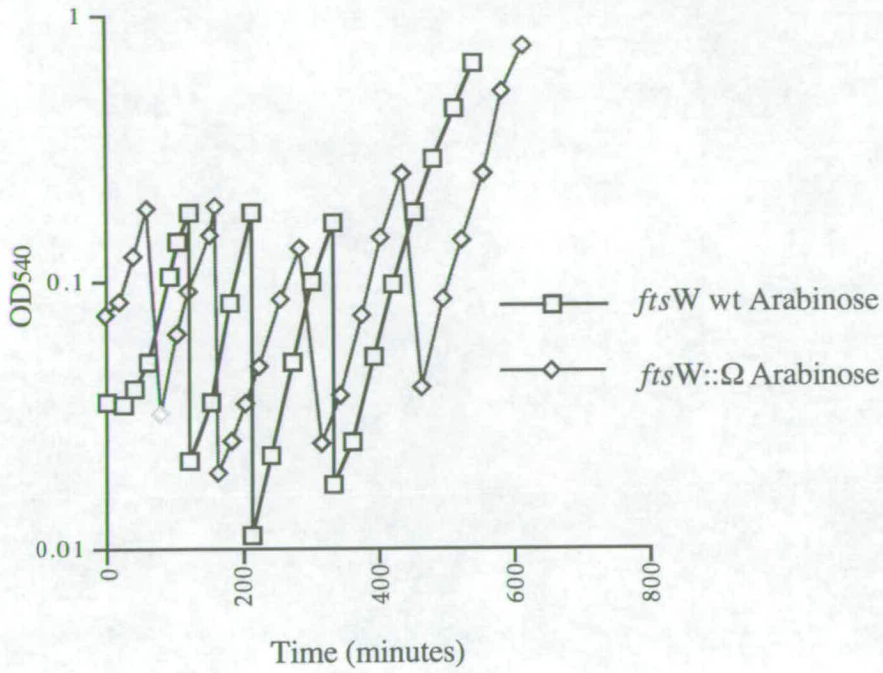
Figure 3.3.24. A comparison of the DBHΩ3/pBBW1 (*ftsW*::Ω) and DSB4/pBBW1 (*ftsW* wild-type) grown in LB 1% arabinose.
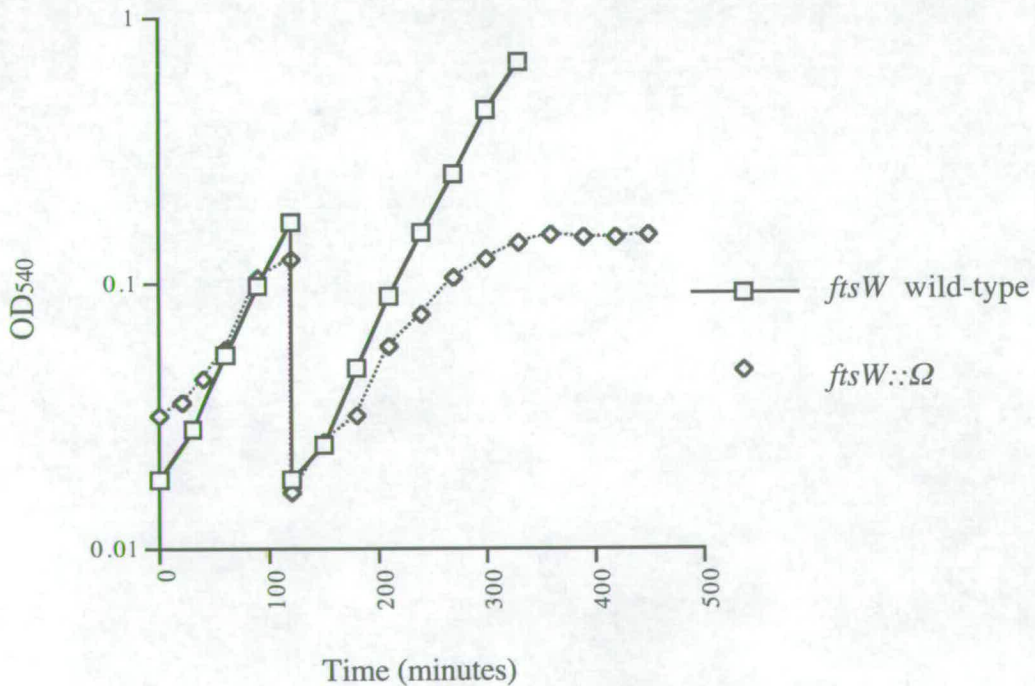

Figure 3.3.25. A comparison of DBHΩ3/pBBW1 and DSB4/pBBW1 grown in LB 1% glucose.
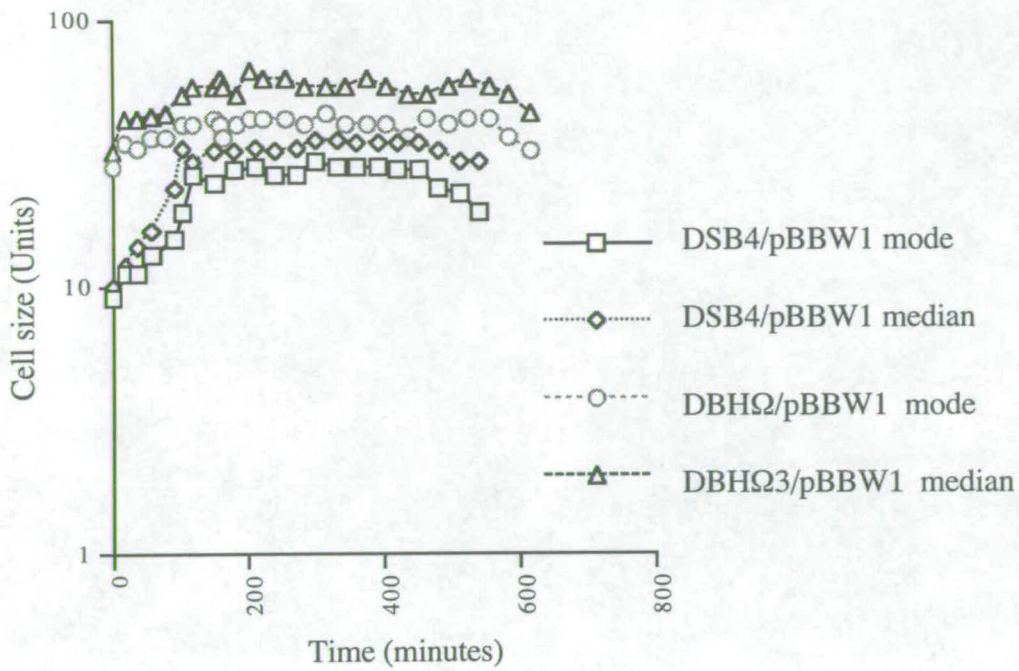
Figure 3.3.26. The mode and median cell size of DBHΩ3/pBBW1 and DSB4/pBBW1 after growth in LB with 1% arabinose. This revealed a significant difference between the cell sizes of the mutant and wild-type cultures. The most common cell size (mode) of both cultures was markedly different. The mutant DBHΩ3/pBBW1 (circles) has a mode nearly three times that of the wild-type DSB4/pBBW1 (squares) at the start of growth. The median reflects this difference in cell size too with the average cell size in the mutant (triangles) being almost treble than that of the wild-type (diamond). These are stationary phase cells from both cultures. As the cultures start to grow and reach log phase growth, the wild type cells increase their cell size threefold. The mutant cells only double in size. During a steady growth rate, the mode and median for both cultures does not fluctuate. Upon entry to the late log phase the decrease in cell size is apparent from the mode and median of both cultures.

Figure 3.3.27. A comparison of the cell number per ml and the OD_{540} of the null mutant and the wild-type strains grown in LB Tet/Spc with 1% arabinose at 37°C. The OD_{540} for both cultures was very similar with both strains exhibiting similar growth rates. The upper graph shows the number of cells per ml of culture at the time of sampling for the OD_{540}. In comparison to the OD_{540} plots there is a difference in the number of cells per ml between the null mutant and the wild-type strain. At the same OD_{540} in log phase growth (legends 1 and 2) the cell numbers per ml are different. The wild-type strain (1) at an OD_{540} of 0.083 was found to contain 1.19×10^7 cells per ml of culture. The null strain (2) at an OD_{540} of 0.085 had 8.9×10^6 cells per ml of culture. The data from the median measurements suggests that the null mutant was larger as the wild-type strain. The count data would indicate that the size of null mutant cells was one and a half times that as the wild-type.
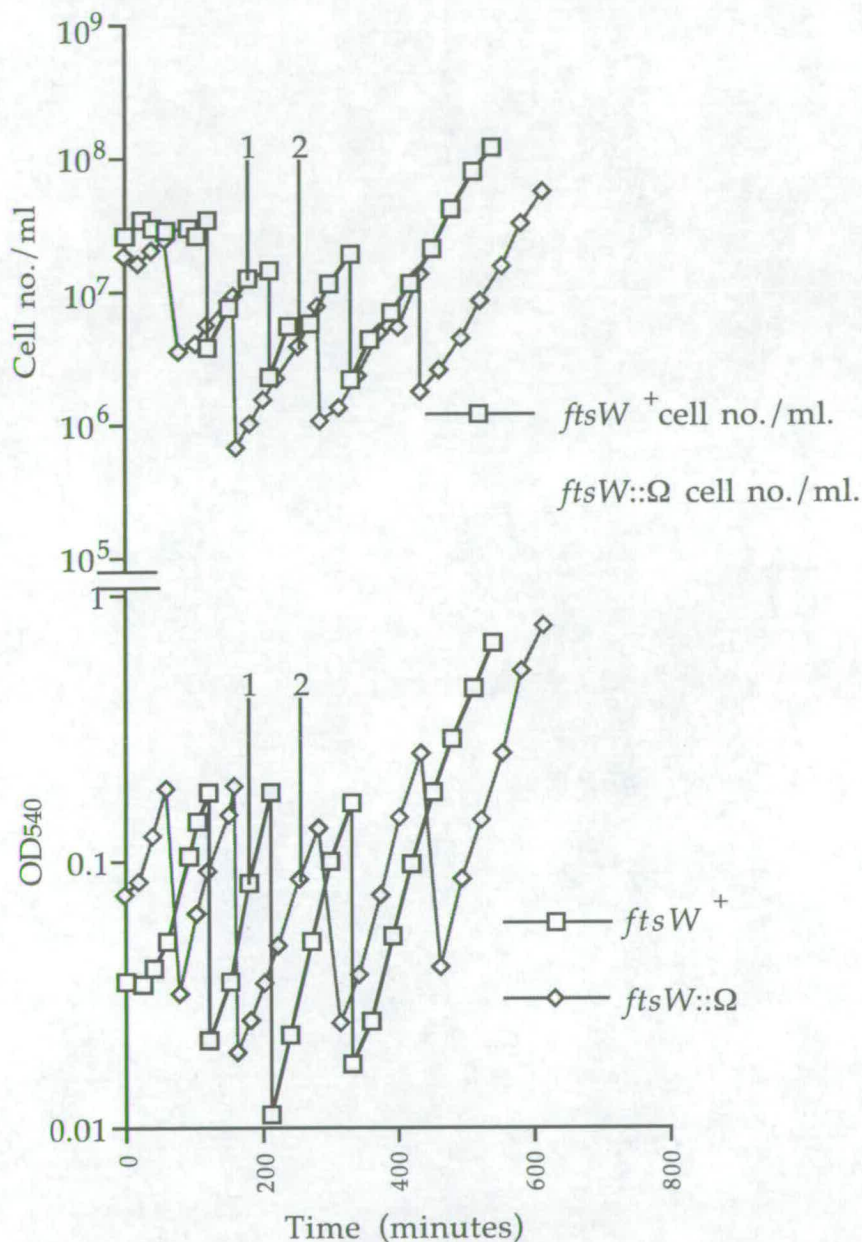
Figure. 3.3.27. The cell number per ml. and the OD540 data of DBHΩ3/pBBW1 and DSB4/pBBW1 cultured in LB supplemented with 1% arabinose.

The growth rates (that is the culture mass doubling times) of the cultures were calculated. The generation time of DBHΩ3/pBBW1 cultured in LB with arabinose was 31 minutes. When DBHΩ3/pBBW1 is grown in LB 1% glucose the doubling time was initially 40 minutes. This slowed further after 180 minutes and stopped at 300 minutes (Figure 3.3.22.). This was in agreement with the data presented in Figure 3.3.21. where the growth rate of DBHΩ3/pBBW1 grown in LB 1% glucose was affected over a similar time scale. A comparison of the wild-type *ftsW* strain with *ftsW*::Ω revealed that the growth rate of the null mutant was immediately affected when introduced to media supplemented with glucose (Figure 3.3.25.). The growth rates of the DSB4 were the same irrespective of the presence of glucose or arabinose in the media (Figure 3.3.21.). The doubling time was calculated to be 30 minutes. This is similar to DBHΩ3/pBBW1 grown in LB arabinose. Therefore expression of *ftsW* from P_{BAD} was sufficient to complement the null chromosomal copy of *ftsW*.

The cell number, mode and median of the fixed cell samples were then determined using the Coulter apparatus. The samples from DBHΩ3/pBBW1 grown in LB 1% glucose could not be measured as debris, presumably from lysed cells, prevented accurate reading of the cell size. Cells from the DSB4 cultures had a similar volume, independent of glucose or arabinose supplementing the media. Figure 3.3.26. shows the initial median cell volume to be ~10 Units. With the onset of log growth the median cell volume trebles to ~30 Units. At the start of the late log phase growth the cell size starts to decrease. This change in cell size during the growth cycle was expected as stationary phase cells when introduced to rich media initially increase in size before starting to divide in log phase growth. The median cell size decreases as the cells reach stationary phase due to a lack of available nutrients and increasing levels of metabolic waste.

The sizing data recorded for DBHΩ3/pBBW1 grown in LB 1% arabinose revealed that the median cell size was greater than for the *ftsW*+ controls. In the mutant strain median cell size is treble that of the wild-type. The difference in cell size between DBHΩ3/pBBW1 and the DSB4 cultures grown in LB 1% arabinose are shown in Figure 3.3.26. Therefore the *ftsW*::Ω allele is having an effect on cell size even when *ftsW* is being expressed. Due to the type of insertion used to disrupt *ftsW*, the increased cell size may be caused by a decrease in the level of FtsZ rather than incomplete suppression of the *ftsW*::Ω allele. Alternatively this phenotype may be a combination of

incomplete expression of both *ftsW* and *ftsZ*. The effects of inhibition of expression of FtsW, from pBBW1, with glucose, is immediately apparent on the growth rate of the cells in DBHΩ3. In addition, the attempts to measure cell size from the mutant glucose culture reveal that cells start to lyse after one mass doubling. When growth of the this culture stopped, the cells were long smooth filaments and either intact or lysed. The expression and repression of *ftsW* from P$_{BAD}$ did not affect the growth rate or the size of the *ftsW* wild-type strain, DSB4. Therefore overexpression of the wild-type sequence of *ftsW* from P$_{BAD}$ does not affect cell growth or morphology.

3.3.17 Construction of an alternative *ftsW* null mutant.

The creation of an *ftsW::Ω* mutant was satisfactory in so far that it proved *ftsW* to be an essential gene involved in cell division, perhaps at an early stage. However, the transcriptional terminator in the Ω fragment may affect the cellular levels of FtsZ and at least part of the filamentous phenotype may be attributable to this. Because pBBW1 is a high copy number vector and FtsW is a protein normally produced in very low amounts, it seems likely that enough FtsW protein is made to complement the *ftsW::Ω* allele. The expression or suppression of *ftsW* from P$_{BAD}$ does not affect the growth rate or size of wild-type cells.

The *ftsW* allele in pDDG1 was again disrupted with another selective marker. The marker chosen was the gene encoding chloramphenicol acetyl transferase (CAT, [confering chloramphenicol resistance]) from pCM4 (Close and Rodriguez, 1982). The CAT fragment was chosen because it contains no promoter or terminator sequences (Close and Rodriguez, 1982) and therefore should not affect the transcription or expression of downstream genes, notably *ftsZ*. pDDG1 was restricted with *Cla*I and the 5' ends 'blunted' using the Klenow enzyme. The CAT fragment was excised from pCM4 by restriction with *Bam*HI and the 5' protruding ends also 'blunted' using the Klenow enzyme. The 0.9 kb CAT fragment was electrophoresed through agarose and recovered by Geneclean and cloned into the blunt ended *Cla*I restricted pDDG1. Clones were selected on LB Cmp/Amp agar. The CAT fragment was cloned in both orientations in pDDG1. A clone was chosen where the CAT fragment was expressed in the same orientation as *ftsW*. This was pDWC1, 8 kb in size (Figure 3.3.28.). This was used to insert the disrupted *ftsW* allele into the partial diploid DSB1. pDWC1 was restricted

with *Bgl*II/*Kpn*I to produce a 4.9 kb fragment containing *ftsW* interrupted with CAT (Figure 3.3.28.). DSB1 was transformed with this linear fragment by electroporation and transformants selected for by plating on LB Tet/Cmp agar, incubated at 37°C. To ensure Cmp^R isolates had not been transformed with uncut pDWC1, the isolates were patched on LB Amp. Cmp^R isolates which were Amp^S were candidate *ftsW*::CAT mutants.
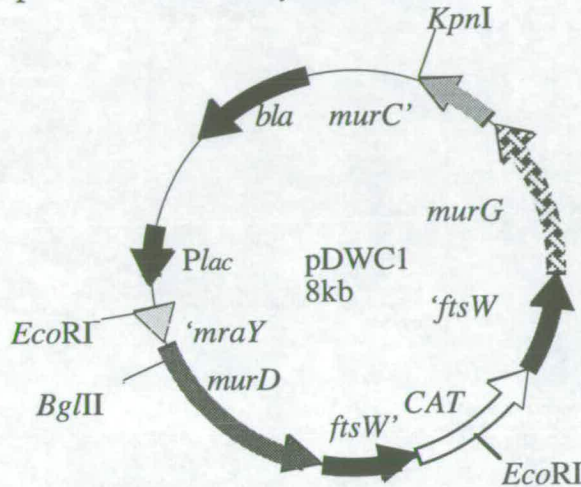

Figure 3.3.28. The vector pDWC1 used as the source for linear DNA containing *ftsW*::CAT used to linear transform the partial diploid DSB1.

A P1 lysate was made on a Cmp^R/Amp^S isolate. The P1 lysate was then used to transduce C600T transformants of pBAD18, pBBW1 and pBADW1. *leu*$^+$ transductants were selected for on fully supplemented minus leucine VB salts Amp agar. This medium was supplemented with 0.5% glycerol and arabinose as the carbon source. The addition of arabinose was necessary to allow complementation of *ftsW*::CAT from pBBW1. Isolates which were *leu*$^+$ were patched on LB Tet and LB Cmp agar, supplemented with 1% arabinose, to ensure that the *leu*::Tn*10* marker had been replaced with *leu*$^+$ and to measure the cotransduction frequency of the *ftsW*::CAT allele with *leu*$^+$.

All of the C600/pBAD18 *leu*$^+$ isolates screened (100 colonies) were Tet^S and Cmp^S. This indicated that the *leu*::Tn*10* had been replaced and that if the *ftsW*::CAT allele was cotransduced the cells did not survive. This is expected as pBAD18 cannot complement *ftsW*::CAT. The *leu*$^+$ transductants containing *ftsW* clones (pBBW1 and pBADW1) were also Tet^S. However, when tested on LB Cmp agar 33% of pBBW1 and 37% of pBADW1 transductants were Cmp^R. This indicated that the *ftsW*::CAT allele was

cotransduced with leu^+ at the predicted cotransduction frequency. This and the lack of Cmp^R cells isolated from the C600/pBAD18 transductants suggested that the $ftsW$::CAT had successfully integrated into DSB1. The Cmp^R isolates of C600/pBBW1 and pBADW1 were then streaked on LB Amp/Cmp agars supplemented either with 1% arabinose or 1% glucose and incubated overnight at 37°C. The pBADW1 transformants grew on both types of media. The pBBW1 transformants only grew on the arabinose containing agar. These results were similar to those obtained previously with the curing of pWAC1 from DBHΩ3. That is pBADW1 can complement an $ftsW$ null mutation irrespective of expression from P_{BAD}.

As a final check on the location of $ftsW$::CAT a P1 lysate was grown on C600 ($ftsW$::CAT)/pBADW1. This was used to transduce DDWΩ1, the partial diploid strain which carries the $ftsW$::Ω allele. As $ftsW$ is an essential gene, the transfer of $ftsW$::CAT into DDWΩ1 by P1 can only be at the expense of the $ftsW$::Ω. Since the cell cannot carry both $ftsW$::Ω and $ftsW$::CAT alleles simultaneously. The P1[$ftsW$::CAT] DDWΩ1 transductants were grown on LB Tet/Cmp agar. Cmp^R isolates were then screened for Spc^S by patching on LB Spc/Tet agar. All of the Cmp^R transductants were Spc^S. Therefore $ftsW$::CAT had replaced $ftsW$::Ω in all the transductants isolated. This was as predicted.

$ftsW$ was again cloned by, this time cutting a PstI 3.3 kb fragment from pDDG1 and cloning it into PstI restricted pUC18. This fragment was cloned in both orientations to make the clones pUCP1 and 2. pUCP1 contained the fragment orientated such that $ftsW$ was expressed from Plac (Figure 3.3.29.). The $ftsW^{ts}$ mutant KH201 (Khattar et $al.$, 1994) was transformed with pUCP1 and 2 and the transformants incubated at 30°C and 42°C on NB Amp agar. At the permissive temperatures both transformants grew but at 42°C only pUCP1 complemented the $ftsW^{ts}$ allele. This showed that the expression of $ftsW$ from the PstI fragment was dependant on an exogenous promoter. pUCP1 was restricted with SmaI and the 4.5 kb fragment re-ligated. This removed the 3' portion of $murG$. This construct was pUPW1, 4.5 kb in size (Figure 3.3.30.). EcoRI/HindIII digestion of pUPW1 released a 1.8 kb fragment which was cloned into pBAD18 restricted with the same enzymes. This made the 6.2 kb plasmid, pBPW1 (Figure 3.3.31.).
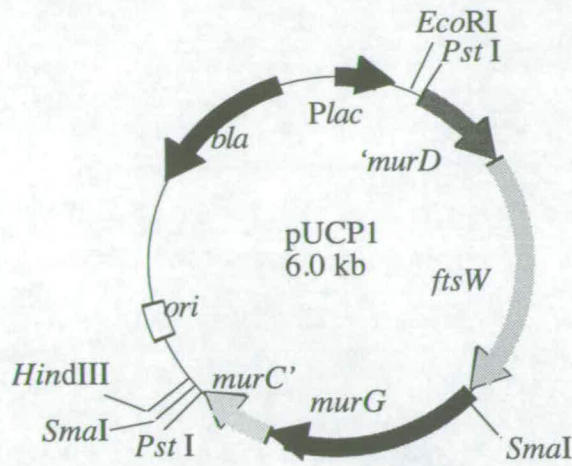
Figure 3.3.29. The 3.3 *Pst*I fragment cloned into pUC18 to create pUCP1.
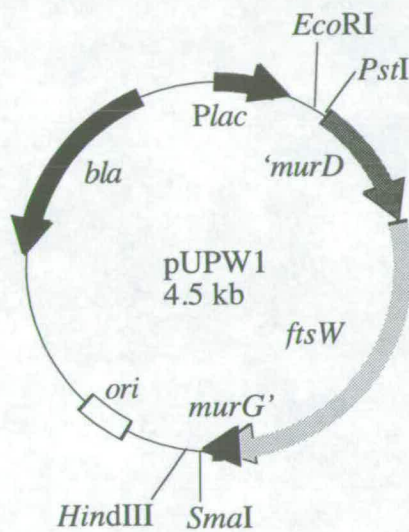

Figure 3.3.30. The *Sma*I truncated clone of pUCP1, pUPW1. This was used to supply the 1.8 kb fragment used to construct pBPW1.

KH201 (*ftsW^{ts}*) was transformed with pBPW1 and Amp^R transformants were screened for growth at 42°C on NB Amp supplemented with either 1% arabinose or 1% glucose. KH201/pBPW1 grew at 42°C only in the presence of arabinose. Therefore the 1.8 kb fragment could complement the *ftsW^{ts}* allele, but only if P_{BAD} expression was induced.
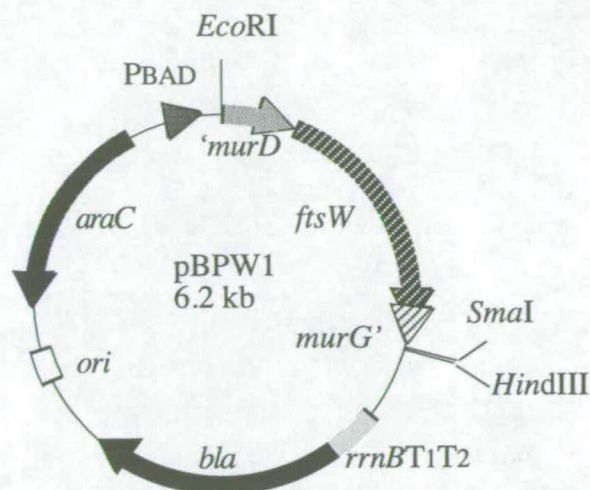
Figure 3.3.31. The plasmid pBPW1 where *ftsW* expression is controlled by P$_{BAD}$. This construct was used to examine the effects of FtsW depletion in an *ftsW*::CAT mutant.

pBPW1 was chosen for complementation studies on the *ftsW*::CAT allele. Before introducing the *ftsW*::CAT allele to the host strain, C600T, was transduced to *pcnB*::Kan. PcnB controls the copy number of certain plasmids including those which have a ColE1 based origin of replication. A *pcnB* mutant reduces the number of pBR325 from 30 to ~10 copies per cell (M. Masters pers. comm.). The *pcnB*::Kan allele was constructed by Masters *et al.* (1993) and is disrupted by a Kan^R cassette. A P1 [$\Delta pcnB$] lysate was used to transduce C600T/pBPW1 with selection for Kan^R transductants on LB Kan/Amp agar. The reduced copy number phenotype of $\Delta pcnB$ transductants can be screened for by growth on LB Amp plates containing 1.5 mg/ml Amp. The reduction in plasmid copy number means less β-lactamase is produced and therefore cell growth is inhibited under conditions of high Amp concentration. A *pcnB*::Kan transductant was isolated in this manner, DSB5 (C600 [*leu*::Tn10 *pcnB*::Kan]). DSB5/pBPW1 was then transduced with a P1 [*ftsW*::CAT *leu*$^+$] lysate and Cmp^R transductants selected for on LB Cmp/Amp/1% arabinose. The Cmp^R isolates were then streaked on both LB Cmp/Amp supplemented with either 1% arabinose or glucose and incubated at 37°C. On arabinose all the Cmp^R isolates grew, whereas on glucose none grew. It was therefore assumed that the *ftsW*::CAT had replaced the *ftsW* wild-type allele and transductants were dependent on *ftsW* expression from pBPW1 for survival.

The cells were examined microscopically to ensure that the low copy number of pBPW1 was sufficient to allow complementation of the mutant phenotype. The cells picked from the arabinose media were predominantly short rods with some filaments. The cells picked from the glucose culture were long aseptate filaments, most of which had lysed. This phenotype was the same as the DBHΩ3/pBBW1 mutant studied previously. This strain was named as DBWC2. The next experiment was to measure the growth rate, cell size and cell number of DBWC2/pBPW1 grown in both arabinose and glucose containing media. All incubations were at 37°C with constant shaking. The culture density was measured at 540 nm and 0.5 millilitre aliquots were stored in an equal volume of fixing buffer at 4°C for subsequent analysis using the Coulter apparatus.

As with DBHΩ3/pBBW1, an overnight culture was first grown in the arabinose containing media until the growth rate reached log phase. Aliquots of cells were pelleted and washed in prewarmed LB containing arabinose or glucose before being used to inoculate the respective media. The culture densities were followed measuring the OD_{540} and dilutions were made when appropriate. An *ftsW* wild-type control strain, DSB5/pBPW1, was used. This was grown in filtered LB Kan supplemented with either 1% arabinose or glucose. The relevant genotype for this strain was *pcnB*::Kan *ftsW*+. The cell number and size of the fixed cell samples were later analysed using the Coulter Counter (Model ZB) and Coulter Channelyser (Model C-1000). The data from the OD_{540} of DBWC2/pBPW1 cultured in arabinose and glucose media were plotted against the sampling time and compared (Figure 3.3.32.).

The culture mass doubling time was calculated to be 35 minutes for the arabinose grown culture. This was constant throughout the growth curve. Upon repression with glucose, the culture grew at a similar rate to the arabinose containing culture for 60 minutes. After this time the cells slowed in growth until the OD_{540} became constant. Examination of the glucose containing culture revealed the phenotype of the cells to be long aseptate filaments which had begun lysis (Figure 3.3.33.). The arabinose containing culture consisted of mainly short rods with occasional long filaments (Figure 3.3.34.). These may be cells which have lost pBPW1, due to the instability of ColE1 based plasmids in a Δ*pcnB* strain, and therefore are unable to complement *ftsW*::CAT.
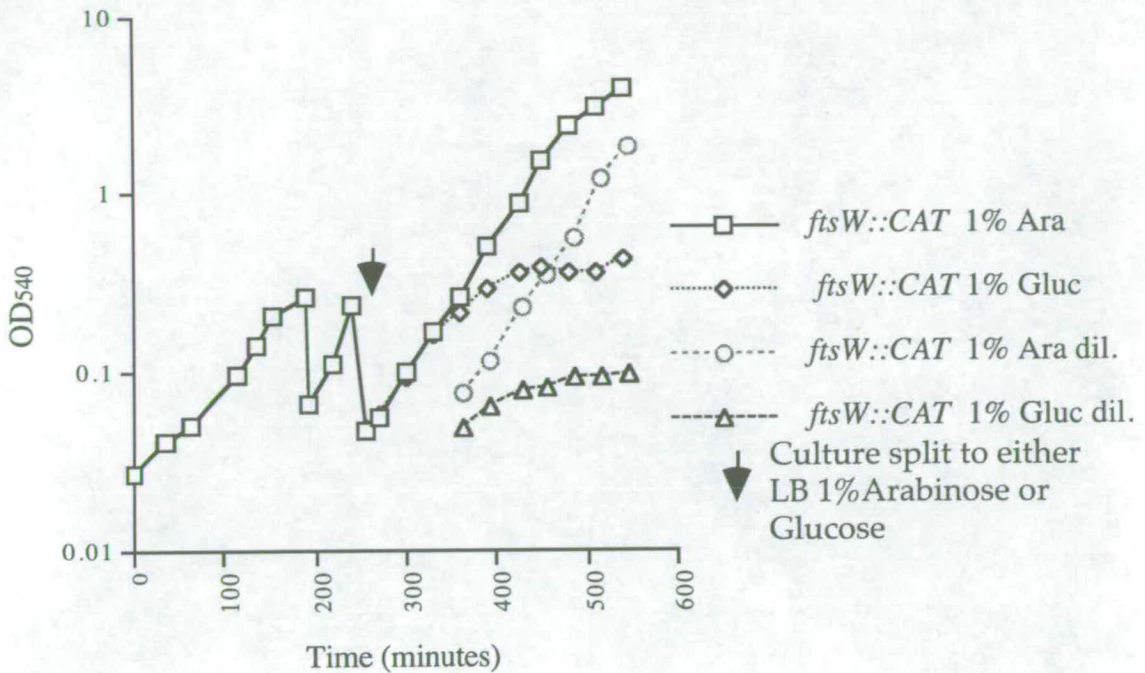
Figure 3.3.32. Growth of DBWC2/pBPW1 in LB supplemented with either 1% arabinose or 1% glucose.

DSB5/pBPW1, the *ftsW* wild-type control, grew at a steady rate in either arabinose or glucose (Figure 3.3.35.). The doubling time of the cultures was identical with the culture mass doubling every 29 minutes. These are faster growth rates than either of the *ftsW*::CAT mutant cultures. The growth rate plots of DSB5/pBPW1 and DBWC2/pBPW1 are compared in Figure 3.3.36. The phenotype of both wild-type cultures was the same, being predominantly short, rod-shaped cells.

The data from the cell sizing revealed that the median cell size of DBWC2/pBPW1 cultured in media containing glucose immediately started to increase upon repression of P_{BAD} expression with glucose. The cells could be sized for the first 70 minutes until cell debris affected the results. The arabinose grown culture maintained a steady median cell size throughout the growth curve until the culture approached stationary phase where a decrease in the median cell volume was observed (Figure 3.3.37.). The median cell size of the DSB5/pBPW1 cultures were very similar. The initial cell size was ~12 units which increased to ~30 units during log growth phase (Figure 3.3.38.). The median cell size of DBWC2/pBPW1 and DSB5/pBPW1 cultured in LB Cmp/Kan containing arabinose was compared

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(Figure 3.3.39.). The data showed that although the median cell size for both cultures was different during the approach to log phase, the median cell size of the cultures was similar during log phase growth. This is in contrast to the previous mutant DBHΩ3/pBBW1 which had a different median cell size when compared with it's control, DSB4/pBBW1. Therefore the disruption of *ftsW* with the CAT cassette does not have a noticeable effect on the size of the mutant cells when grown in arabinose containing media.

Figure 3.3.35. The plot shows the OD_{540} of the wild-type *ftsW* controls cultured in LB medium containing either 1% arabinose or 1% glucose. The doubling time for both cultures was calculated to be ~29 minutes. Therefore the induction or repression of expression from P_{BAD} does not affect the growth of the culture.

Figure 3.3.36. The OD_{540} of the mutant, DBWC2/pBPW1, and the wild-type strain, DSB5/pBPW1, when both were cultured in media containing arabinose. The culture mass doubling time of the mutant was ~36 minutes whilst the wild-type faster with a doubling time of ~29 minutes. This suggests incomplete suppression of the *ftsW*::CAT allele for growth. However other data presented suggest that the complementation was complete with respect to cell shape, size and number per millilitre.
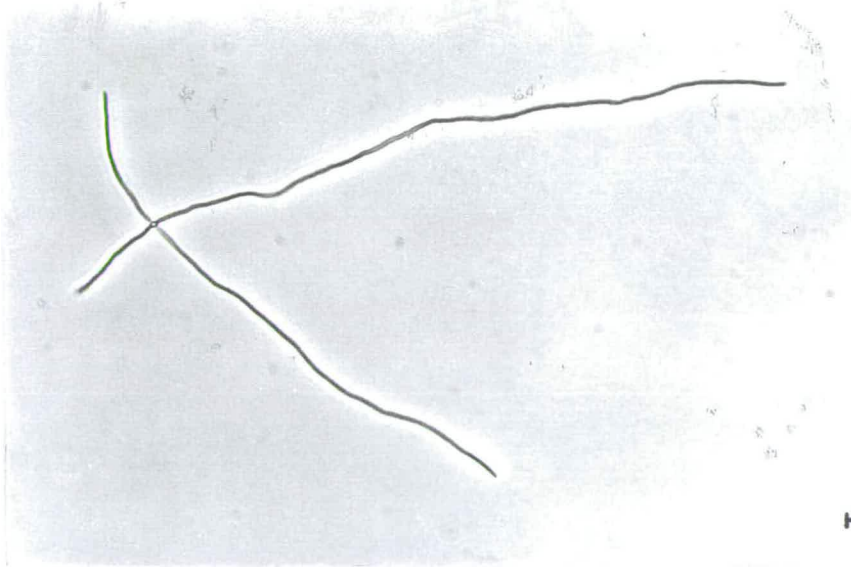
Figure 3.3.33. DBWC2/pBPW1 cells after 180 minutes of culturing in LB Kan/Cmp/1% gluc at 37°C with shaking. Bar is equal to 10 μm.
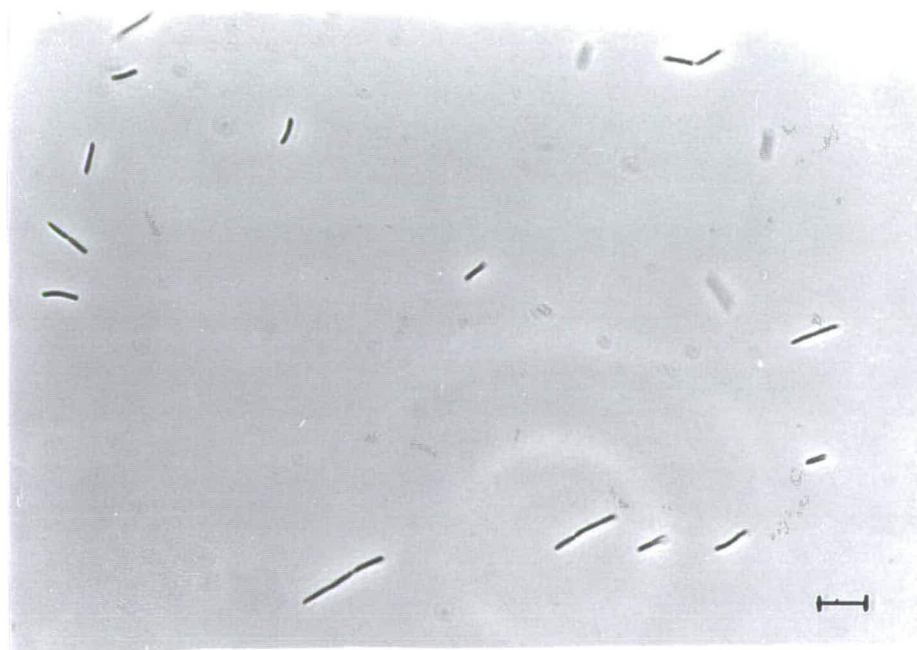

Figure 3.3.34. DBWC2/pBPW1 cells after 180 minutes of culturing in LB Kan/Cmp/1% ara at 37°C with shaking. Bar is equal to 10 μm.
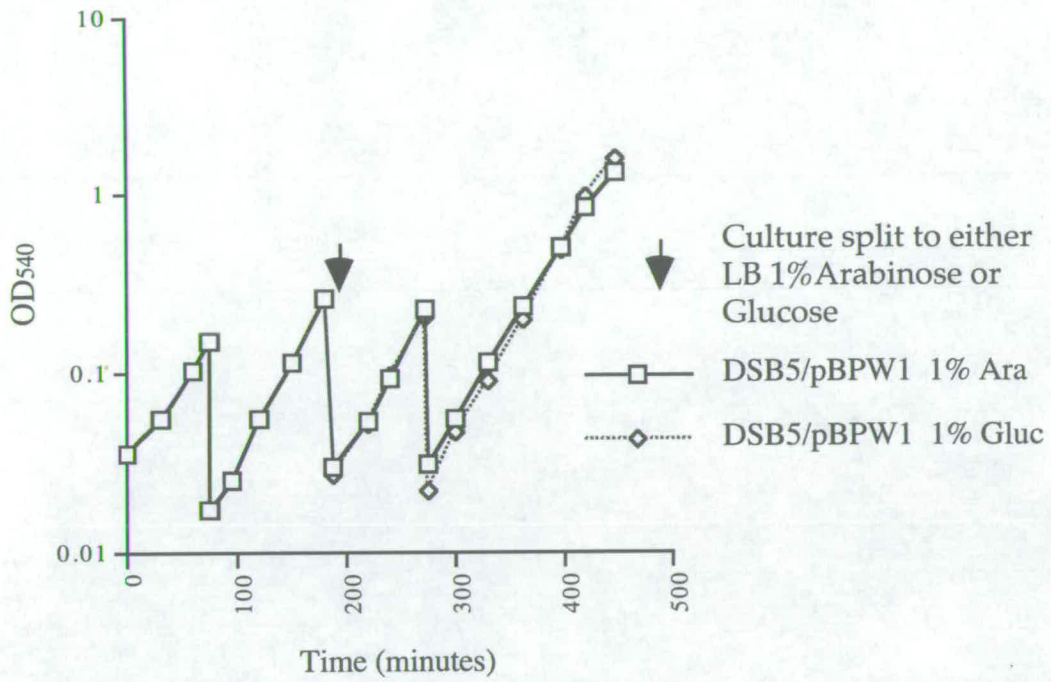
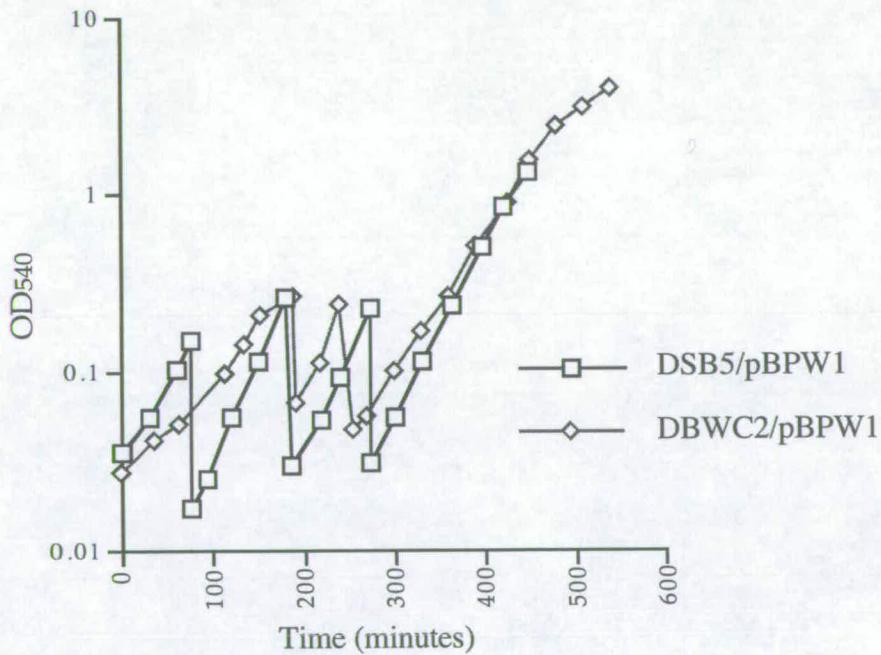**Figure 3.3.35.** The growth of the *fts*W wild-type control in either LB 1% arabinose or 1% glucose.

Figure 3.3.36. The growth rates of the mutant and wild-type strains in LB 1% arabinose.

Figure 3.3.37. The median cell size of DBWC2/pBPW1 grown in LB containing arabinose does not fluctuate during the growth of the culture until late log phase. At this point cell size decreased with the onset of stationary phase. When cultured in LB with 1% glucose the mutant immediately started to increase in size. This increase was continuous until the readings could no longer be taken. Therefore the depletion of FtsW from actively growing cells had an immediate effect on the size and therefore division of cells.

Figure 3.3.38. The median cell volume of the wild-type strain when grown in LB with 1% arabinose was unaffected by a switch to growth in LB 1% glucose. The median cell size from both cultures remained constant throughout log growth with a decrease in the median cell volume as the cultures approached stationary phase. Therefore the levels of FtsW produced from the pBPW1 do not affect the median cell volume.
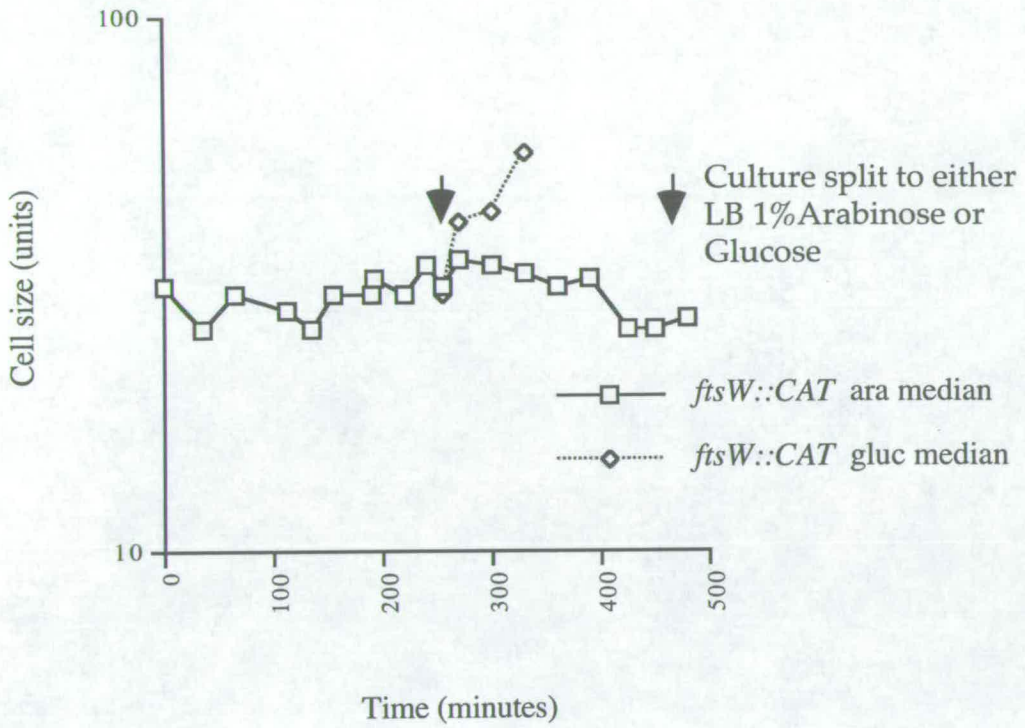
Figure 3.3.37. The median cell size of DBWC2/pBPW1 cultured in LB with either arabinose or glucose.
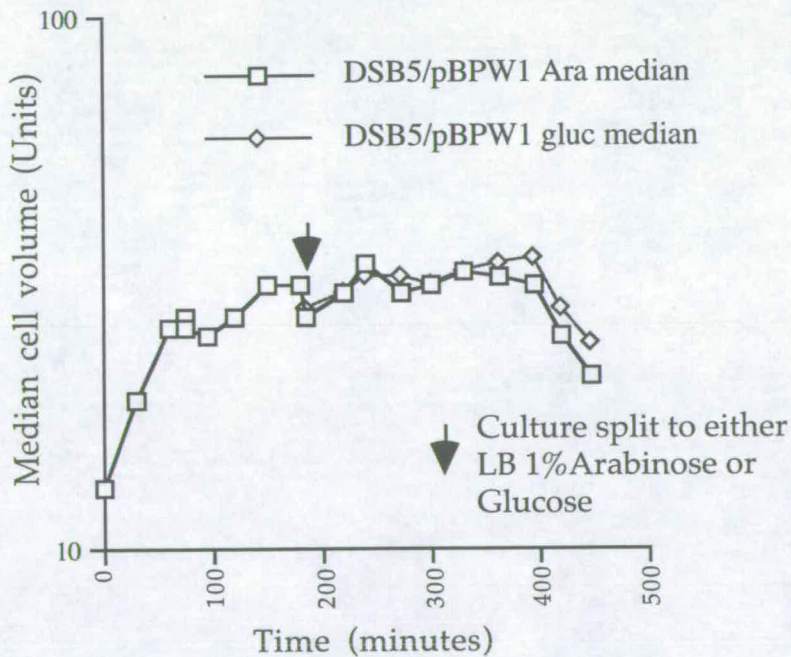

Figure 3.3.38. The median cell size of DSB5/pBPW1 cultured in LB with either 1% arabinose or glucose.

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Figure 3.3.39. The similarity of the median cell sizes of both the mutant and wild-type strains grown with LB 1% arabinose reflects that complementation of *fts*W::CAT was complete when expression from pBPW1 is induced. The initial median reading for the mutant suggests that the cells in stationary phase are large. However, at a later stage in the growth curve, the median cell size of the mutant initially decreased at a similar rate to the wild-type strain.

Figure 3.3.40. The number of cells per millilitre of culture are compared for the mutant grown in LB containing either 1% arabinose or glucose. The initial growth of the mutant in LB arabinose shows the cell number remains static. This is because of the cells increasing in size before starting log phase growth. Upon changing to LB glucose medium, the suppression of *fts*W expression is immediately apparent. The number of cells does not increase at a rate similar to the LB arabinose grown culture. Instead there is a slight rise followed by a decrease in the cell numbers. This would indicate lysis of the culture. The subsequent increase in counts could be due to the increase in cell debris as the lysed cells disintegrate.
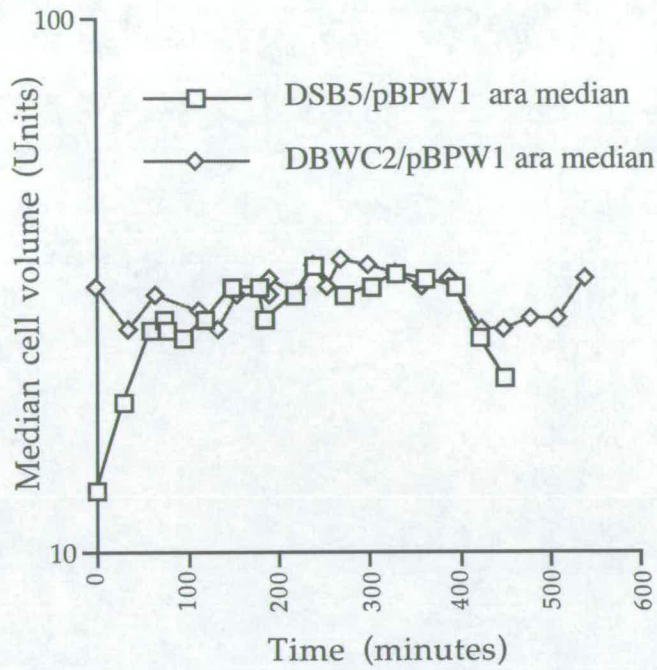
Figure 3.3.39. A comparison between the median cell sizes of DBWC2/pPBW1 and DSB5/pBPW1 grown in LB 1% arabinose.
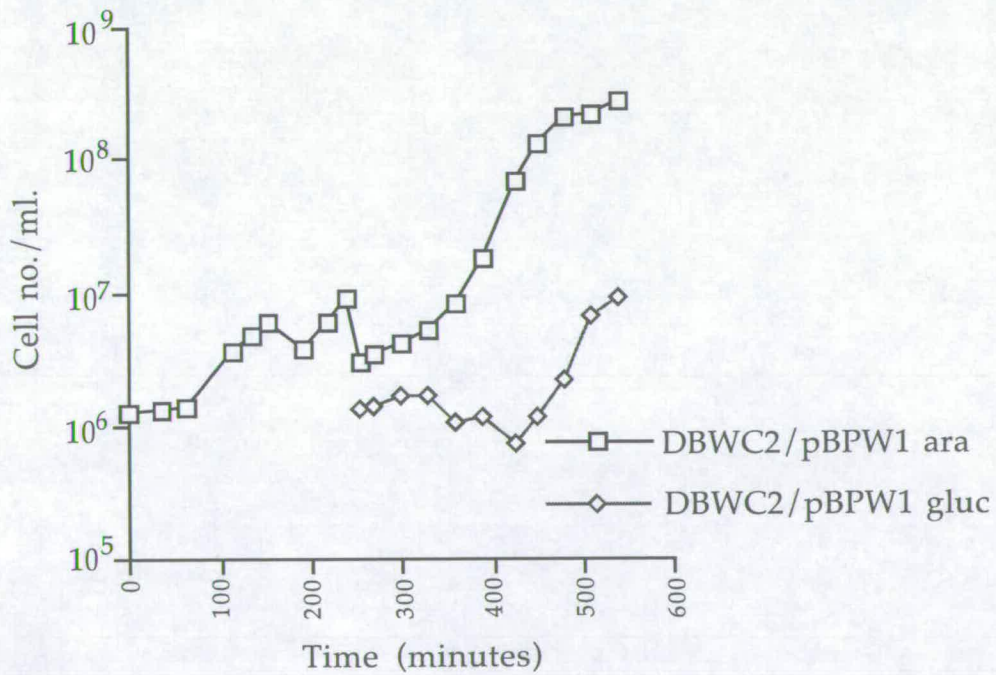
Figure 3.3.40. A comparison showing the cell no./ml of the *ftsW* mutant cultured in LB containing either arabinose or glucose.

Figure 3.3.41. A comparison between the mutant, DBWC2/pBPW1 and the wild-type, DSB5/pBPW1, cultured in LB Kan/Cmp with 1% arabinose. The upper graph reflects the cell number per ml whilst the lower shows the OD_{540} at the time of sampling. The cell number per ml of the mutant culture was very low compared to the wild-type. Upon the onset of log growth the number of cells per ml per unit OD was similar. This was confirmed by the median cell size of both cultures being the same.

The cell number per ml of culture for DBWC2/pBPW1 was calculated for both cultures. Upon inoculation the arabinose culture cell number was static until the cells started to divide as the culture entered log phase growth. Surprisingly the median remained constant, this is expected to increase as cells increase in size prior to log growth. The number of cells per ml in the glucose grown culture remained relatively static (Figure 3.3.40.). This, in conjunction with the OD_{540} and cell median data shows that the cells stop dividing once growing in the glucose medium. The OD_{540} increases, as does the median cell size but the number of cells in the culture only marginally increases. Therefore the increase in culture density is by growth without division.

DBWC2/pBPW1 cultured in medium containing arabinose has a similar cell number per ml to that of the wild-type DSB5/pBPW1 in log growth. In stationary phase the DSB5/pBPW1 culture has a greater cell number per ml but the median cell size is only a third of that of the DBWC2/pBPW1 culture. The cell populations from both cultures become more uniform as both reach log growth phase and the cell medians of both cultures are similar. That is if the OD_{540} and cell medians of both strains, grown in arabinose, are similar then the cell number per ml must also reflect this.

When the data from DBWC2/pBPW1 and DBHΩ3/pBBW1 grown in media containing either arabinose and glucose are compared there are several apparent differences. Both plasmids confer complete suppression of *ftsW* null alleles with respect to the growth rates of the cultures when grown in arabinose. These were barely distinguishable from the wild-type controls grown in similar media. There is however a difference in the time for the effects of FtsW depletion to affect the growth rate of the culture. With the *ftsW*::Ω allele the growth rate slows for 2 hours and finally ceases after another 3 hours culturing. During this time the cells in the culture immediately filament and cell lysis begins at an early stage. With the *ftsW*::CAT allele the growth of the culture stops in less than 3 hours after initial inoculation in glucose. The rate of cell lysis is less but the immediate effect of FtsW depletion can be clearly seen in the increase of the cell median and the stasis in the cell numbers. The size of the *ftsW*::CAT mutant is similar to that of the wild-type during exponential growth. With the *ftsW*::Ω allele the median of the mutant cells is always much greater than the wild-type cells. Therefore the CAT disruption is having a lesser effect on cell size.

This was expected as levels of FtsZ are not expected to be so greatly affected by the CAT fragment inserted in *ftsW* compared to *ftsW*::Ω. The presence of *pcnB*::Kan indicates that the levels of FtsW necessary to complement *ftsW*::CAT are low, given the proposed inefficient translation of the *ftsW* transcript and the low copy number of the complementing plasmid. This indicates that a low concentration of FtsW protein is necessary to permit normal cell growth. The effect upon repression of *ftsW* expression by glucose confirms this and shows that FtsW is not recycled by the cell. There may be a critical threshold concentration of FtsW for division or perhaps the FtsW protein is unstable.

3.3.18. Analysis of a *rodA/ftsW* double mutant.

The control of different stages in cell division was demonstrated by Begg and Donachie (1985). In their work they constructed double mutants carrying a (ts) allele of an elongation gene (*rodA* or *pbpA*) with ts alleles of each of the cell division genes *ftsI, ftsQ ftsA* and *ftsZ*. The effects of the double mutations revealed two morphogenetic phenotypes. The *rodA* (ts) or *pbpA* (ts) alleles in conjunction with *ftsI, ftsQ* and *ftsA* ts alleles produce long swollen cells with regularly spaced constrictions when cultured at the restrictive temperature. The *ftsZ* (ts) allele in either a *rodA*^{ts} or *pbpA*^{ts} background did not produce constricted swollen filaments when cultured at the restrictive temperature. Instead, the cells grew as prolate elipsoids, 'lemon shaped' large cells rather rather than a chain. A similar phenotype was exhibited by the single *rodA or pbpA* mutants when cultured in the presence of Nalidixic acid. This induces the SOS response where FtsZ is inhibited by the SulA protein. These also produced the lemon shaped cells. Therefore Begg and Donachie (1985) concluded that FtsZ is required for the initiation of constriction. However, as the *fts* I, Q, and A (ts) alleles produced constricted filaments their proteins are involved in the stages after the initiation of constriction.

The isolation of a *ftsW* ^{ts} mutant allele in this laboratory meant that this too was tested in the same manner (Khattar *et al.*, 1994). The morphology of a *rodA/ftsW* double mutant was lemon shaped cells. Therefore FtsW is also required at an early stage in division. A strain containing *rodA*^{ts} and *ftsW*::CAT was constructed in C600/pBBW1. The *rodA* allele was linked to a Tet^R marker (a gift from K. Begg). A (ts) mutant was

selected by screening Tet[R] colonies on LB Tet agar at 30°C and 42°C. The C600 *rodA* (ts) strain (DSB 8) was transformed with pBBW1 and the transformant strain transduced with a P1 (*ftsW*::CAT) lysate and Cmp[R] transductants selected for on LB Tet/Amp/Cmp 1% arabinose agar plates incubated overnight at 30°C. Transductants were screened for growth on LB Tet/Amp/Cmp 1% glucose agar with incubation at 30°C overnight. A glucose sensitive Cmp[R] isolate was chosen. The strain DSB9/pBBW1 now contains the *rodA* (ts) allele and the *ftsW*::CAT allele complemented by pBBW1, when in the presence of arabinose.

DSB9/pBBW1 was grown in LB Tet/Amp/Cmp 1% arabinose at 30°C until the cells reached a steady growth rate. The cells from several 1 ml aliquots of this were pelleted and washed in LB prewarmed to 30°C and these were used to inoculate LB Tet/Cmp with either 1% arabinose or 1% glucose. The incubation temperature was 42°C with shaking. After several hours of incubation, strain DSB9/pBBW1 cultured in glucose started to produce the lemon shaped cells described by Begg and Donachie (1985) and Khattar *et al.* (1994). The cells from the arabinose culture assumed a spherical shape; the *rodA* (ts) mutant phenotype. The lemon-shaped cells are shown in Figure 3.3.42.. Therefore the data from this experiment is in agreement with that of Khattar *et al.* (1994), that the *ftsW* gene product is involved at the initiation of division.
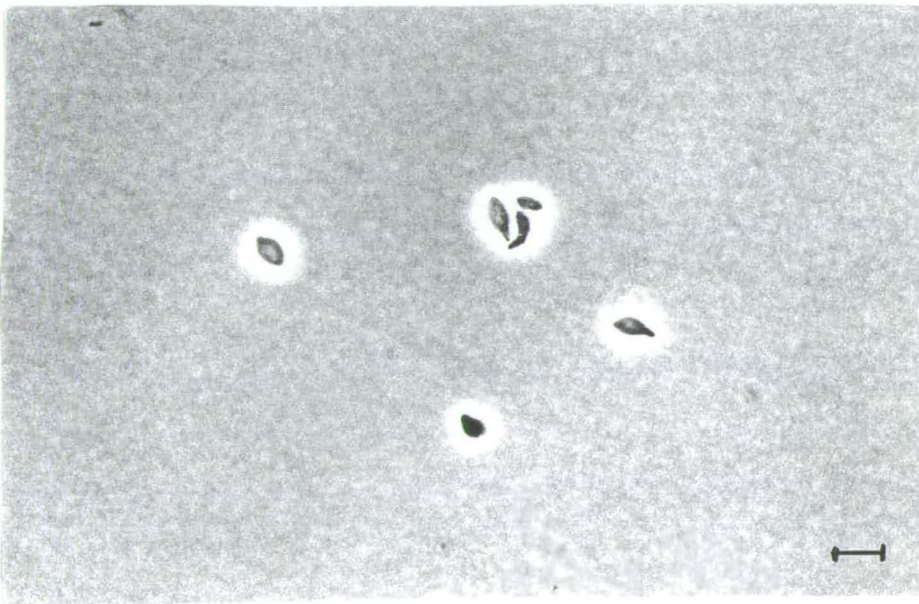

Figure 3.3.42. DSB9/pBPW1 cultured in LB Cmp/Tet with 1% glucose at 42°C. Bar is equal to 10 μm.

3.3.19 β–galactosidase assay of a potential *ftsW* promoter region.

Previous experiments in this thesis studying the complementation of the disrupted *ftsW* with plasmid based copies of the wild type *ftsW* allele revealed that DNA upstream from the *Bgl*II site in *murD* was required for complementation of *ftsW*::CAT in the absence of expression from a plasmid based promoter. If the DNA upstream was 424 bp longer, that is to the *Eco*RI site in *mraY*, then *ftsW*::CAT could be complemented by independant expression from the DNA upstream. Therefore the 424 bp *Eco*RI/*Bgl*II fragment may contain a promoter region which can express *ftsW* independently from plasmid based promoters. The expression from this region was monitored by measuring β–galactosidase activity using the method described by Miller (1972). The vectors used to make the 'operon' *lac*Z fusion were those described by Simons *et al.* (1987).

By using the method devised by Simons *et al.* (1987), the region of interest is initially cloned into a multicopy plasmid vector and then transferred to a λ phage by homologous recombination *in vivo* (Figure 3.2.43.). The vector/phage system, has several distinct advantages over existing β–galactosidase assay vectors (Simons *et al.*, 1987). Most importantly it can detect very weak transcriptional signals. The plasmid used in this study, pRS551 (Figure 3.3.44.), contains four tandem copies of T1 from the *rrnB* terminator sequence upstream of the cloning site (Brosius *et al.*, 1981). This prevents transcription of *lac*Z from other promoters upstream of the cloned insert. The insertion of the plasmid-borne clone into a prophage (λRS45) removes undesirable characterisitics associated with plasmid-borne *lac*Z fusions. These include variability in copy number, gene dosage, titration effects and high levels of expression.
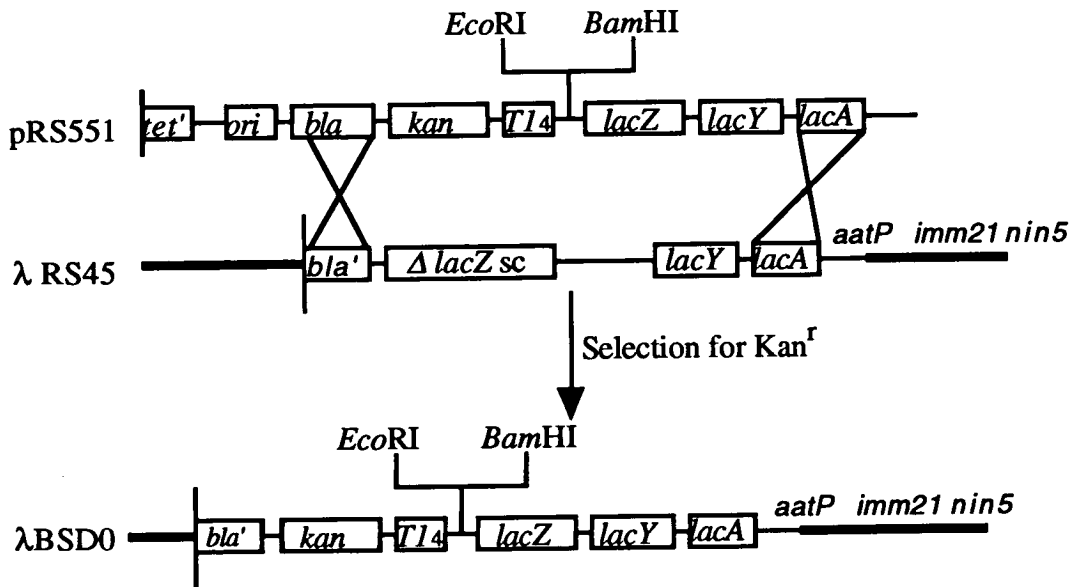
Figure. 3.3.43. The *in vivo* recombination between homologous regions of pRS551 and the single copy prophage λRS45.

The 424 bp *Eco*RI/*Bgl*II fragment was cut from pDDG1 (Figure 3.2.4.) and cloned into the *Eco*RI/*Bam*HI sites in pRS551. This made pBSD30, 12.9 kb in size (Figure 3.3.45.). The cloned insert and surrounding DNA was then transferred to prophage λRS45 by homologous recombination *in vivo*, to make the λ vector, λBSD30. This was achieved by transforming TP8503 (λRS45) with pBSD30 and then culturing in LB Kan at 37°C. The recombinant phage was recovered by UV induced lysis. Simons *et al.* (1987) calculate that 1 in 10^4 phage produced are recombinants. The recombinant phage is selected by screening for Kan^R lysogens. The recombination event also transfers Kan^R from the plasmid in addition to the cloned region and *lacZYA* into the prophage (Figure 3.3.43.).
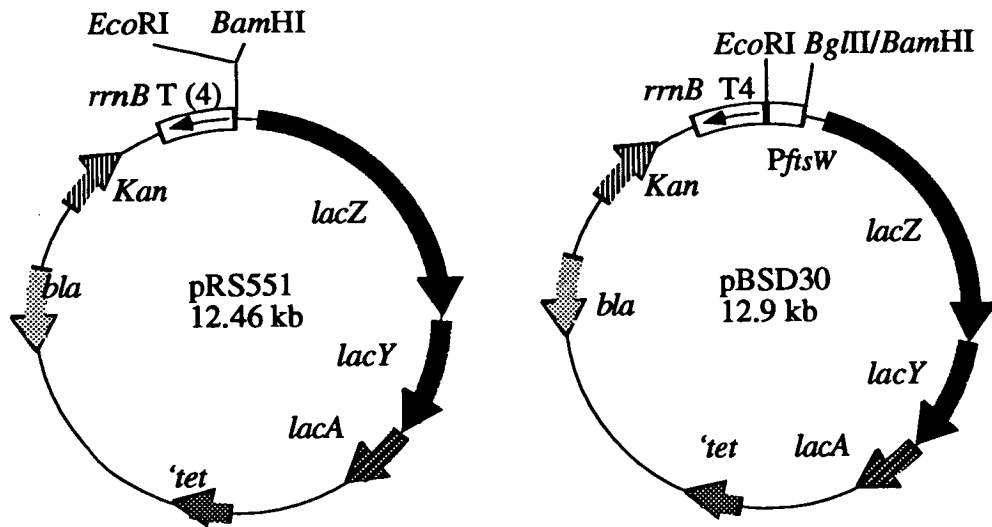
Figures 3.3.44. and 45. The host vector pRS551 and the clone containing the putative *ftsW* promoter region, pBSD30.

A Kan^S/Δ*lac* host (TG1) was infected with the lysate and Kan^R lysogens selected for on LB Kan at 37°C. A lysate of the recombinant λBSD30 was then made from a Kan^R lysogen, again using the UV induction method; this was stored at 4°C until further use. A promoterless recombinant λ phage control was made by selecting for Kan^R lysogens isolated from a lysate induced from a pRS551/ λRS45 culture. This was known as λBSD0. In this case the original vector, pRS551, was used to make a λ prophage by recombination with λRS45. Therefore λBSD0 lysogens are also Kan^R but contain no cloned insert upstream from *lacZ*.

The host strain used to make the lysogens was TP8503. This was infected with λBSD0 and λBSD30 lysates. Lysogens were selected for on LB Kan at 37°C overnight. As an initial screening for β–galactosidase activity, five Kan^R lysogens from each were then streaked on LB Kan/X-Gal agar and incubated overnight. All of the TP8503 (λBSD30) lysogens tested positive, producing blue colonies. The TP8503 (λBSD0) lysogens produced colourless colonies when grown on the same media, therefore the cloned insert does induce the expression of β–galactosidase.
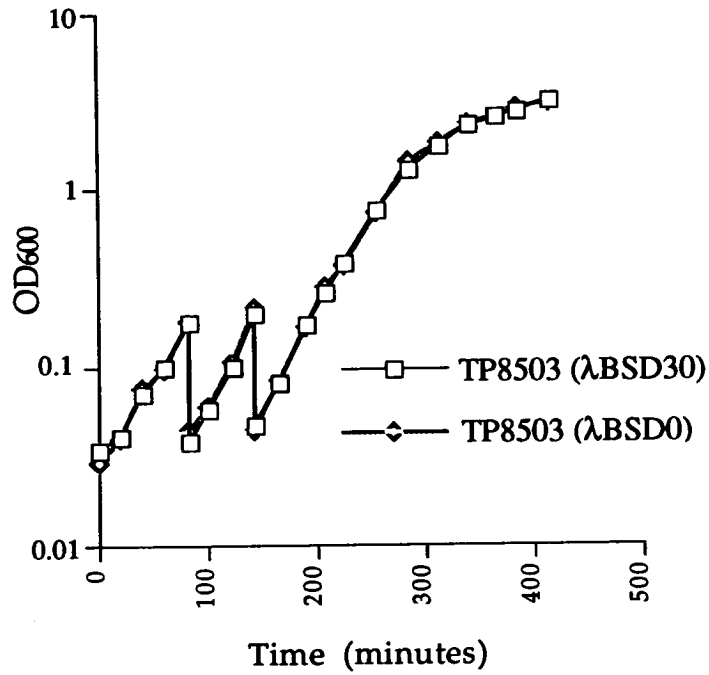
Figure 3.3.46. The growth of the lysogens used in the measurement of β-galactosidase activity from P$_{ftsW}$.
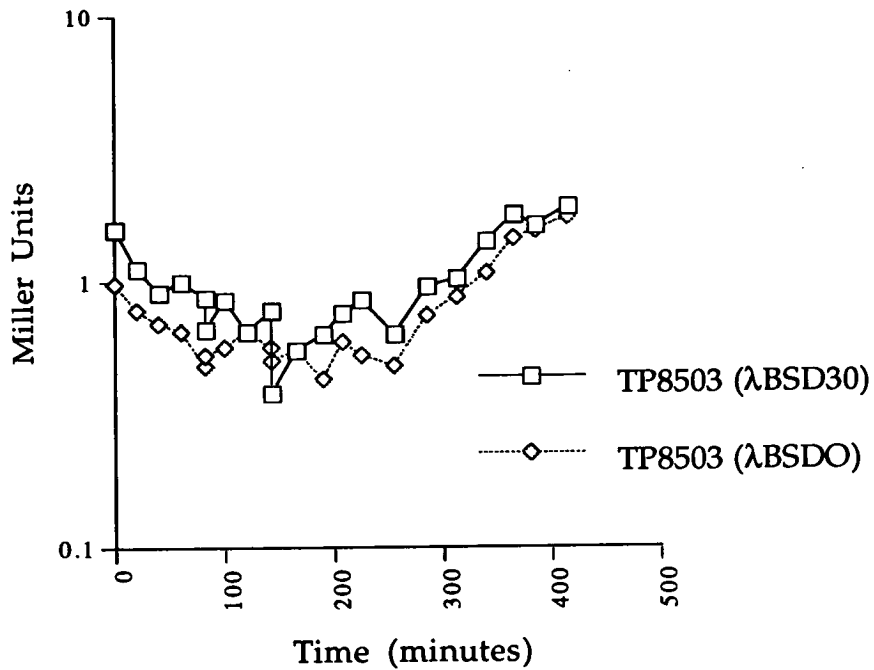

Figure 3.3.47. The β-galactosidase activity expressed from the reporter lysogens.

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Overnight cultures of the two lysogens TP8503 (λBSD30) and TP8503 (λBSD0) were used to inoculate LB Kan and incubated at 37°C. Samples were taken regularly and the culture density read at 600 nm. At each sampling, aliquots were prepared for the β–galactosidase assay described by Miller (1972). The β–galactosidase activities of the two lysogens were calculated in Miller Units and plotted against time (Figure 3.3.47.). The activities were measured for the cultures from the lag phase, through log phase and into stationary phase.

From the data presented in Figure 3.3.47. it is apparent that the β–galactosidase activity produced from TP8503 (BSD30) is only double that of the promoterless control TP8503 (BSD0). The experiment was repeated several times with the same results. The same reagents were used by Smith (1995) to measure the expression from the *ftsZ* P3, P2 and P1 promoters, using the same single copy λ prophage method. In that experiment β–galactosidase activity was markedly higher, reaching up to 500 Miller Units (Smith, 1995). Therefore the promoter activity from the 424 bp *EcoRI/BglII* fragment may be spurious, given that there was such little activity expressed by the phage based fusion. Simons *et al.* (1987) reported that any DNA fragment cloned in the single copy reporter system will produce some β–galactosidase activity. This is presumably due to promoter-like sequences. The lack of activity from the transcriptional fusion may be due to the upstream mRNA produced being susceptible to degradation by endonucleases if stem loop or double stranded RNA structures are formed. This may affect the translation of *lacZ*. The translation of the upstream mRNA could affect the translation of *lacZ* transcript reducing the activity of the clone. A translational fusion between the N-terminal region of MurD and LacZ may produce a more accurate measurement of promoter activity as it will not be susceptible to the interference of upstream RNA affecting the promoter fusion transcript.

However, in their studies on complementation of a *murD^ts* mutant, Mengin-Lecreulx *et al.* (1989) also reported the presence of a promoter located after the *EcoRI* site in *mraY*. They showed that a pUC18 based clone, containing *murD* sequence and upstream DNA to the *EcoRI* site in *mraY*, would complement the *murD^ts* mutant even if the fragment was cloned in the opposite orientation to P*lac* in pUC18. The complementation of the *ftsW* null alleles was similar to this in that the 2845 bp sequence from *EcoRI* in *mraY* to the *SmaI* site in *murG*, cloned in the arabinose promoter vector pBAD18

(pBADW1), would complement *ftsW* null alleles when expression from PBAD was repressed by glucose.

Promoter	-35	Spacing	-10
σ70	TTGACA	17 (+/- 1 bp)	TATAAT
σ32	TCTCnCCCTTGAA	~15 bp	CCCCAT(t)TA
σF	TAAA	15 bp	GCCGATAA
σE	GAACTT	~16 bp	TCTG(G/A)T

Figure 3.3.48. Promoter consensus sequences searched for in the 424 bp fragment (from Helman and Chamberlin, 1988)

The sequence of the 424 bp *EcoRI/BglII* fragment was examined for similarity to the -35 and -10 consensus sequences for the σ70 promoter (Figure 3.3.48.; Helmann and Chamberlin, 1988). Several possible -10 regions were identified but only one had a suitable -35 consensus sequence. This was identified to be CTGTCA (-35) and TTTAAA (-10). 17 bp separates the -35 and -10 domains. Other promoter consensus sequences for σ32 (*rpoH*), σF (σ28), and σE (*rpoE*) were searched for (Figure 3.3.48.), but no similarities were observed (Helman and Chamberlin, 1988).

*Eco*RI

GTCAGGAATTCCTGCTGGTGATTATGGGGGGCGTGTTCGTGGTAGAAACGCTTTCTG
TCATCCTGCAGGTCGGCTCCTTTAAACTGCGCGGACAACGTATTTTCCGCATGGC
ACCGATTCATCACCACTATGAACTGAAAGGCTGGCCGGAACCGCGCGTCATTGTGC
GTTTCTGGATTATTTCGCTGATGCTGGTTCTGATTGGTCTGGCAACGCTGAAGGTACG
TTAATCATGGCTGATTATCAGGGTAAAAATGTCGTCATTATCGGCCTGGGCCTCACC
GGGCTTTCCTGCGTGGACTTTTTCCTCGCTCGCGGTGTGACGCCGCGCGTTATGGATA
CGCGTATGACACCGCCTGGCCTGGATAAATTACCCGAAGCCGTAGAACGCCACACG
GGCAGTCTGAATGATGAATGGCTGATGGCGGCAGATCTGA

                                    *Bgl*II

Figure 3.3.49. The 424 bp *Ec o*RI/*Bgl*II sequence with restriction sites underlined. Also shown underlined are the σ70 -35 and -10 consensus sequences. The exact matches are shown outsize in bold(-35 T, G, C and A; -10 T, T, A and A).

The similarity of the proposed -35 and -10 regions to the consensus sequence of the σ^{70} promoter were both 67%. The -35 and -10 are separated by 17 bp. The regions therefore probably provide expression for *murD* and *ftsW* (this work and Mengin-Lecreulx *et al.*, 1989). It may be that since the clones were in high copy number plasmids there was sufficient expression to complement the mutations. The *ftsW* clone, pBADW1 (Figure 3.3.16) however, complemented the *ftsW*::CAT allele in a strain which contained a null *pcnB* allele. The *pcnB*::Kan allele reduces the plasmid copy number to ~10 plasmids/cell (M. Masters, pers. comm.). The levels of FtsW required for cell division are very low, probably 20-50 molecules per cell (M. Khattar pers. comm.). Therefore there was enough promoter activity from the 424 bp fragment to produce enough FtsW to permit an Δ*ftsW* strain to grow. Ideally, a single copy clone of the 2845 bp *Eco*RI/*Bgl*II fragment would need to be constructed to detemine whether the 424 bp region actually contains the promoter for *ftsW*. It is possible that *ftsW* expression is directed from a promoter located much further upstream. This has been reported for two other genes involved in cell division, namely *ftsI* (Hara and Park, 1993) and *ftsZ* (Dai and Lutkenhaus, 1991).

Discussion.

The initial aim of this study, that of identifying the FtsW peptide was unsuccessful. Khattar *et al.* (1994) demonstrated that, to obtain expression, it was necessary to remove the non coding DNA upstream from the proposed translational start site (Ikeda *et al.*, 1989). If the larger ORF was cloned into a vector with a suitable RBS then the larger FtsW peptide was efficiently expressed *in vivo* (Khattar *et al.*, 1994). The region proposed to code for *ftsW* also contains a smaller ORF coding for a peptide with a molecular weight of 43 kDa. This was not produced from the *gene* 10 *ftsW* fusion, only the larger peptide of 46 kDa was expressed (Khattar *et al.*, 1994; see Chapter 3.1.). The hypothesis by Ikeda *et al.* (1989) that the products of *ftsI* (PBP3) and *ftsW* (FtsW) may interact to create a division complex may be correct when the levels of protein produced by either gene are compared. The levels of peptides produced are barely detectable even when heavily transcribed using T7 RNA polymerase (Khattar *et al.*, 1994; this work, chapters 4 and 5). However, it has been shown genetically that *ftsI* has to be involved at a later

stage of the division process than *ftsW* (Begg and Donachie, 1985; Khattar *et al.*, 1994).

Khattar *et al.* (1994) have shown that the region encoding the 46 kDa peptide complements the thermosensitve *ftsW* mutant KH201 when expressed from a suitable plasmid based promoter. Until now, no null allele for *ftsW* has been reported. The work reported in this chapter produced two null alleles of *ftsW* which were used to confirm that *ftsW* is an essential gene required for cell division. Depletion of FtsW resulted in filamentation of cells and their eventual lysis. This work also showed that the ORF for the larger FtsW peptide complements the *ftsW* null mutant. The testing of the smaller *ftsW* ORF remains to be done. To obtain complementation of the null mutant without expressing *ftsW* from a plasmid based promoter, an extra 1684 bp of DNA upstream of *ftsW* was required.

The putative promoter was found to be located in the upstream DNA within a 424 bp *Eco*RI/*Bgl*II fragment which spans the 3' end of *mraY* and the start of the *murD* ORF. Searches within the 424 bp sequence revealed two regions with partial homology to the sigma 70 -10 and -35 consensus sequences. These were located within *mraY*. Studies on the complementation of a thermosensitive *murD* mutant by Mengin-Lecreulx *et al.* (1989) showed that when the *murD* ORF was cloned with upstream DNA to the *Eco*RI site, the mutant strain could grow at the restrictive temperature. This indicates that both *murD* and *ftsW* may share the same promoter.

The 424 bp region was studied for promoter activity in a single copy phage reporter vector. β-galactosidase assays on cells hosting the clone did not show promoter activity from this region. These results were in contradiction to the complementation studies carried out in this work on the *ftsW*::Ω and *ftsW*::CAT null mutants and also the *murD^ts^* studies by Mengin-Lecreulx *et al.* (1989). Therefore for these reasons and in conjunction with the identification of a suitable sigma 70 promoter consensus sequence in *mraY*, it is possible that transcription from the proposed promoter region is so innefficient that it cannot be quantified when using a single copy *lacZ* reporter vector. An alternative method for measuring the expression of *ftsW* would be to measure the CAT activity from either the partial diploid *ftsW*::CAT mutants, DBWC1, or the haploid *ftsW*::CAT mutant, DBWC2/pBPW1. There are other methods which could be used to resolve the issue of the putative promoter. The sites with homology to the sigma 70 consensus sequences could be mutagenized by site directed mutagenesis or

removed by nucleases and the altered or shortened DNAs tested for complementation of the *ftsW*::CAT mutant.

The formation of 'lemon-shaped' cells in the *ftsW*::CAT *rodA^{am}* double mutant suggests that FtsW is required at a very early stage in the division process. This result confirms the work of Khattar *et al.* (1994) with a ts allele of *ftsW*, OV2WR. A similar phenotype is observed when a *rodA* null is combined with the *ftsZ*84 ts allele (Begg and Donachie, 1985). The absence of constrictions in the *ftsZ rodA* double mutant suggest that the Z ring does not contract or is not formed. Therefore FtsW may play a role in either the localization of the FtsZ to the cell midpoint or in the constriction of the ring once it has formed. The equipment and techniques necessary to study the localization of FtsZ rings within the cell by immunofluorescence were not available in the laboratory at the time of study (Bi and Lutkenhaus, 1992). However, immunofluorescence could be used to examine the *ftsW*::CAT *rodA^{am}* double mutant for the presence of the FtsZ ring structure. If the FtsZ ring was not present then it could be deduced that FtsW is required for the localization or polymerization of FtsZ rings. Alternatively, if the FtsZ rings were present, the function of FtsW may be as the link between the constricting FtsZ ring at the inner face of the cytoplasmic membrane and the septal synthesizing complex comprising PBP3 and other proteins in the periplasm (Holtje, 1993; M. Khattar, pers. comm.). That is, coordination between FtsZ ring driven invagination of the inner membrane with concommitant synthesis of the septal murein ensuring the successful formation of two new daughter cell poles at the midpoint of a dividing cell (M. khattar, pers. comm.).

The *ftsW*::Ω null allele construct provided further evidence for the complex organisation of the *mra* region. The presence of transcriptional terminators in the Ω fragment appeared to effect the expression of *ftsZ*. The *ftsW*::Ω allele could only be transduced into haploid strains (containing complementing plasmids for *ftsW*) when an extra copy of *ftsZ* was present. As the insertion of Ω was 7 kb upstream from the *ftsZ* ORF, it suggests that there may be an *ftsZ* transcript starting upstream from *ftsW*. Earlier studies on the RNA transcripts that include *ftsZ* have shown that there are many species (Dewar *et al.*, 1989; Cam *et al.*, 1994).

On the other hand it has been proposed that the *mra* region may be expressed as a single transcript as there are no terminators from *mraW* until downstream of *envA* (Dai and lutkenhaus, 1991; Hara and Park, 1993;

Donachie, 1993). The amount of the growth and division proteins may be controlled by post transcriptional processing of this transcript as well as by the production of smaller transcripts from promoters located along the *mra* region. Another large operon, the *amiB-mutL-miaA-hfq-hflX-hflK-hfflC* superoperon in *E. coli* (Tsui and Winkler, 1994) has been shown to have complex controls at the transcriptional and post transcriptional level (Tsui *et al.*, 1994). Another important regulatory factor is the efficiency with which the individuals are translated (Mukherjee and Donachie, 1989; Dai and Lutkenhaus, 1992; Khattar *et al.*, 1994; this work, Chapter 5). If the *mra* region is transcribed as a single transcript it has not yet been detected. This may be due to its rarity as the 5' end may be rapidly processed into smaller transcripts whilst transcription continues towards *envA*. As yet there is no experimental evidence for a massive transcript being detected from the *mra* region.

Chapter 4.

Analysis of *mraY*.

4.1.1. Introduction.

Ikeda *et al.* (1990) identified an open reading frame (ORF) lying between *murF* and *murD* in the *mra* cluster at 2 minutes on the *E.coli* chromosome. Sequencing revealed an ORF of 1080 bp, designated *mraY*, which codes for a 360aa peptide with a predicted molecular size of 39874 Da (Figure. 4.1.1., Ikeda *et al.*, 1990). The *murF* gene overlaps the start of the *mraY* sequence by 7 bp and *murD* starts 5 bp after the stop codon of *mraY*. *In vitro* enzymatic assays of extracts of cells carrying high copy number *mraY* clones suggest that the MraY protein catalyses the first reaction in the lipid cycle reactions for the biosynthesis of cell wall murein, (Ikeda *et al.*, 1991; see Chapter 1.1.2.). It was suggested that *mraY* encodes UDP-N-acetylmuramoyl-pentapeptide: undecaprenyl-phosphate phospho-N-acetylmuramoyl-pentapeptide transferase. This enzyme catalyses the transfer reaction where cytoplasmic UDP-MurNAc-pentapeptide is bound to the lipid carrier molecule, undecaprenol-phosphate, in the cytoplasmic membrane. The later stages of the lipid cycle involve the addition of GlucNAc to create a disaccharide-pentapeptide lipid complex, Lipid II (Mengin-Lecreulx *et al.*, 1991) and the subsequent transfer across the membrane and release of the disaccharide-pentapeptide into the periplasm for incorporation into the murein sacculus.

UDP-MurNAc-pentapeptide + undecaprenyl-phosphate

$$\rightleftharpoons$$ undecaprenyl-pyrophosphoryl (PP)-MurNAc-pentapeptide + UMP
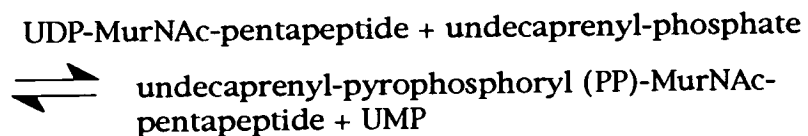
Figure 4.1.2. The reaction catalysed by the MraY protein.

The MraY protein is highly basic with an isoelectric point (pI) of 10.5. This indicates that the protein is hydrophobic and therefore probably is located in the cytoplasmic membrane. The hydropathy profile of the peptide sequence also suggests that MraY is membrane bound as it is predominantly hydrophobic with several membrane domains (Figure.4.1.3.). Analysis of the peptide sequece by TopPred II (Claros and von Heijne, 1994) using the Kite and Doolittle algorithm topology predictions of the secondary structure show ten membrane spanning domains (Figure 4.1.4.). There are several homologues of *mraY* which have been identified in prokaryotes. There has been no actual research performed on the putative genes. All have been identified by DNA sequencing and subsequent

computational analysis of the predicted peptide similarity with MraY from *E.coli*. The homologues have been identified in *Rhizobium melioti* (Leach *et al.*, 1994), *B. subtilis* (Daniel and Errington, 1993) and *H. influenzae* (Fleischmann *et al.*, 1995). The aims of this study were to identify the 39874 Da peptide produced by *mraY* and to investigate the role of MraY by the isolation of an *mraY* conditional mutant.

```
    aggtagtacgcgctttacaggagaatgggacatgttagtttggctggccgaacatttggt
  1 ---------+---------+---------+---------+---------+---------+
                                         M  L  V  W  L  A  E  H  L  V

    caaatattattccggcttttaacgtcttttcctatctgacgtttcgcgccatcgtcagcct
 61 ---------+---------+---------+---------+---------+---------+
     K  Y  Y  S  G  F  N  V  F  S  Y  L  T  F  R  A  I  V  S  L

    gctgaccgcgctgttcatctcattgtggatgggcccgcgtatgattgctcatttgcaaaa
121 ---------+---------+---------+---------+---------+---------+
     L  T  A  L  F  I  S  L  W  M  G  P  R  M  I  A  H  L  Q  K

    actttcctttggtcaggtggtgcgtaacgacggtcctgaatcacacttcagcaagcgcgg
181 ---------+---------+---------+---------+---------+---------+
     L  S  F  G  Q  V  V  R  N  D  G  P  E  S  H  F  S  K  R  G

    tacgccgaccatgggcgggattatgatcctgacggcgattgtgatctccgtactgctgtg
241 ---------+---------+---------+---------+---------+---------+
     T  P  T  M  G  G  I  M  I  L  T  A  I  V  I  S  V  L  L  W

    ggcttacccgtccaatccgtacgtctggtgcgtgttggtggtgctggtaggttacggtgt
301 ---------+---------+---------+---------+---------+---------+
     A  Y  P  S  N  P  Y  V  W  C  V  L  V  V  L  V  G  Y  G  V

    tattggctttgttgatgattatcgcaaagtggtgcgtaaagacaccaaagggttgatcgc
361 ---------+---------+---------+---------+---------+---------+
     I  G  F  V  D  D  Y  R  K  V  V  R  K  D  T  K  G  L  I  A

    tcgttggaagtatttctggatgtcggtcattgcgctgggtgtcgccttcgccctgtacct
421 ---------+---------+---------+---------+---------+---------+
     R  W  K  Y  F  W  M  S  V  I  A  L  G  V  A  F  A  L  Y  L

    tgccggcaaagacacgcccgcaacgcagctggtggtcccattctttaaagatgtgatgcc
481 ---------+---------+---------+---------+---------+---------+
     A  G  K  D  T  P  A  T  Q  L  V  V  P  F  F  K  D  V  M  P

    gcagctggggctgttctacattctgctggcttacttcgtcattgtgggtactggcaacgc
541 ---------+---------+---------+---------+---------+---------+
     Q  L  G  L  F  Y  I  L  L  A  Y  F  V  I  V  G  T  G  N  A

    ggtaaacctgaccgatggtctcgacggcctggcaattatgccgaccgtatttgtcgccgg
601 ---------+---------+---------+---------+---------+---------+
     V  N  L  T  D  G  L  D  G  L  A  I  M  P  T  V  F  V  A  G

    tggttttgcgctggtggcgtgggcgaccggcaatatgaactttgccagctacttgcatat
661 ---------+---------+---------+---------+---------+---------+
     G  F  A  L  V  A  W  A  T  G  N  M  N  F  A  S  Y  L  H  I

    accgtatctgcgacacgccggggaactggttattgtctgtaccgcgatagtcggggcagg
721 ---------+---------+---------+---------+---------+---------+
     P  Y  L  R  H  A  G  E  L  V  I  V  C  T  A  I  V  G  A  G

    actgggcttcctgtggtttaacacctatccggcgcaggtctttatgggcgatgtaggttc
781 ---------+---------+---------+---------+---------+---------+
     L  G  F  L  W  F  N  T  Y  P  A  Q  V  F  M  G  D  V  G  S

    gctggcgttaggtggtgcgttaggcattatcgccgtactgctacgtcaggaattcctgct
841 ---------+---------+---------+---------+---------+---------+
     L  A  L  G  G  A  L  G  I  I  A  V  L  L  R  Q  E  F  L  L

    ggtgattatggggggcgtgttcgtggtagaaacgctttctgtcatcctgcaggtcggctc
901 ---------+---------+---------+---------+---------+---------+
     V  I  M  G  G  V  F  V  V  E  T  L  S  V  I  L  Q  V  G  S

    ctttaaactgcgcggacaacgtattttccgcatggcaccgattcatcaccactatgaact
961 ---------+---------+---------+---------+---------+---------+
     F  K  L  R  G  Q  R  I  F  R  M  A  P  I  H  H  H  Y  E  L

    gaaaggctggccggaaccgcgcgtcattgtgcgtttctggattatttcgctgatgctggt
1021 ---------+---------+---------+---------+---------+---------+
      K  G  W  P  E  P  R  V  I  V  R  F  W  I  I  S  L  M  L  V

    tctgattggtctggcaacgctgaaggtacgttaatcatggctgattatcagggtaaaaat
1081 ---------+---------+---------+---------+---------+---------+
      L  I  G  L  A  T  L  K  V  R  *
```

Figure 4.1.1. The nucleotide and amino acid sequences of the *mraY* ORF as predicted by Ikeda *et al.* (1990). Shown in bold is the ribosome binding site (ggaga) located upstream from the proposed start codon at 31 bp in the nucleotide sequence, also highlighted in bold.

Full Window 21   1
Core Window 11   Critical Length 60
Charge-pair Energy   0.0

Figure 4.1.3. The hydropathy profile of the proposed MraY peptide produced from the TopPred II program (Claros and von Heijne, 1994).

Figure 4.1.4. The transmembrane domains of the MraY peptide as predicted by the TopPred II program (Claros and von Heijne, 1994).

4.2.1. Construction of clones for the identification of the *MraY* peptide

Previous attempts to overexpress *mraY* and radiolabel the peptide *in vivo* failed to identify the expected 40 kDa peptide (Mengin-Lecreulx *et al.*, 1989; Ikeda *et al.*, 1991). The aim of my work was to identify the MraY peptide using the T7 RNA polymerase-driven expression vectors pT7-4 and pT7-6 (Figure 4.2.1.) developed by Tabor and Richardson (1986).
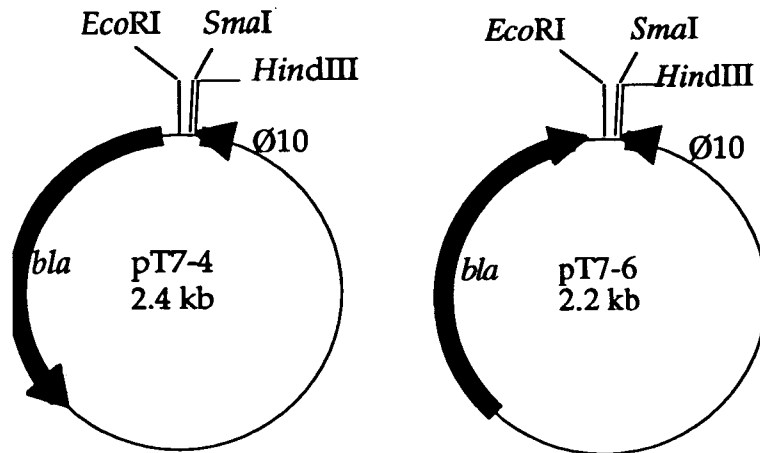

Figure 4.2.1. The T7 RNA polymerase-driven expression vectors pT7-4 and pT7-6.

Three clones of *mraY* were constructed in pT7-4 (Figure 4.2.2.). These clones were pDBY4 ('*murF mraY murD*'), pDEFY4 ('*ftsI murE murF mraY murD*') and pDIEFY4 ('*ftsL ftsI murE murF mraY murD*'). pDBY4 contains the 2.7 kb *Eco*RV fragment from pDEG1 cloned into the *Sma*I site in pT7-4. pDEFY4 was constructed by subcloning a 4.4 kb *Hind*III/*Bgl*II fragment from pDEG2 (see chapter 3.2.1.) into pT7-4. *ftsI* was introduced into pDEFY4 from pT7-4I. PT7-4I is an *ftsI* clone in pT7-4, a gift from T. Ogura. By restricting both pT7-4I and pDEFY4 with *Hind*III and *Kpn*I, a 1.9 kb fragment from pT7-4I was cloned into pDEFY4 to make pDIEFY4 ('*ftsL* to *murD*', see Figure 4.2.2.).
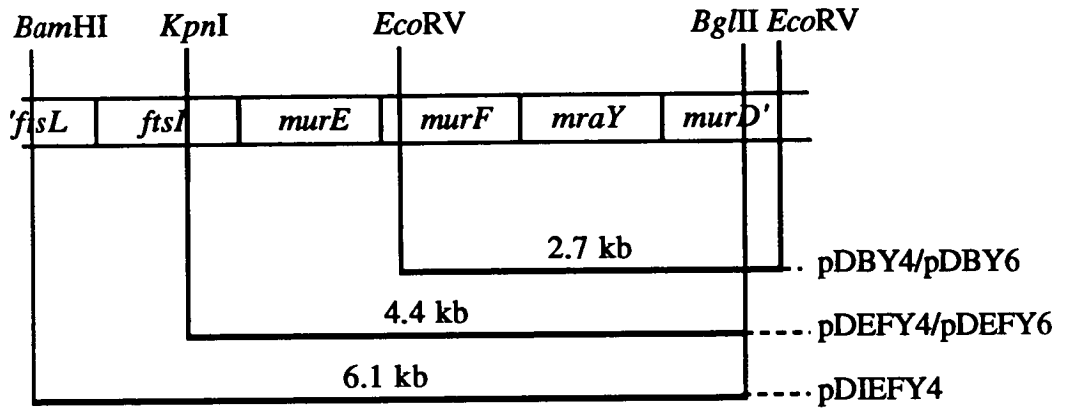
Figure 4.2.2. Clones for *mraY* overexpression (not to scale).

The pT7-6 vector also contains the gene10 promoter (see Figure 4.2.1.). It differs from pT7-4 in that the *bla* gene is orientated against the promoter; and therefore when transcription from the gene10 promoter is activated, it does not transcribe *bla* in the presence of rifampicin. Should the mobility of the MraY peptide in SDS-PAGE be similar to that of β-lactamase, then the use of pT7-6 for the overexpression of MraY would ensure that MraY was not masked by the products of the efficiently expressed *bla* gene. pDBY6 and pDEFY6 were constructed by subcloning the 2.7 kb and 4.4 kb inserts from pDBY4 and pDEFY4 respectively (Figure. 4.2.3. and 4.2.4).
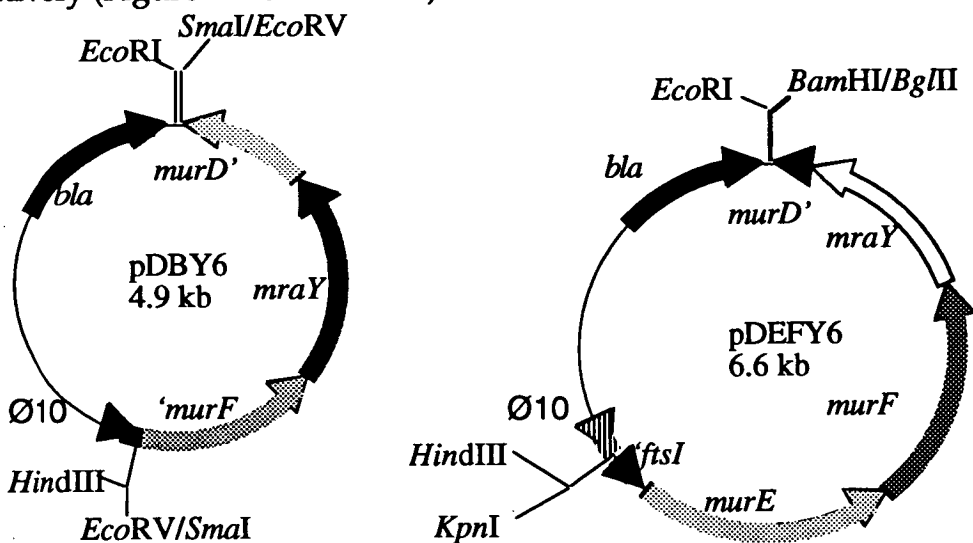
Figures 4.2.3. and 4.2.4. The pT7-6 clones used for overexpression of *mraY*, pDBY6 and pDEFY6.

4.2.2. Expression from T7 clones.

The host strain used for the over-expression of the various *mraY* clones, described in Figures 4.2.2. and 3., was BL21 (λDE3)/pLysS. The methods for expression and labelling are described in Chapter 2. Labelled protein samples, in SDS-PAGE sample buffer, were treated either by boiling for 5 minutes or incubation at 37°C for 1 hour. Samples were analysed on 10% SDS-PAGE gels. Controls with extracts of cells carrying pT7-4 and pT7-6 were used where appropriate. Figures 4.2.5.-13. show the results of these experiments. The clone pDBY4 did not produce any peptide with the predicted molecular size of 40 kDa (Figures 4.2.5. and 4.2.6.). No other bands, either faster or slower running were present. There was no apparent difference between protein profiles of the samples that had been boiled and those treated mildly at 37°C. Clone pDEFY4 produced two labelled peptides corresponding to the predicted molecular sizes for MurE and MurF proteins, respectively. Again there was no band which could correspond to the MraY peptide in either boiled or 37°C treated samples (Figures 4.2.7. and 4.2.8.). Similar results were obtained using pDIEFY4, except for an additional band at ~60 kDa which corresponds to PBP3, encoded by *ftsI*. (Figures 4.2.9. and 4.2.10.).

The use of pDBY6 and pDEFY6 to produce the MraY peptide also failed (Figures 4.2.11.-13.). The absence of the β-lactamase protein from the SDS-PAGE gels reveals that MraY does not have an unusual migration rate in SDS-PAGE whereby it is obscured by the presence of other labelled peptides. The results from the labelling of pDBY6 can be seen in Figures 4.2.11. and 12. Labelled protein samples from BL21 (λDE3)/pLysS/pDEFY6 also did not produce a labelled band expected for MraY. Bands corresponding to 53 kDa and 47 kDa were detected after SDS-PAGE analysis, these are for the MurE and MurF peptides respectively (Figures 4.2.13.). Once more the denaturing conditions did not affect the peptide profile. The presence of the MurE and MurF peptides from pDEFY6 shows that overexpression and labelling of peptides was efficiently carried out by the cells in this experiment.

The overproduction of MraY using these vectors was therefore unsuccessful irrespective of the denaturing conditions used. The presence or absence of long sequences of upstream DNA has no effect on the production of MraY polypeptides.
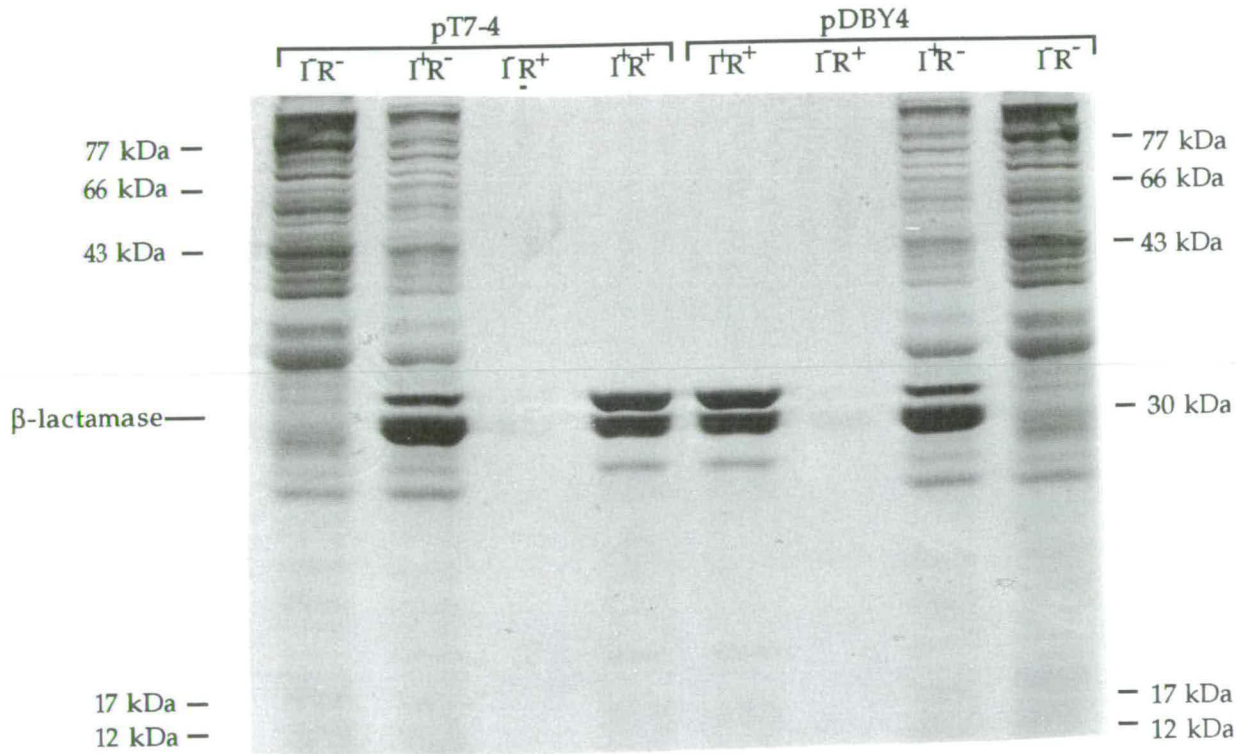
Figure 4.2.5. The SDS-PAGE analysis of pDBY4 samples treated at 37°C. The lanes 1-4 show the pT7-4 control and 5-8 are the pDBY4 samples. Labelled lanes 1, 2, 7 and 8 where rifampicin was not added show general labelling of the peptides produced by the cell. In lanes 3 and 6 rifampicin has been added to the culture but there is no induction with IPTG. This is reflected by the absence of any labelled peptides. In lanes 4 and 5 there are labelled peptide bands at ~31 kDa and ~29 kDa. These are the precursor and mature β-lactamase proteins, respectively. In lane 5 there are no other bands, notably at ~40 kDa. If MraY had been labelled it should also be visible unless it migrated the same as the β-lactamase proteins.
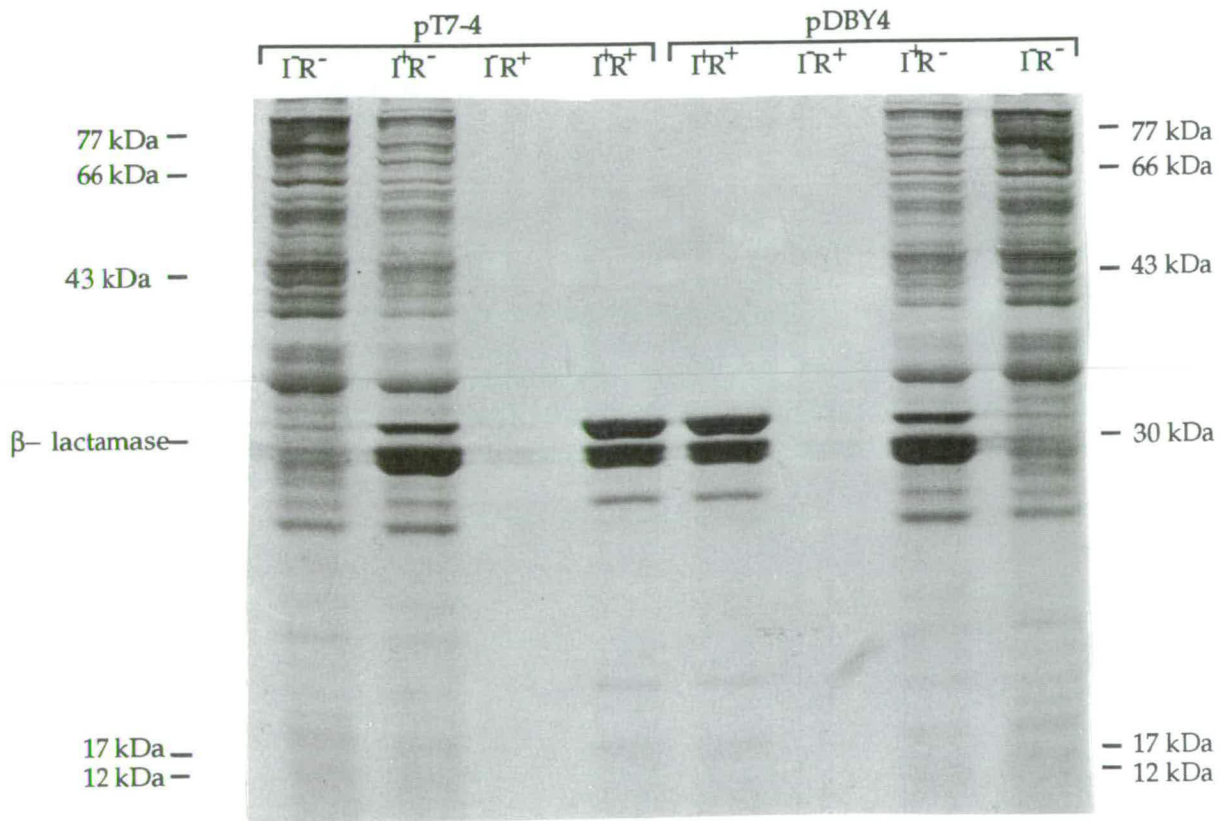
Figure 4.2.6. The 100°C treated samples from pT7-4 (lanes 1-4) and pDBY4 (lanes 5-8) after SDS-PAGE analysis. The figure shows both sets of samples have the same profile as the samples treated at 37°C in figure 4.3.5.. There is no difference between the induced and Rif treated pT7-4 and pDBY4 samples shown in lanes 4 and 5. The presence of the β-lactamase bands shows over-expression and labelling was achieved but that the predicted 40 kDa MraY was not produced using pT7-4.
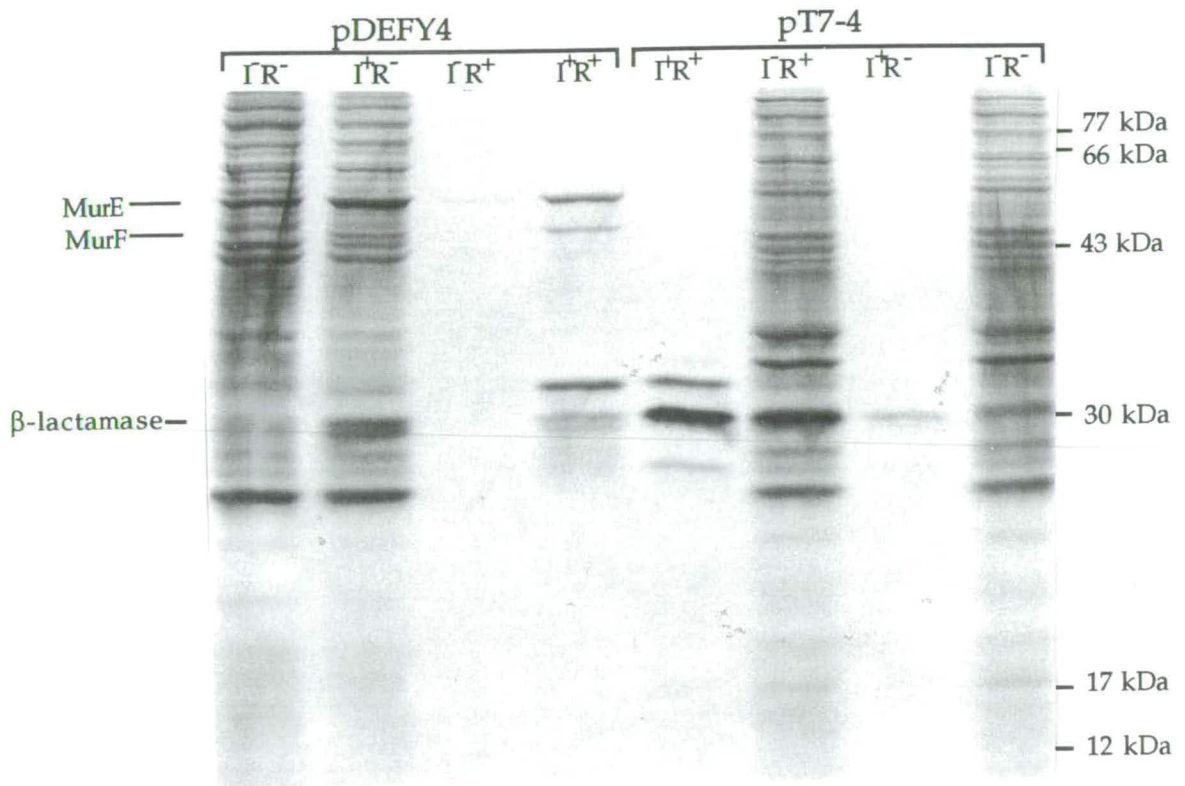
Figure 4.2.7. The over-expression and labelling of pDEFY4 and pT7-4 analysed by SDS-PAGE after 37°C treatment of samples. Lanes 1-4 contain pDEFY4 samples and lanes 5-8, the pT7-4 samples. The rifampicin free cultures show the labelled cell extracts in lanes 1, 2, 6 and 8. The uninduced samples treated with rifampicin (lanes 3 and 7) show no labelled peptides. The induced and Rif. treated (I^+R^+) pDEFY4 (lane 4) has bands corresponding to the β–lactamase proteins and two heavier peptides at 53 kDa and 47 kDa. These are the MurE and MurF peptides, respectively. By comparison pT7-4 only shows the two forms of β–lactamase. Therefore there was expression from gene 10 and efficient labelling of the peptides although the 40 kDa MraY peptide is not present.
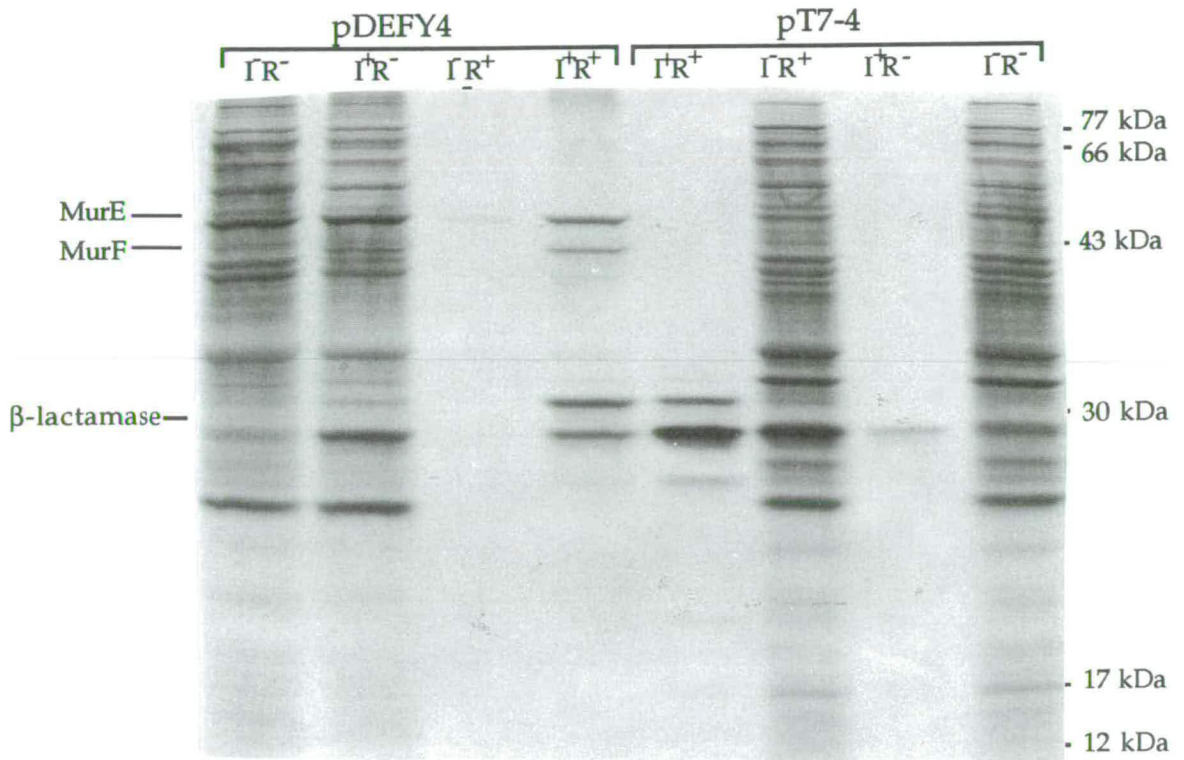
**Figure 4.2.8.** The 100°C treated samples of pDEFY4 and pT7-4 after SDS-PAGE analysis. Lanes 1-4 contain the pDEFY4 samples and 5-8 the pT7-4 samples. The profile is almost identical to Figure 4.2.7., where the samples were treated at 37°C. The peptides visible from the induced and Rif treated samples are the same. That is β–lactamase, MurE and MurF are produced and labelled from pDEFY4 in lane 4. There is no peptide at ~39 kDa. pT7-4 only produced β–lactamase (lane 5)
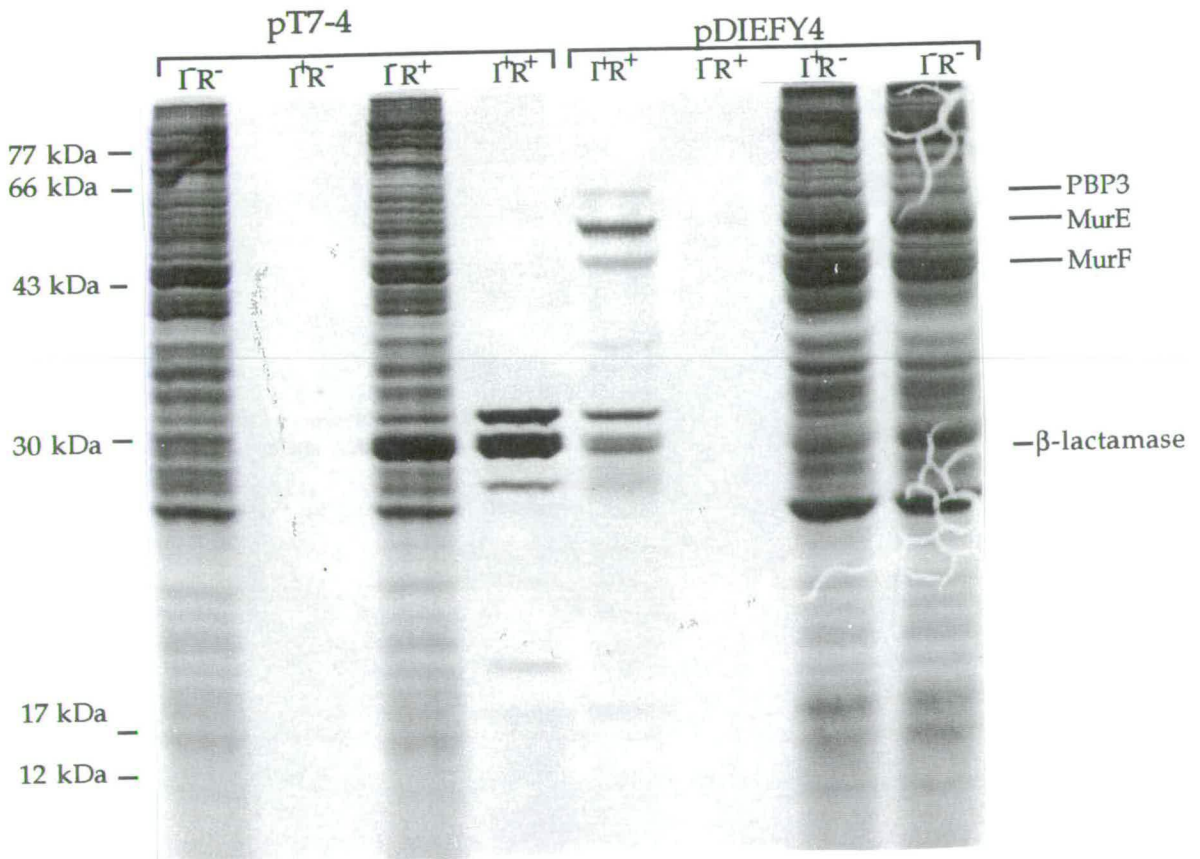
Figure 4.2.9. SDS-PAGE analysis of pDIEFY4 and pT7-4 samples treated at 37°C. The samples of the control pT7-4 are in lanes 1-4 and pDIEFY4 in lanes 5-8. The gel is essentially similar to the pDEFY4 samples treated at 37°C, with the induced and Rif treated samples producing proteins corresponding to the β–lactamase peptides and the MurE and MurF peptides at 29 kDa, 31 kDa, 53 kDa and 47 kDa respectively. Another band at 62kDa is present in lane 5. This is PBP3, produced from *ftsI*.
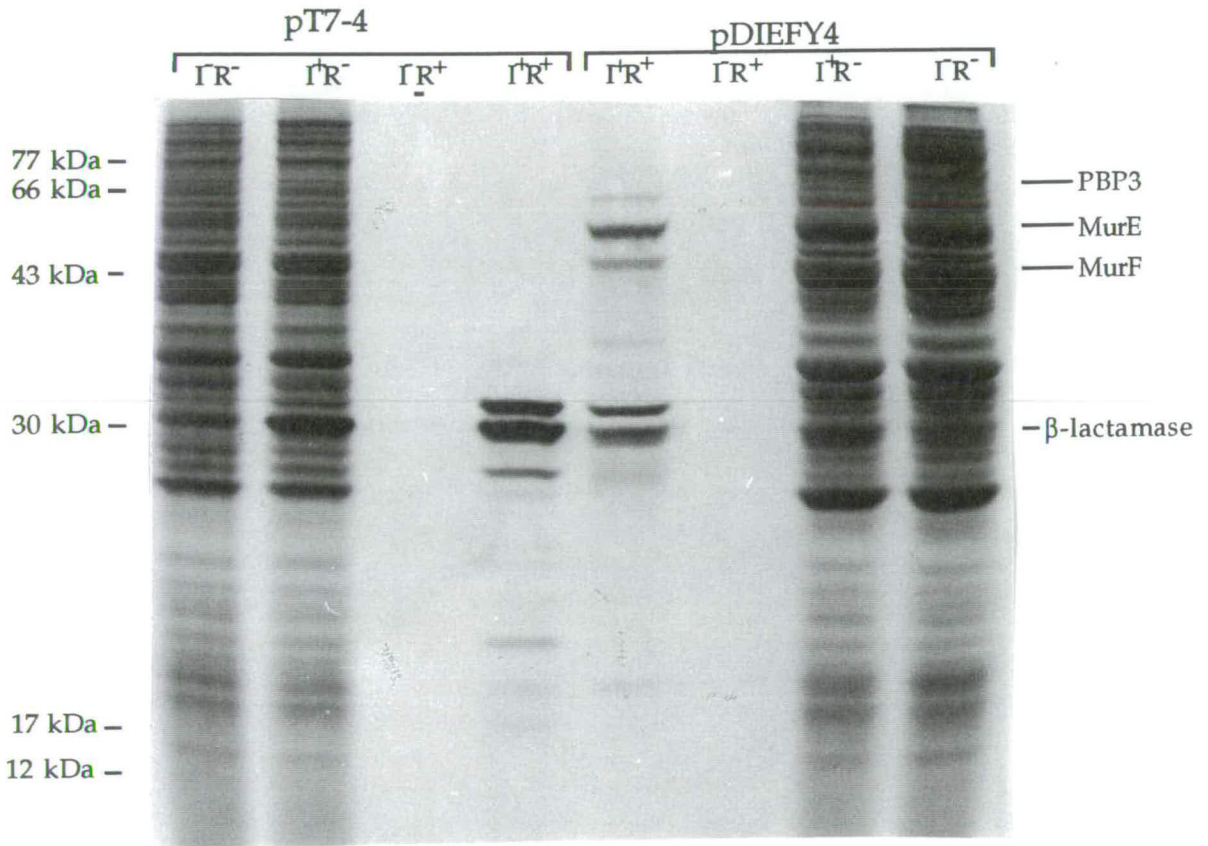
Figure 4.2.10. The 100°C treated samples of pDIEFY4 and pT7-4 analysed by SDS-PAGE. pT7-4 samples are in lanes 1-4 and pDIEFY4 are in lanes 5-8. The 100°C treated pDIEFY4 samples when analysed by SDS-PAGE were the same as the 37°C samples. That is β–lactamase, MurE, MurF and PBP3 peptides were isolated from the induced and Rif treated sample in lane 5. There was no peptide corresponding to the predicted size for MraY.
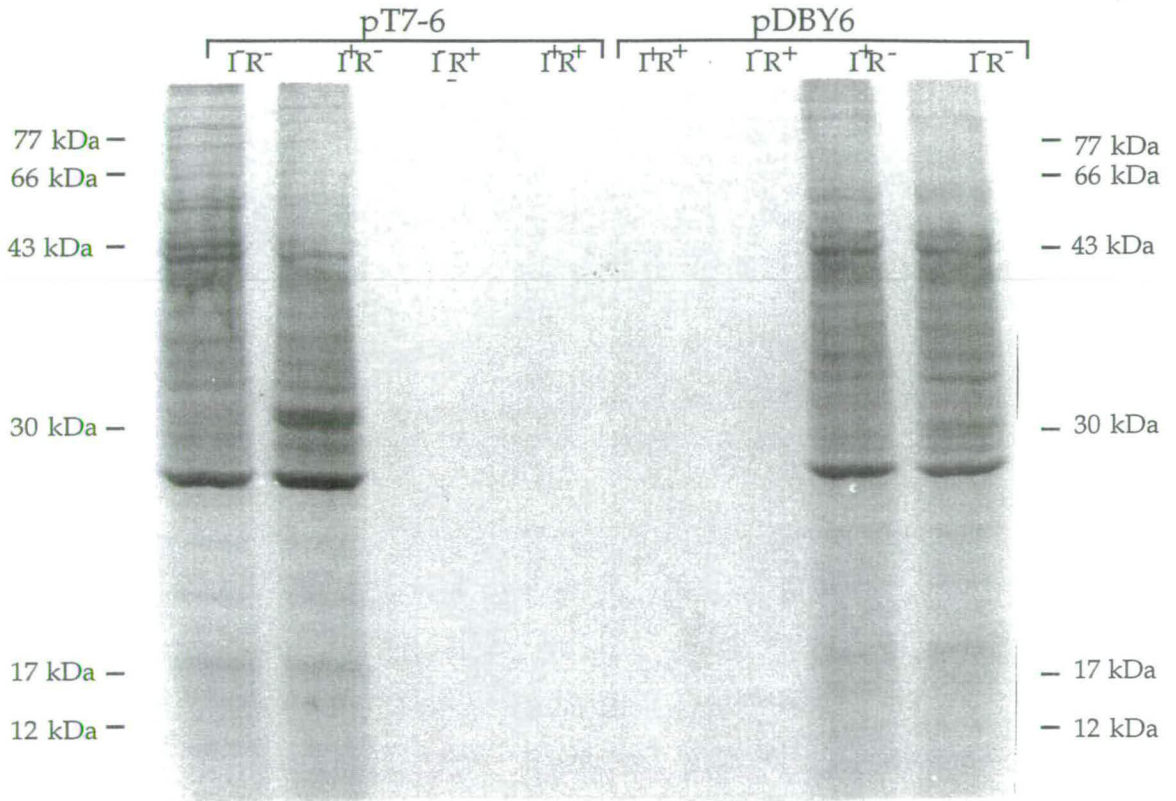
Figure 4.2.11. The SDS-PAGE analysis of the pT7-6 (lanes 1-4) and pDBY6 (lanes 5-8) samples treated at 37°C. The samples not treated with Rif are labelled (lanes 1, 2, 7 and 8). The uninduced samples are free of any peptide (lanes 3 and 6). The induced and Rif treated samples also show no evidence of a peptide being produced with either pDBY6 or pT7-6 (lanes 4-5).
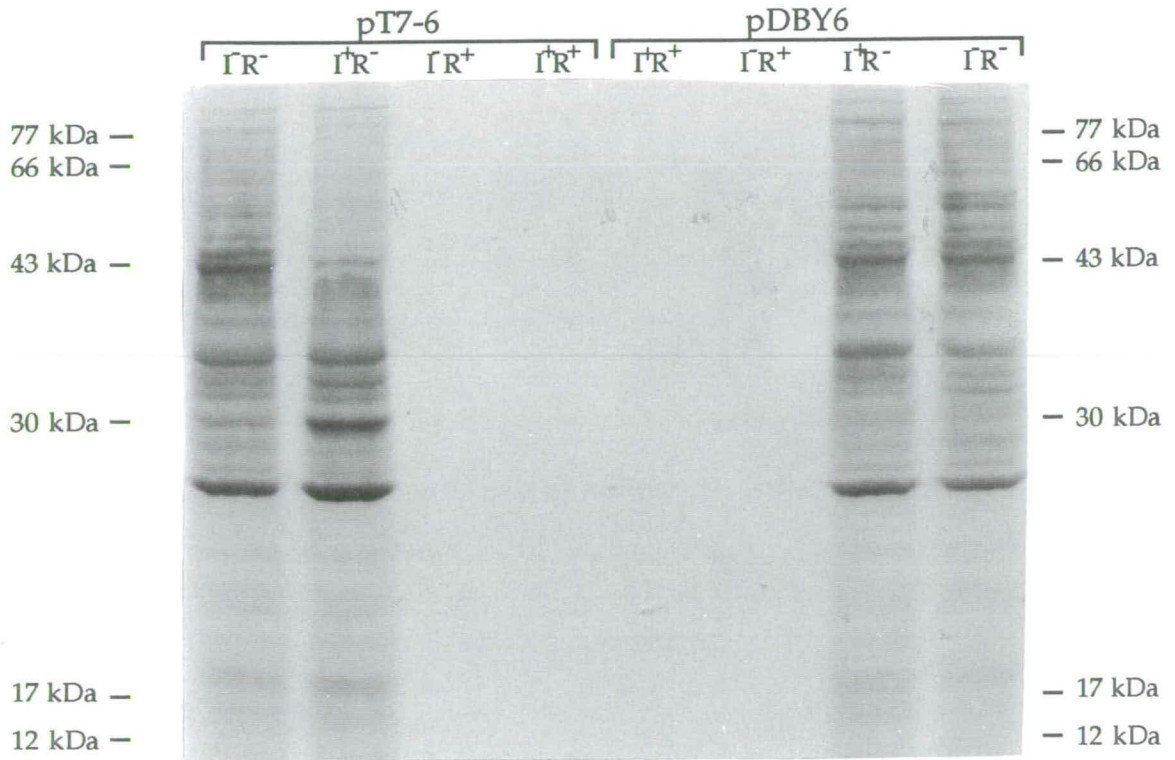
Figure 4.2.12. The samples of pDBY6 and pT7-6 treated at 100°C and analysed by SDS-PAGE. Both pT7-6 (lanes 1-4) and pDBY6 (lanes 5-8) samples treated at 100°C did not produce any labelled peptides from the profile of the induced and Rif treated samples (lanes 4 and 5 for pT7-6 and pDBY6 respectively). The results are the same as for the 37°C treated samples (Figure 4.2.11) with no MraY peptide visible in the induced and Rif treated pDBY6 sample (lane 5).
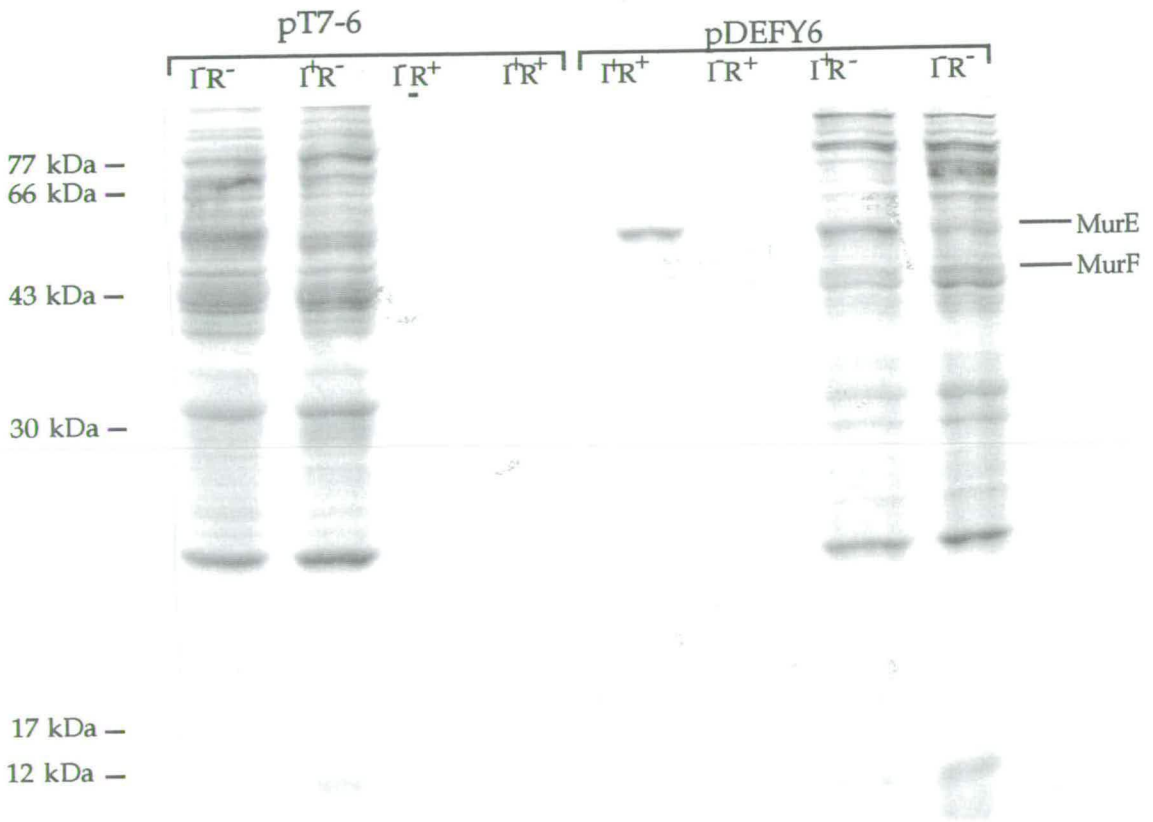
Figure 4.2.13. The SDS-PAGE analysis of 37°C treated samples of the control pT7-6 (lanes 1-4) and pDEFY6 (lanes 5-8). The induced and Rif treated sample of pDEFY6 showed two bands (lane 5) corresponding to 53 kDa and 47 kDa in size. These are the MurE and MurF peptides respectively. The similar treated PT7-6 control did not produce any labelled peptides (lane 4). Therefore the MraY peptide was not obscured by either of the two forms of β–lactamase produced.

4.2.3. pET-3c *mra*Y fusions to improve expression.

The gene 10 protein, the major capsid protein of T7, is synthesized more rapidly than any other protein during infection and at a rate higher than any host protein (Rosenberg *et al.*, 1987). The vector pET-3c (plasmid Expressing T7 RNA polymerase, Rosenberg *et al.*, 1987) contains the promoter region and the ribosome binding site (RBS) and the first 11 codons of T7 gene 10 (Figure.4.2.14.). An *Nde*I site at the gene 10 start codon allows the cloning of coding sequences of interest such that a fusion between gene 10 and the sequence of interest is created. A fusion of the *mra*Y open reading frame to the rbs region of gene 10 of T7 phage was constructed as follows.

```
RBS              NdeI                                            BamHI
TAA GAA GGA GAT ATA CAT ATG GCT AGC ATG ACT GGT GGA CAG CAA ATG GGT CGG ATC
                     M   A   S   M   T   G   G   Q   Q   M   G   R   I
```

Figure 4.2.14. The nucleotide and amino acid sequence of the gene 10 fusion region in pET-3c.

An *Nde*I site was introduced at the *mra*Y start codon using a primer with the altered sequence, DAV2 (Figure 4.2.15.). Another primer was made for the opposing strand 1286 bp downstream, DAV3. This is at the *Bgl*II site in *murD* (Figure 4.2.15).

```
                        NdeI
DAV2     5' GGA GAA TGG CAT ATG TTA GTT TGG  3'


                        BglII
DAV3     5' CAA TCA GAT CTG CCG CCA  3'
```

Figure 4.2.15. Primers DAV2 and DAV3 used to amplify the open reading frame of *mra*Y. DAV2 is a mutagenic primer resulting in the introduction of *Nde*I recognition sequence at the start of *mra*Y. Substituted nucleotide bases are in bold.

The two oligonucleotides, DAV2 and DAV3, were used to amplify the open reading frame (ORF) of *mra*Y using the polymerase chain reaction (PCR) as described in Chapter 2. DAV2 was designed to introduce an *Nde*I recognition sequence at the start of *mra*Y whilst DAV3 overlaps the *Bgl*II restriction site in the

downstream gene, *murD*. The 1.3 kb fragment was amplified using pDEG1 as a template, and was restricted with *Nde*I and *Bgl*II. The restricted PCR fragment was then cloned into the *Nde*I and *Bam*HI sites of pET-3c. The cloning produced a 5.7 kb plasmid, pETY3c. BL21 (λDE3)/pLysS was used as the host for overexpression of pETY3c as the pET-3c gene 10 promoter is recognized by T7 RNA polymerase. The cloned insert was overexpressed and cell extracts were prepared as described in materials and methods. The samples were again treated at 37°C or 100°C prior to SDS-PAGE analysis. Neither treatment produced a ~40 kDa peptide (Figures 4.2.16. and 4.2.17.). The expression and SDS-PAGE analysis was again repeated with another 4 different pETY3c clones and once more no peptide was apparent. This was repeated with the same result.

The pETY3c clones, however, did have an effect on the growth rate of the cells. The BL21 (λDE3)/pETY3c strain grew as small colonies (0.5 mm in size) after overnight incubation on LB Cmp/Amp agar plates. Control cultures of BL21(DE3)/pLysS carrying pET3c produced larger colonies (1-1.5 mm). When the pETY3c BL21 (DE3) transformants were grown in either LB or Spizizen's broth the growth rate slowed by half. That is BL21 (DE3)/pLysS with pET3c had a doubling time of 30 minutes in LB Amp/Cmp whilst the pETY3c transformants had a doubling time of 60 minutes (Figure 4.2.18.). There were no morphological differences between cells from the two cultures, even in the presence of the IPTG inducer. Host strains which did not produce T7 RNA polymerase were unaffected by harbouring pETY3c.
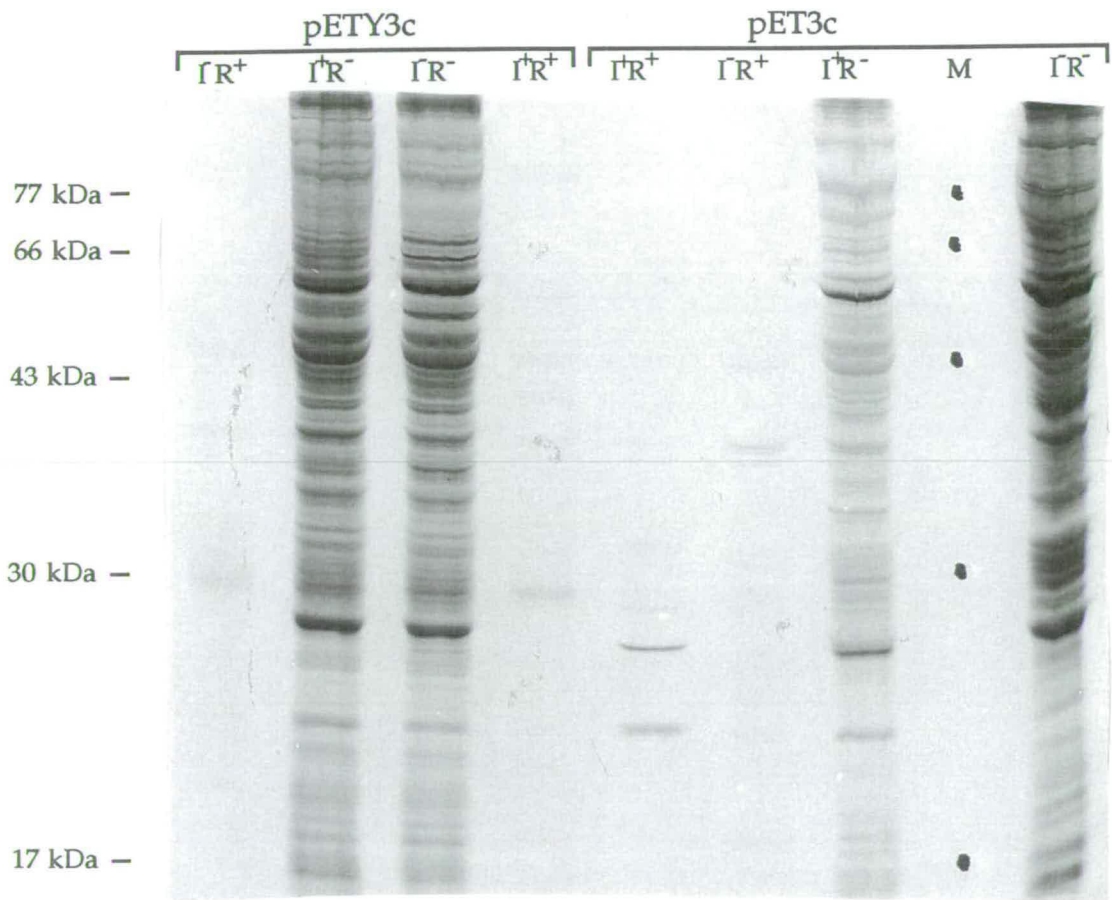
Figure 4.2.16. The SDS-PAGE analysis of the pETY3c 37°C samples. These are shown in lanes 1-4. The pET3c controls are in lanes 5-7 and 9. Marker proteins were run in lane 8. The absence of Rif allows the expression and labelling of all transcripts present in the cell (lanes 2, 3, 7 and 9). The use of Rif without induction produces no peptides in either sample (lanes 1 and 6). With induction and Rif there are no peptides which can be seen in lane 4 of the pETY3c sample. Therefore MraY has not been labelled. The induced and Rif treated pET3c sample (lane 5) produced two small peptides with migration rates of 25 and 21 kDa proteins. These may be "run on" peptides produced from the gene 10 start codon until the T7 RNA polymerase terminator sequence downstream.
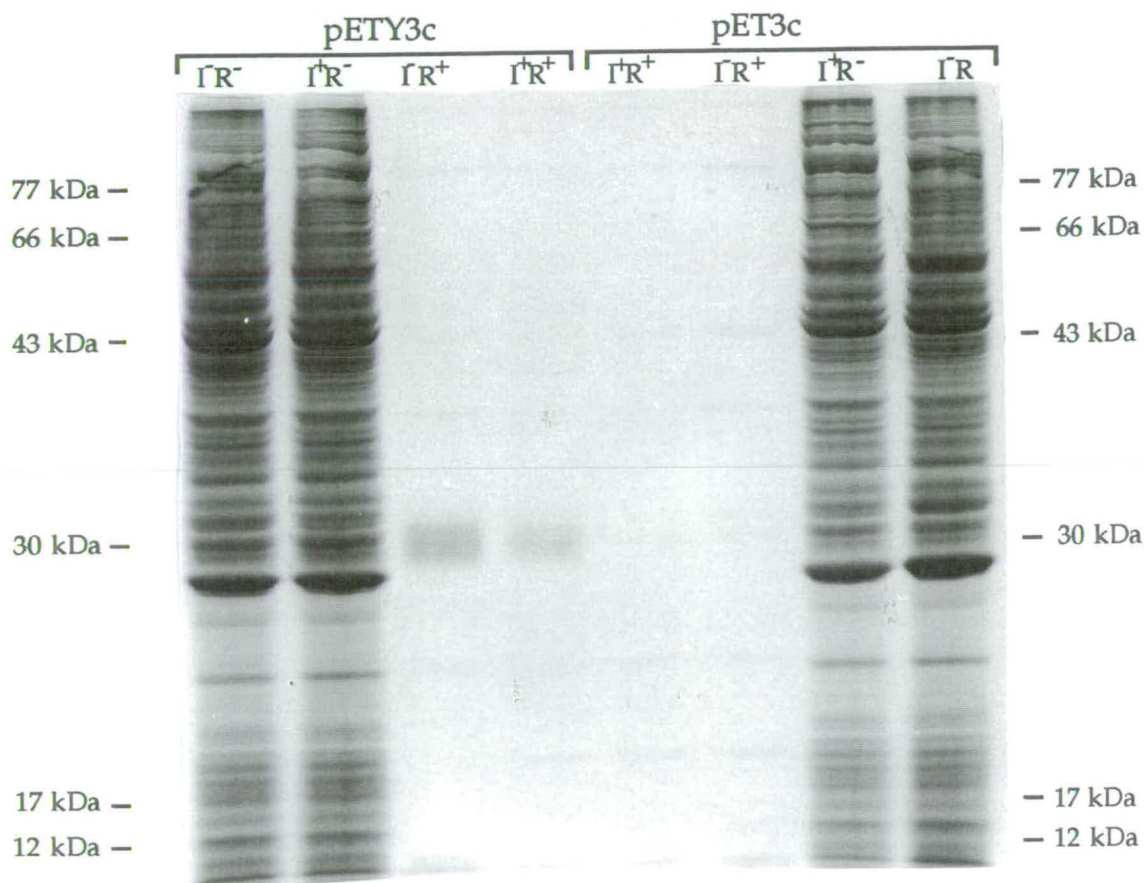
Figure 4.2.17. The SDS-PAGE analysis of the pETY3c samples treated at 100°C prior to analysis. These are shown in lanes 1-4. The pET3c controls are in lanes 5-8. The absence of Rif allows the expression and labelling of all transcripts present in the cell (lanes 1, 2, 7 and 8). The use of Rif without induction produces no peptides in the pET3c control in lane 6. With pETY3c, in lane 3 there is a smeared band at 30 kDa. The induced and Rif treated pETY3c sample also produced the same band (lane 4). As this band is in both induced and uninduced samples pETY3c it cannot be the MraY protein. The induced and Rif treated pET3c control lane has no labelled proteins. The peptides of 25 and 21 kDa visible in the Figure 4.2.16 are not present. Therefore, these are probably degraded by the boiling of the samples.
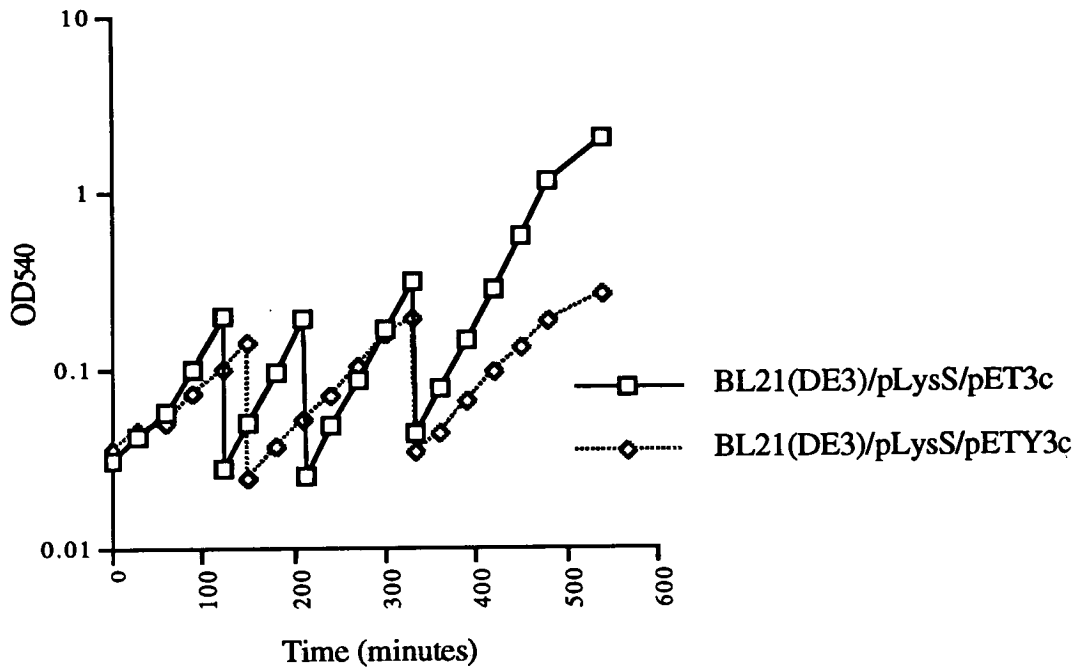
Figure 4.2.18. The effect on growth rate of pETY3c when grown in BL21(DE3)/pLysS.

4.2.4. Sequencing of the gene 10/*mraY* fusion junction.

Nine different pETY3c clones had failed to produce any peptide and therefore the junction at the gene10/*mraY* fusion was determined by sequencing to ensure that the expression of *mraY* was not curtailed by an error in the oligonucleotide sequence or that the PCR product was not in frame with the gene *10* start in pET3c. A 1.5 kb *BglII/PstI* fragment from pETY3c was cloned into *BglII/PstI* restriction sites in the polylinker of M13mp19. This was MY19. Single stranded DNA was prepared (see Chapter 2) and sequenced using universal primer 1201 (New England Biolabs). The region of the fusion between the gene10 rbs and *mraY* was sequenced and no irregularities were found in the 140 bp region sequenced (Data not shown).

4.2.5. Construction of a gene 10/MraY hybrid protein.

Overexpression of the gene 10/*mraY* fusion in pET3c did not produce the MraY peptide. However, the clones did have a deleterious effect on the growth rate of the cells and sequencing over the junction of the fusion has shown that the sequence was inserted correctly; that is *mraY* is cloned in frame with the gene 10 start codon. It is clear that the overexpression of *mraY*, therefore, is deleterious to the cell and the MraY peptide might be subject to proteolytic degradation. It was thought that the construction of other fusions might overcome the deleterious effects of MraY overproduction and might lead to stabilization of the peptide.

Figure A   5' C G **G A T C C** 3'
              3' G C C T A G **G** 5'

Figure B   5' C G **G A T C G C T** 3'
             3' G C C T A G **C G A** 5'

Figure 4.2.19. The *Bam*HI site in pET-3c (Figure A). Figure B represents the 5' end of the *Sau*3AI truncation (bold) cloned into the *Bam*HI site of pET-3c.

*Nde*I   *Bam*HI/*Sau*3AI

CAT ATG GCT AGC ATG ACT GGT GGA CAG CAA ATG GGT CGG ATC GCT CGT TGG
  M   A   S   M   T   G   G   Q   Q   M   G   R   I   A   R   W

Figure 4.2.20. Nucleotide and amino acid sequences at the protein start point in pE'Y3c. The '*mraY* nucleotide and aa sequence is in bold.

A 900 bp *Sau*3AI fragment containing the C terminus of *mraY* and the N-terminus of *murD* was subcloned into the *Bam*HI site in pET-3c (Figures 4.2.19. and 4.2.20.) from pDBY4. This clone was pE'Y3c. This creates a translational fusion between the N-terminus of gene 10 (11 aa) and the C-terminus of MraY (232aa). The resulting gene should encode a hybrid protein of 241aa with a molecular weight of 28 kDa. A second peptide of 12 kDa was expected from this clone; this is the N-terminus of MurD.

BL21 (λDE3)/pLysS was transformed with pE'Y3c and the colony size was the same as those carrying the control vector pET3c, that is 1-1.5 mm. The culture grew at the same rate as the pET3c transformants and therefore the deleterious effect, observed with BL21 (λDE3)/pLysS/pETY3c, was lost with this

particular clone. Radiolabelled cell extracts were prepared as before and samples treated by incubation at 37°C for one hour. The samples were analysed on 10% SDS-PAGE gels with pET3c used as a control. The gel show bands at 30 kDa and 16 kDa (Figure 4.2.21.). The smaller band represents the truncated MurD peptide (the MurD' peptide). The larger band at 30 kDa is probably the gene 10-MraY peptide although the rate of migration is less than expected for a polypeptide with a calculated molecular weight of 28 kDa.
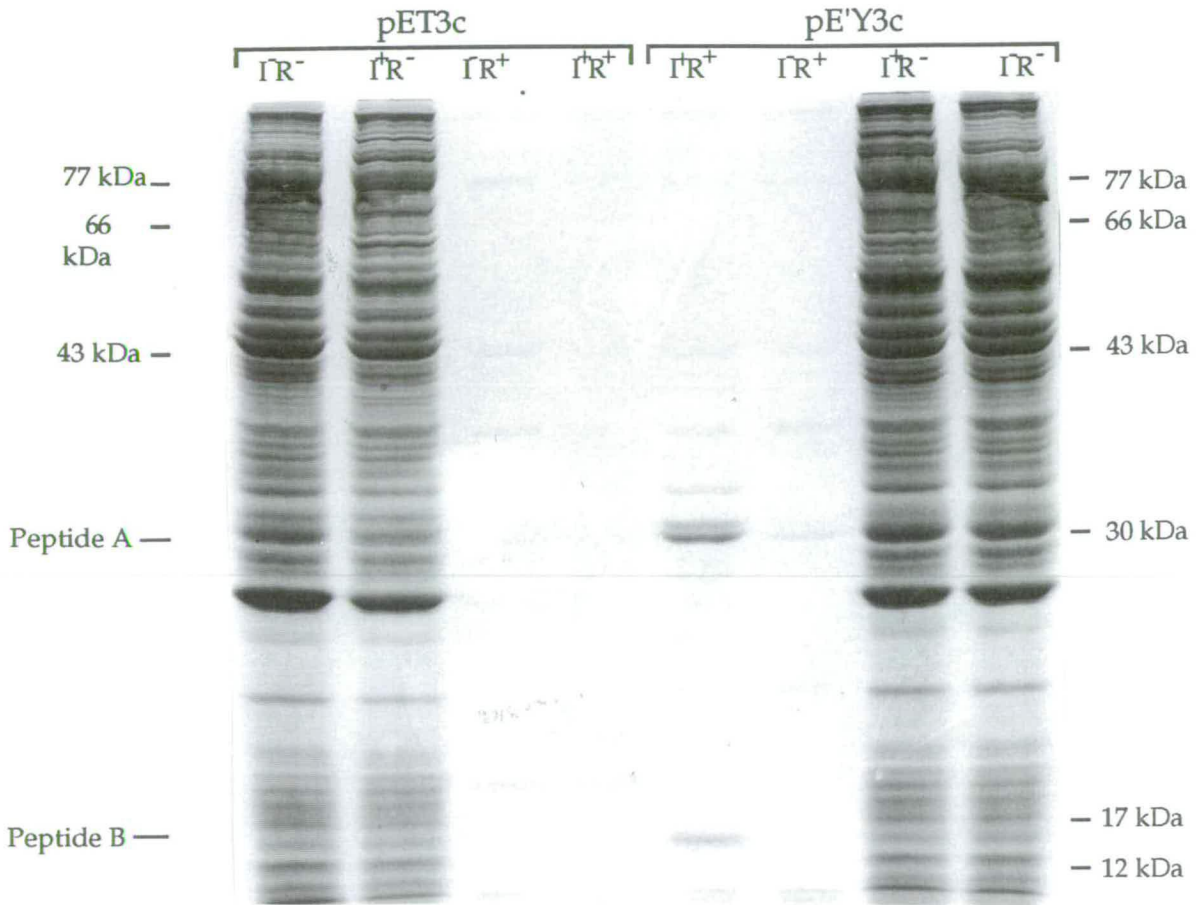
Figure 4.2.21. SDS-PAGE analysis of pE'Y3c samples treated at 37°C (lanes 5-8). As a control pET3c was used (lanes 1-4). The induced and Rif treated sample of pE'Y3c in lane 5 shows two peptides present. The larger band migrates as a ~30 kDa peptide, the calculated size for the fusion peptide was 28 kDa. Therefore this may be the fusion product. However, these bands do have a striking similarity to the β-lactamase proteins. A smaller peptide of ~16 kDa is also visible, this may be the truncated form of the MurD peptide. The pET3c control did not produce any labelled peptides.

4.2.6. The stability of the MraY peptide.

The various attempts to overexpress *mra*Y have not resulted, so far, in the visualisation of a peptide. There are several possible events that might prevent detection or expression of any peptide. The first is instability of the mRNA although Novagen report that target mRNA substantially accumulates when using T7 RNA polymerase. Alternatively the peptide itself may affect the viability of the cell. Although all of the other *mra*Y clones tested did not appear to affect the growth rate of the host culture. pETY3c has been shown to affect the growth rate of cells (Figure 4.3.18.), although these cells appeared normal when observed microscopically. Another possibility is that the production of the peptide may prevent further translation. A fourth possibility is that the cell may degrade the protein (although BL21 is a double mutant for both Lon and OmpT proteases). An important factor in determining the stability of protein is the the second amino acid at the N-terminus (Hirel *et al.*, 1989). That is the aminoacyl residue after the start methionine residue. The penultimate amino acid determines whether the N-terminal Met is removed. This cleavage is catalysed by methionyl aminopeptidase. The rate of cleavage of Met decreases inversely to the size of the side chain of the second amino acid (Hirel *et al.*, 1989). The relationship between protein stability and the primary N-terminal amino acid in bacteria was examined by Tobias *et al.* (1991). For proteins with Leu, Trp, Lys, Phe, Arg and Tyr as the second N-terminal amino acids, the protein half life was only 2 minutes. Examination of the MraY peptide sequence reveals that Leu is the second N-terminal amino acid in both the wild type sequence and the PCR fusion sequence (Figures 4.2.22. and 4.2.23.). The predicted effect of this is two fold. Leu has a long (4C) side chain and therefore it is likely that the Met is cleaved from the peptide. This in turn leaves Leu as the N-terminal residue which could lead to rapid proteolytic degradation.

5' ACA GGA GAA TGG GAC ATG TTA GTT TGG CTG 3'

                                  M   L   V   W   L

Figure 4.2.22. The nucleotide sequence (rbs underlined) and N-terminus of *mra*Y.

5' TAA GAA GGA GAT ATA CAT ATG TTA GTT TGG CTG 3'

                      M   L   V   W   L

Figure 4.2.23. The nucleotide sequence (rbs underlined) and N-terminus of the gene 10/*mraY* fusion.

4.2.7. Construction of a His-tag fusion of *MraY*

The presence of Leu as the second amino acid may be the reason why MraY is not being detected in the expression/labelling experiments. An alternative approach to the production of MraY would be to have a different second amino acid. This could be achieved by mutagenizing the coding sequence to insert a different amino acid (e.g. Serine) or alternatively make a fusion protein where Leu is not the second amino acid. The latter process was chosen and the PCR product of *mraY* with the *Nde*I site at the start site was cloned into the His-tagging vector pET16b (Novagen). This vector permits an N-terminal fusion to a polyHis-tag. The promoter of this vector is, once again, the gene 10 promoter recognized by T7 RNA polymerase. By using this vector the second amino acid is a glycine residue from the start of T7 gene10 (Figure.4.2.24.). In addition the MraY peptide produced should be purifiable by binding the His-tag to a nickel-agarose column (Hoffman and Roeder, 1991). A 1.6 kb *Nde*1/*Hind*III fragment from pETY3c was therefore cloned into pET16b to create pEHY16 (size 7 kb).

          rbs   *Nco*I

5' TTA AGA AGG AGA TAT ACC ATG GGC CAT (CAT)$_7$ CAC AGC AGC GGC

                                         M   G   H   (H)$_7$ H   S   S

                      *Nde*I

CAT ATC GAA GGT CGT CAT ATG TTA GTT TGG CTG 3'

 H   I   E   G   R   H   **M**   **L**   **V**   **W**   **L**

Figure 4.2.24. Nucleotide and peptide sequence at the start of pEHY16 showing the polyHis-tag and the start of the MraY peptide(in bold).

BL21 (λDE3)/pLysS was transformed with pEHY16. The transformants were then used for labelling experiments and the labelled cell extracts were incubated at 37°C for one hour. The samples were analysed on 10% SDS-PAGE gels and examined for the presence of any peptides. BL21 (λDE3)/pLysS transformed with pET16b was also induced and labelled as a control. There were no bands to be seen on the gels after exposure (Figure 4.2.25.). The absence of a peptide suggests that instability resulting from the Leu at position 2 is not the cause of any instability. The purification of the peptide by collection in a nickel agarose column was not attempted as no protein had been detected by radiolabelling. Instead another *mraY* fusion peptide was engineered, on this occasion with *murF*.
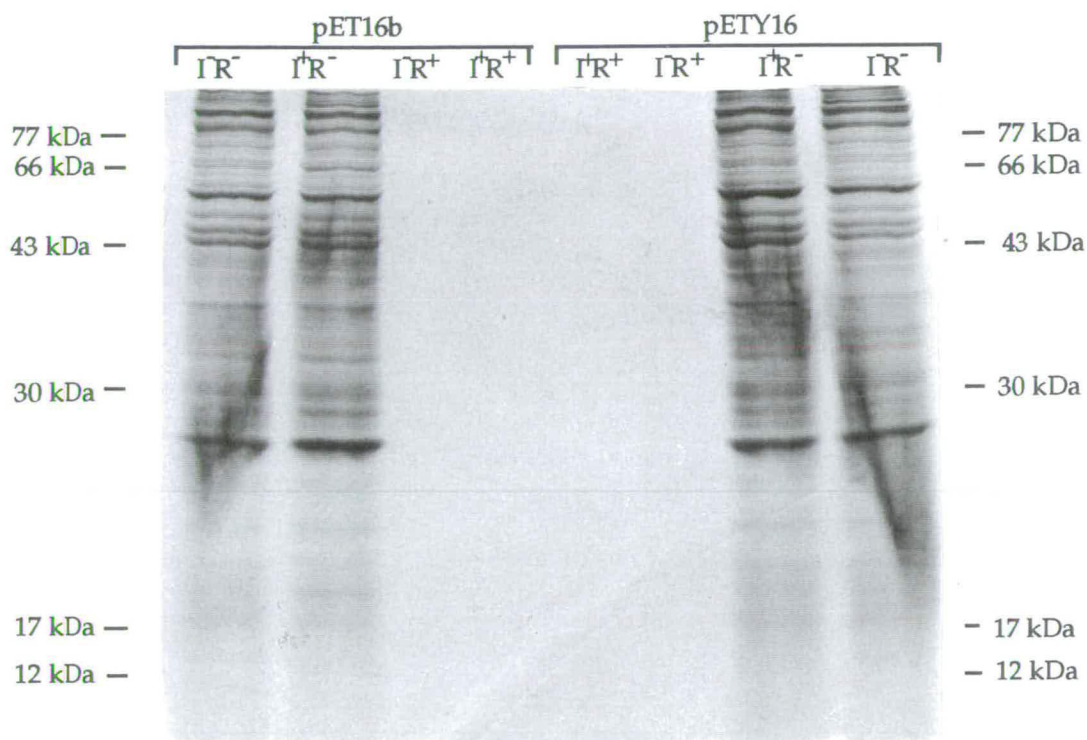
Figure 4.2.25. The results of SDS-PAGE analysis of pET16b control (lanes 1-4) and pETHY16 (lanes 5-8) when treated at 37°C. It is apparent thast no peptide has been produced from the *in vivo* labelling of pETY16b. The induced and Rif treated sample (lane 5) shows no peptide bands. Similarly the pET16b control also shows no peptides when induced and treated with Rif (lane 4).

4.2.8. Construction and overexpression of a *murF-mraY* fusion.

The expression and radiolabelling of pDEFY4 in BL21 (λDE3)/pLysS reveals two peptides of 53 kDa and 47 kDa when the products are separated by SDS-PAGE. The larger peptide is MurE and the smaller, MurF. In this clone there is a unique *Nco*I site at 1313 bp in the *murF* reading frame. The *mraY* His-tag fusion has an *Nco*I site (Figure 4.2.24.) which if cloned into the *murF Nco*I site remains in frame with *murF*. Therefore pDEFY4 was restricted with *Nco*I and *Eco*RI, and pEHY16 was partially digested with the same enzymes. A 1.7 kb fragment from pEHY16 was subcloned into the corresponding sites in pDEFY4. This created pDEFHY1, a plasmid of 7.1 kb (Figure 4.2.26). The new fusion gene has an open reading frame of 2460 nucleotides, coding for a peptide of 820aa with a calculated molecular size of of 88 kDa. The MurE protein is also expressed within this plasmid and serves as an internal control for expression from the construct. BL21 (λDE3)/pLysS was transformed with pDEHFY1 and pDEFY4. The cells were induced, labelled and the protein samples denatured in sample buffer by treating at 100°C for 5 minutes or at 37°C for one hour. The samples were analysed on 7.5% SDS-PAGE gels and exposed to film (Figures 4.2.26. and 4.2.27.).
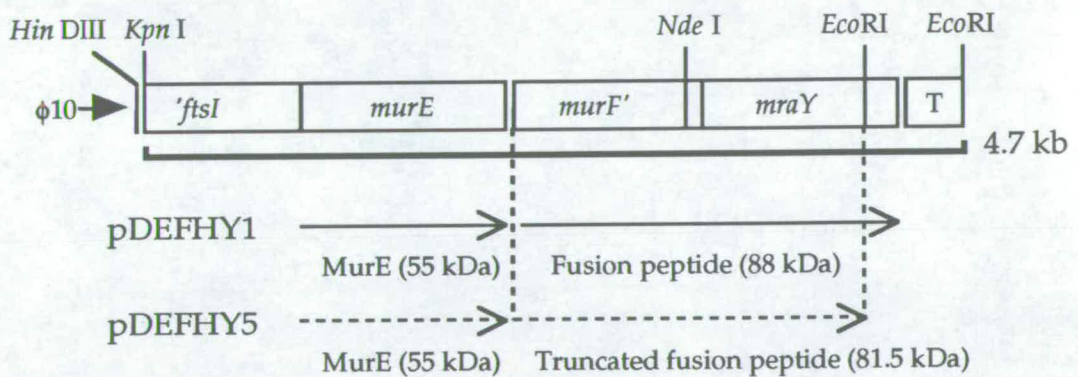

Figure 4.2.26. A schematic diagram showing the 4.7 kb region containing *'ftsI murE* and the *murF polyHis mraY* chimera cloned in pT7-4, pDEFHY1 (not to scale). The φ10 promoter (filled arrowhead), polyHis tag (shaded) and the φ10 terminator region (T) are also shown. The calculated molecular weights of the two peptides expressed from this clone are also shown. Also presented above are the two peptides (dashed arrows) expressed from the cloned region in pDEFHY5. These are MurE and a truncated chimera (81.5 kDa) which lacks the C-teminus of MraY.
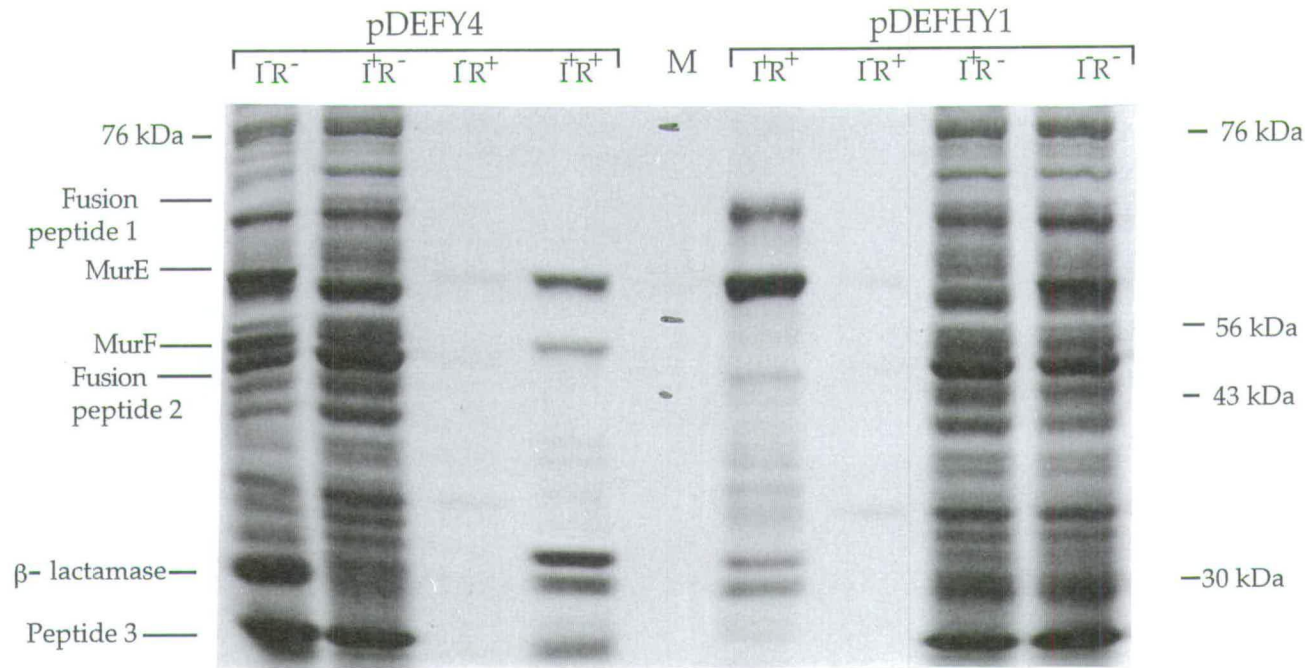
Figure 4.2.26. The SDS-PAGE analysis of the control pDEFY4 (lanes 1-4) and pDEFHY1 (lanes 6-9) samples treated at 37°C. The induction and labelling of pDEFHY1 produces the MurE peptide at 53 kDa on the gel (lane 6). Also present are the β–lactamase bands at 31 kDa and 29 kDa. In addition there is the peptide at ~66 kDa. This was assumed to be the MurF-MraY fusion peptide. The calculated weight for the entire MurF-MraY fusion is 88 kDa. Also present is the peptide at 44 kDa. This may be a degradation product from a larger peptide, for example the 66 kDa truncated fusion. In lane 4 there is a peptide of ~25 kDa. It is not known what this constitutes. The induced and Rif treated pDEFY4 sample treated at 100°C does not have this peptide. This is not present in the pDEFHY1 sample seen in lane 6.
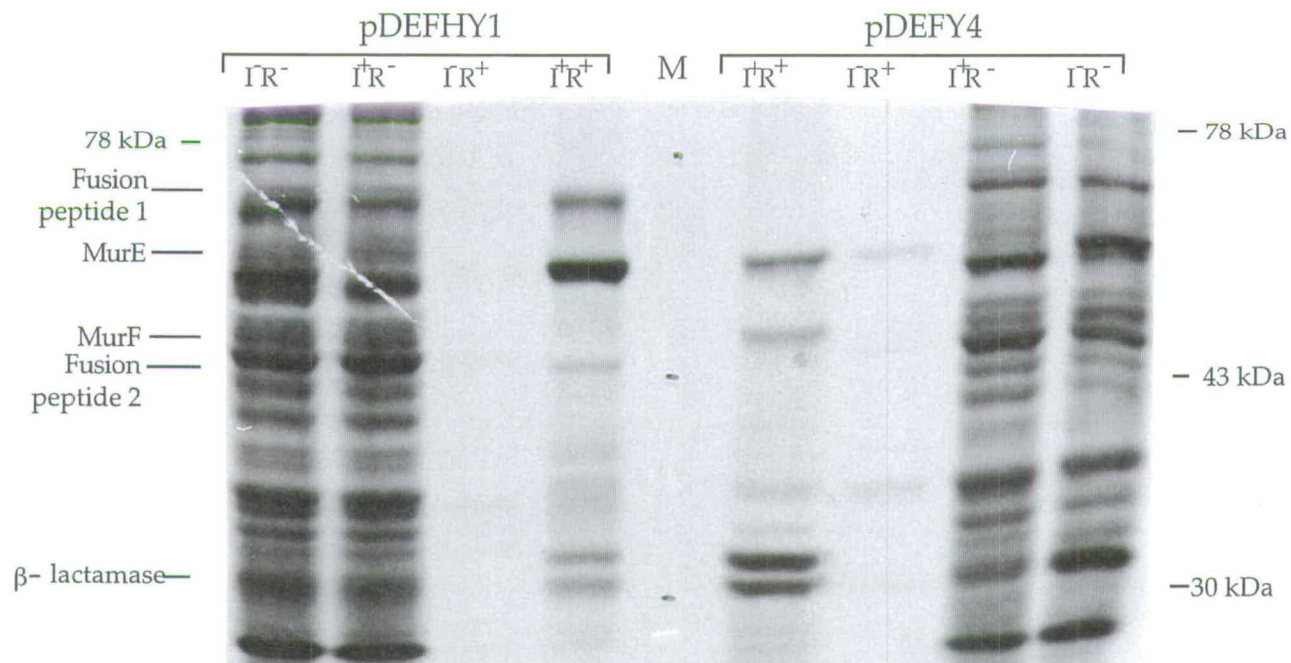
Figure 4.2.27. The SDS-PAGE analysis of pDEFHY1 (lanes 1-4) and pDEFY4 (lanes 6-9) samples treated at 100°C. The induced and Rif treated sample in lane 4 shows five labelled peptides. Two of these migrated as if they had molecular weights of ~64 kDa and 53 kDa. These correspond to the MurF-MraY hybrid peptide and the MurE peptide, respectively. There is a faint band at ~40 kDa. It is not known what this is. Another two low intensity peptide bands are located at 31 and 29 kDa. These are the β–lactamase peptides. These are produced in low levels compared to MurF-MraY and MurE due to the T7 transcriptional terminator present after the *murF-mraY* fusion. The induced and Rif treated pDEFY4 (lane 6) control produced both MurE at 53 kDa and MurF at 47 kDa. Also present are the β–lactamase peptides.

The induced samples of pDEFY4 at 100°C revealed bands at 53, 47, 31 and 29 kDa. These corresponded to the labelled MurE, MurF, preformed and mature β–lactamase proteins respectively. The 100°C treated samples from cells carrying pDEFHY1 shows MurE at 53 kDa and faint bands for β–lactamase at 31 and 29 kDa. The poor translation of β–lactamase is due to a T7 RNA polymerase transcriptional terminator sequence downstream of the cloning sites in pET vectors which was carried over in the construction of pDEFHY1. The largest protein visible is ~64 kDa in size. This is presumed to be the MurF-polyHis-MraY fusion product. It does however have an unusual migration rate through SDS-PAGE. This phenomenon has been reported for other *E.coli* proteins, namely RodA and FtsW (Pratt and Spratt, 1983; Khattar *et al.*, 1994). Treatment at 37°C did not produce a different profile of peptides. pDEFY4, however, produced a band at ~25 kDa, which had not been seen in previous labelling experiments (Figures 4.2.7. and 4.2.8.) and which is not present in the boiled extracts (Figure. 4.2.26.). This could be a degraded form of the *mraY* product.

The *murF*-polyHis-*mraY* fusion was truncated by cleaving with *Eco*RI. Plasmid pDEFHY1 was restricted with *Eco*RI to remove a DNA fragment of 860 bp, extending from the *mraY* ORF to the *Eco*RI site in the polylinker of pT7-4, followed by re-ligation of the backbone of the plasmid to create pDEFHY5, 6.3 kb in size (see Figure 4.2.26.). This makes a smaller fusion of 2283 nucleotides coding for a peptide of 761aa with a predicted molecular size of 81.5 kDa. BL21 (λDE3)/pLysS was transformed with pDEFHY5 and the proteins overexpressed as described before. Samples were treated at 100°C for 5 minutes and at 37°C for one hour. The samples were analysed on 7.5% SDS-PAGE gels together with samples of pDEFHY1 for comparison of the peptide sizes (Figures 4.2.28. and 4.2.29.). Both sets of samples revealed MurE and β–lactamase, the latter being produced in greater amounts from pDEFHY5 due to the removal of the T7 RNA polymerase terminator. What is clear from the gels is that the truncated fusion is smaller than MurF-polyHis-MraY by ~5 kDa, that is MurF'-polyHis-MraY' is ~60 kDa in size. Thus the putative fusion peptide has become smaller as predicted. The removal of the C-terminal portion of MraY may be involved in altering the mobility of the peptide in SDS-PAGE. Both the 100°C and 37°C samples of pDEFHY5 produced a smaller peptide of ~27 kDa. It is not known what this is. It might perhaps be a proteolytic product of the fusion peptide.
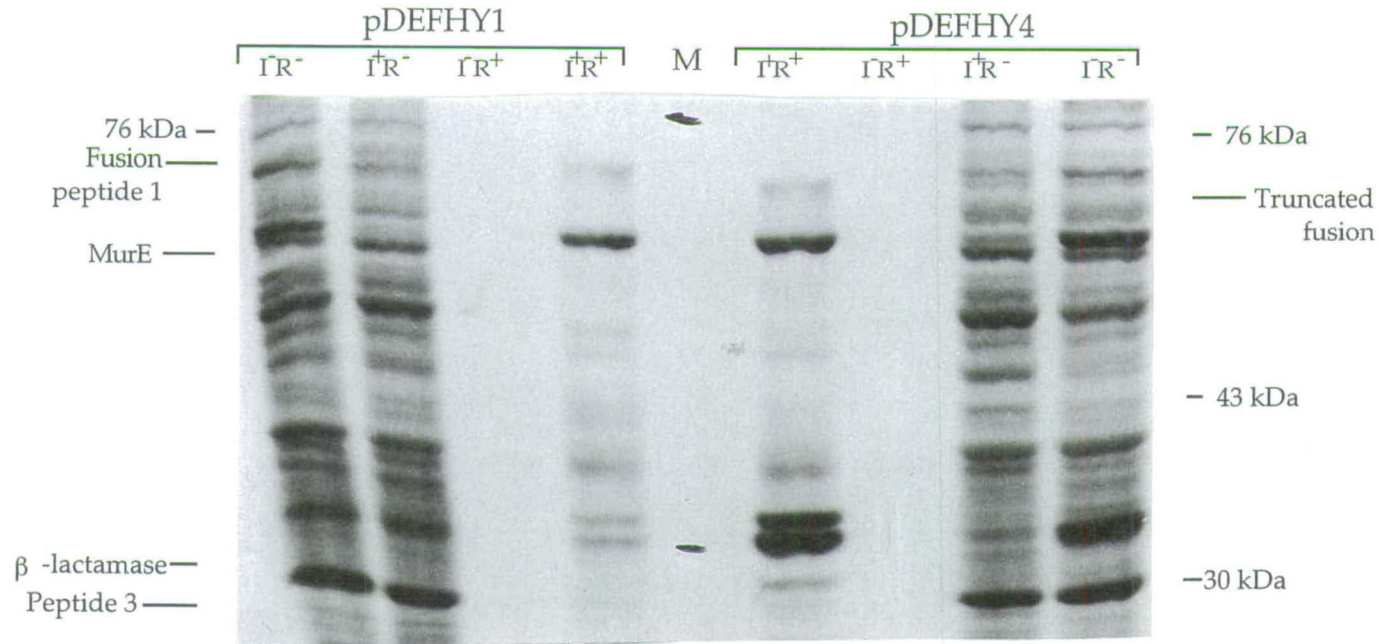
Figure 4.2.28. SDS-PAGE analysis of the 37°C treated samples of pDEFHY1 and pDEFHY4. The induction and labelling of pDEFHY4 produces the MurE peptide at 53 kDa on the gel (lane 6). Also present are the β–lactamase bands at 31 kDa and 29 kDa. In addition there is a large peptide at ~60 kDa. This was assumed to be the truncated MurF-MraY fusion peptide. This is 6 kDa lighter than the size of the entire MurF-MraY fusion present in lane 4, expressed from pDEFHY1. The calculated size of the peptide made from the truncation was 81.5 kDa. This is 7.5 kDa lighter than the calculated weight for the entire MurF-MraY fusion which is 88 kDa. Also visible is a smaller peptide with a migration rate of a 27 kDa peptide. It is not known what this constitutes. This is not present in the pDEFHY1 sample seen in lane 4. It may be a degradation product from a larger peptide, for example the 60 kDa truncated fusion.
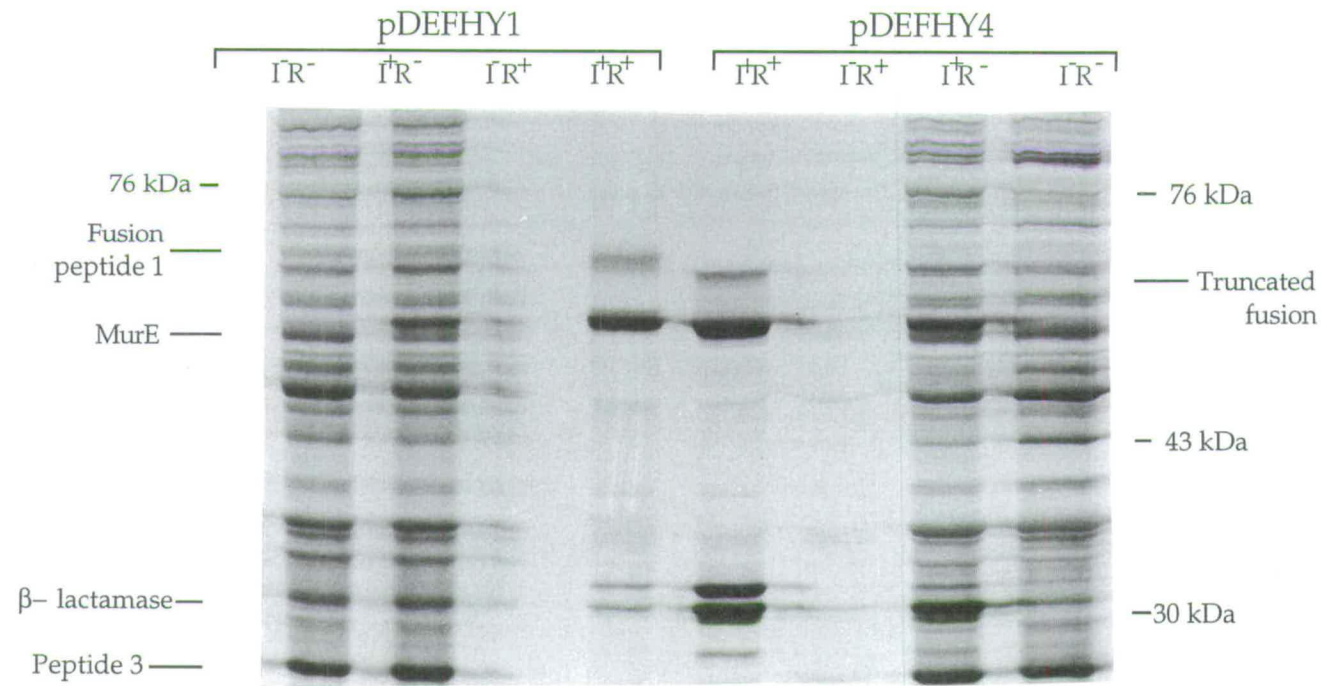
Figure 4.2.29. This shows the same type of samples as used in Figure 4.2.28. but in this instance the samples were treated at 100°C. The peptides visible in the induced and Rif treated pDEFHY4 sample (lane 5) are the same as seen for the 37°C sample in Figure 4.2.28. This is also true for pDEFHY1 (lane 4). Therefore the migration of the peptides are not affected by the denaturing conditions. Again the migration rate of the truncated MurF-mraY fusion is that of a ~60 kDa peptide. The small peptide at ~27 kDa is also present in the pDEFHY4 sample in lane 5.

4.2.9. *in vitro* translation of *mraY*.

The identification of the complete MraY peptide by *in vivo* expression had failed, although hybrid peptides were produced. The failure to visualize the MraY peptide may be due to the very rapid degradation of the peptide. The expression of *mraY* from plasmid pETY3c was attempted using an *in vitro* translation kit supplied by Promega. To this end the gene had to be subcloned into a vector which had a bacterial promoter since the T7 gene 10 promoter sequence would not be recognized by the bacterial RNA polymerase used in the kit. The vector chosen was pJF118EH (Furst *et al.*, 1986) where expression is controlled by a P*tac* promoter upstream of the polylinker. The gene 10-*mraY* fusion was subcloned from pETY3c within a 1.6 kb *Xba*I/*Hind*III fragment into pJF118EH that had been restricted with *Xba*I/*Hind*III. This made the plasmid pJFY3c which was 6.7 kb in size. pJFY3c DNA was prepared using density gradient isopycnic centrifugation (Chapter 2). The *in vitro* labelling experiment was performed according to Promega protocol (Chapter 2).

Samples were analysed on 10% SDS-PAGE gels and the presence of labelled peptides detected using autoradiography (Figure. 4.2.30.). The labelling experiment shows the presence of a band at ~38 kDa. This is produced only from the pJFY3c samples. It was suspected that this might be the MraY peptide. A faint band can be observed at ~39 kDa in the pJF118EH samples (see Figure 4.2.30.). Examination of other open reading frames in pJF118EH revealed that *lac*Iq is also expressed. This produces a peptide of 38 kDa. Therefore the band at ~38 kDa on the autoradiogram may only be LacI or a mixture of LacI and MraY.

An attempt to resolve the presence of LacI obscuring the MraY peptide was made by using an *in vitro* translation kit which uses linear, rather than circular DNA, as a template. pJFY3c was digested with *Bst*EII which restricts the plasmid at a unique site in *lac*Iq. Linear pJFY3c DNA was used for the *in vitro* translation. The LacI protein was now truncated, and MraY would not be masked by LacI. The labelled samples were analysed on 10% SDS-PAGE and products identified by autoradiography. There were problems encountered in the use of the kit supplied. The linearized pJFY3c DNA did not produce translation products when used with this kit. The control DNA supplied with the kit did, however, produce translation products and therefore the components of the kit were functional. Samples of pJFY3c DNA were prepared by isopycnic centrifugation and by the minipreparation method. The linear DNAs were isolated by agarose electrophoresis after restriction with *Bst*EII. Different

concentrations of the linearized pJFY3c (2, 4, and 6 µg per reaction) were used but none produced either of the expected peptides, namely β-lactamase and MraY. The presence and absence of both IPTG inducer and RNase inhibitor also did not produce translation products *in vitro*. The incubation of the reaction mixtures at room temperature for longer periods of time also did not produce translation products. This data has not been presented. There was insufficient time to continue the experiment on the *in vitro* translation of *mraY*. Another method for the *in vitro* translation of *mraY* will be proposed in the discussion (4.4.10.)
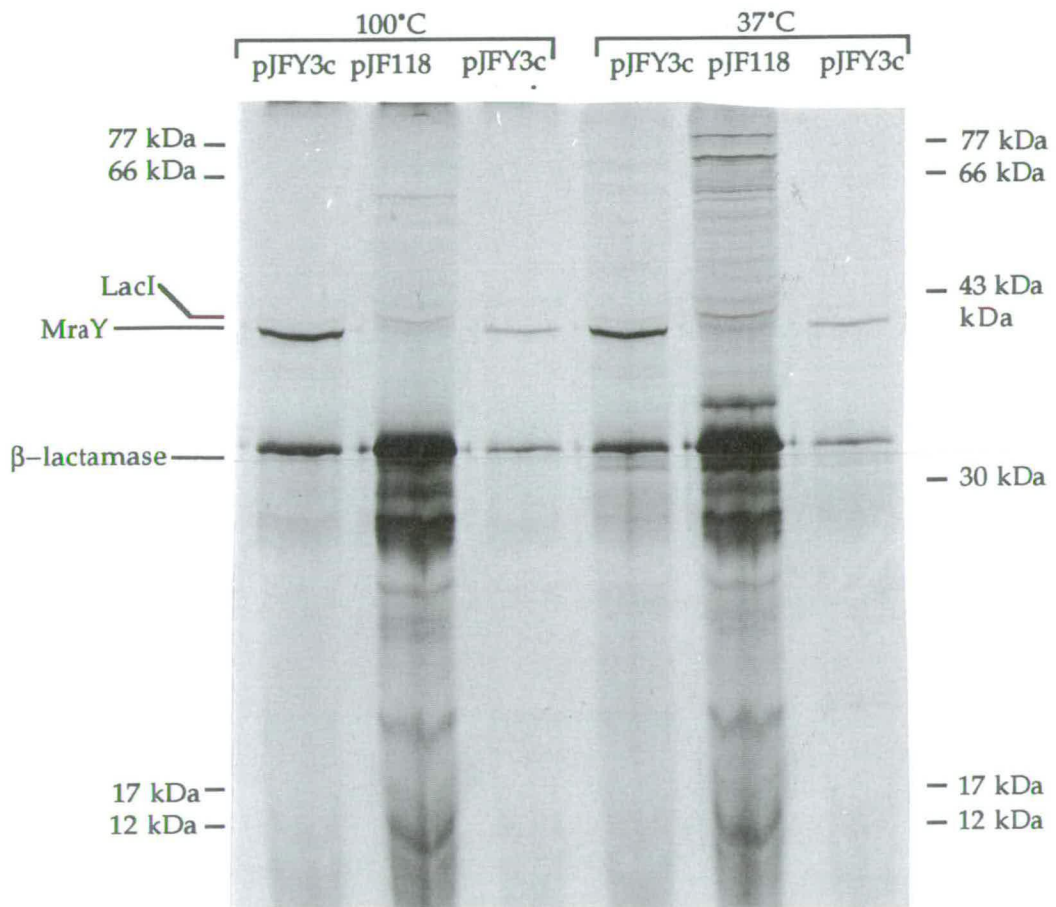
Figure 4.2.30. The SDS-PAGE analysis of products from *in vitro* translation reactions involving the *mraY* clone pJFY3c (lanes 1, 3, 4 and 6) and the vector only control pJF118EH (lanes 2 and 5). The samples were treated by either boiling for (lanes 1-3) or by incubation at 37°C for one hour (4-6). For the pJF118EH control, 4 μg of DNA was used per reaction. The pJFY3c reactions used either 2.5 μg (lanes 3 and 6) or 4 μg of DNA (lanes 1 and 4). The *in vitro* translation of the vectors produced proteins in each reaction. In each lane there were bands at 30 kDa which correspond to the β-lactamase protein. In lanes 1, 3, 4 and 6 there is a labelled peptide which migrates as a peptide of ~38 kDa. This may be the MraY protein. There are faint bands in the pJF118EH which migrate the same distance. This is the LacI peptide.

4.3.1. Mutagenesis of *mra*Y.

As attempts to produce the entire MraY protein had either failed or produced possible fusion peptides or degradation products, it was thought that a mutant of *mraY* would prove useful for determining the open reading frame for *mraY*. *mraY* is also the only gene in the *mra* cluster which does not have a mutant allele. Therefore the aim of this work was to determine the open reading frame of *mraY* by complementation of a mutant strain deleted for *mraY*. An *mraY* null strain was created and attempts were made to isolate a ts mutant.

4.3.2. Localized mutagenesis of the mra region.

A P1 lysate prepared from C600T was mutagenized using the method described by Gibbs *et al.* (1992). This was used to transduce OV2 and W3110 to Tet[R] by selection on NB Tet with incubation at 30°C. Transductants were then screened for lethal ts alleles by patching cells on NB Tet and incubating at 30°C and 42°C overnight. Isolates which were ts at 42°C were then patched onto NB plates and again tested for ts at 42°C. This was to ensure the Tet[R] marker had not become ts. Using this method, M. Khattar isolated 62 ts mutants, of which, 42 were in an W3110 background (labelled MMK) and 20 in an OV2 background (labelled KH). A further 12 OV2 Tet[R] ts mutants (labelled DB) were isolated by me, using a mutagenized P1 lysate of C600T made by M. Khattar. Microscopic examination of the of the mutants incubated at 42°C showed that either growth or division of the cells was inhibited.

Plasmid	Complementation
pDIEFY4	*ftsI murE murF mraY*
pDEG1	*murE murF mraY murD ftsW murG*
pDDC1	*murD ftsW murG*

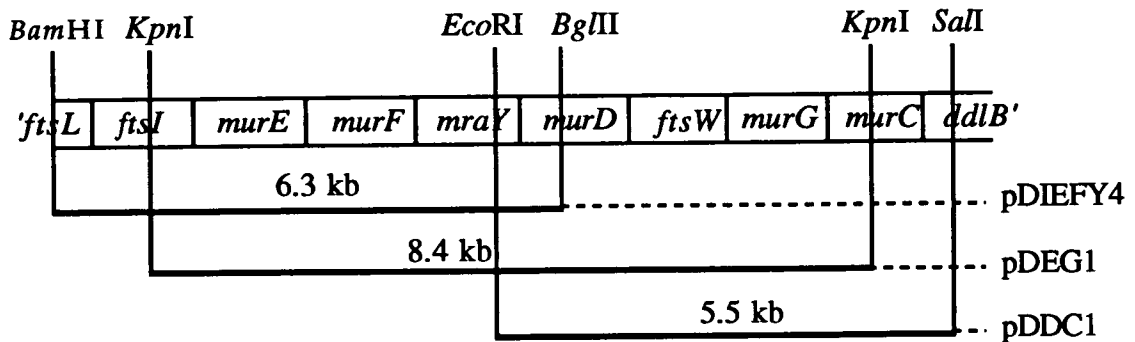**Table 4.3.1.** Plasmids used to complement ts mutants.

Figure 4.3.1. A schematic diagram showing the restriction sites and size of the fragments used in the construction of clones pDIEFY4, pDEG1 and pDDC1.

Three plasmids were used to investigate complementation of the ts alleles at 42°C. The genes they complement are listed in Table 4.3.1. The actual fragments cloned into the vectors are shown in Figure 4.3.1. The vectors used to construct the clones were pUC18 (pDEG1 and pDDC1) and pT7-4 (pDIEFY4). Both of these vectors are present in high copy number in *E.coli*. The P*lac* promoter in pUC18 increases expression of the cloned inserts whilst expression of genes present in the cloned insert in pT7-4 rely solely upon their own promoter sequences. This is because pT7-4 does not contain a bacterial promoter region upstream of the polylinker.

It was presumed that these clones could complement any single mutation lying between *ftsI* and *murC*. The clones pDEG1 and pDIEFY4 were specifically used to complement *mraY* mutants which may have been present, although pDIEFY4 can also complement *ftsI, murE* and *murF*. The construction of pDIEFY4 is described in 4.2.1. The construction of pDEG1 is described in Chapter 3.2.1. pDEG1 has been shown to complement all ts mutant alleles from *murE* until *murG* (Mengin-Lecreulx *et al.*, 1989; M. Khattar pers. comm.) with the obvious exception of *mraY*. pDDC1 was used to screen for any mutations from *murD* to *murC*. It was constructed by subcloning a 2445 bp *SalI* fragment, restricted from λ110 DNA, into pDDG1 (see Chapter 3.2.1.) which also was restricted with *SalI*.

The ts mutants were transformed with selection on NB Tet/Amp at 30°C. Amp^R transformants were then streaked on NB Tet/Amp and incubated overnight at 30°C and 42°C. The screening of the transformants revealed seven ts mutant alleles, none of which were *mraY*. Four of these

were in the W3110 background and three in the OV2 background. See Table
4.3.2.

Strain	Plasmid complementation at 42oc					Mutant allele(s)
	pDEG1	pDDC1	pUC18	pDIEFY	pT7-4	
MMK129	—	—	—	+	—	*ftsI*
MMK441	—	—	—	+	—	*ftsI*
MMK704	—	—	—	+	—	*ftsI*
MMK1888	+	+	—	—	—	*murD*
MMK2628	+	—	—	+	—	*murE*
KH2157	—	—	—	+	—	*ftsI*
KH2219	—	—	—	+	—	*ftsI/murE*
KH2690	—	—	—	+	—	*ftsI*

Table 4.3.2. Complementation of ts mutants and identification of the ts alleles.

Location of the ts alleles. Three of the W3110 mutants (MMK129, 441 and 704)
were complemented only by pDIEFY4. The strains were then transformed
with pT7-4I, a clone of *ftsI* in pT7-4 which can complement the TOE23 *ftsI* ts
mutant isolated by Begg and Donachie (1978)(M. Khattar, pers. comm.). This
too allowed growth at 42°C. The phenotype of these strains at 42°C was
filamentous when wild type *ftsI* was not present in extra copy. The pT7-4I
transformants grew to form single colonies at 42°C but the cells still retained
a filamentous phenotype. The pT7-4, vector only control did not
complement these mutants. The mutation in MMK1888 was found to be
located to *murD ftsW* and *murG*. Only pDEG1 and pDDC1 MMK1888
transformants grew at 42°C. Both of these clones contain *murD, ftsW* and
murG. The phenotype of the mutation was one for growth, that is the cells
had lysed whilst retaining a rod shaped morphology. Ikeda *et al.* (1989);
Ishino *et al.* (1989) and Khattar *et al.* (1994) have described the phenotype of
ftsW ts alleles to be filamentous. Therefore the ts mutation was considered to
be in either *murD* or *murG*. The phenotypes of ts mutants for *murD* and
murG have been described by Mengin-Lecreulx *et al.* (1989) and Salmond *et
al.* (1980) respectively. Both are growth mutants, that is at the restrictive
temperature the cells become swollen and lyse.

Two additional clones were used to complement either *murD* or *murG* (Figure 4.3.2.). To screen for *murD* complementation, pDBD3 was used. This is a pT7-3 (Tabor and Richardson, 1986) clone with a 1958 bp *Eco*RI/*Cla*I fragment containing *'mraY, murD* and *f'tsW*. For *murG*, pDBG3 was used. This contains a 2456 bp *Cla*I/*Kpn*I fragment also cloned into pT7-3. The genes carried in this fragment are *'ftsW murG murC'* . Although pT7-3 has no bacterial promoters, the promoters for the expression of both *murD* and *murG* are also present within the cloned fragment (Mengin-Lecreulx *et al.,* 1989). MMK1888 was transformed with both clones and at 42°C on NB Tet/Amp agar only pDBD3 transformants could grow, on NB Tet/Amp agar at 42°C.
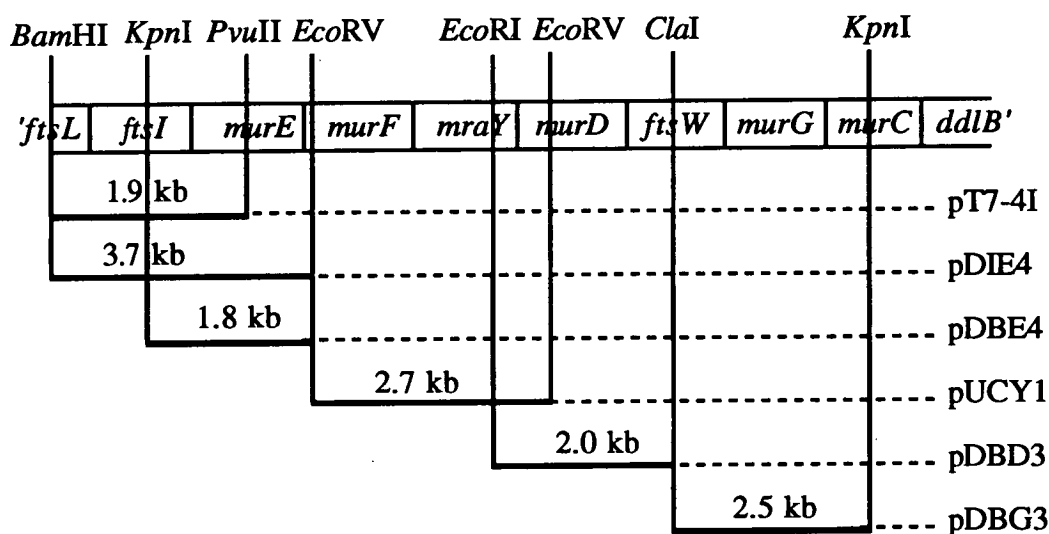

Figure 4.3.2. Clones used to identify the ts mutant alleles. Shown are the sizes of fragments cloned (not to scale) and the nomenclature of the clones.

MMK2628 was complemented by both pDEG1 and pDIEFY4. The phenotype of the cells at 42°C suggested once more that it was a growth mutant. Both clones have the entire sequences for *murE, murF* and *mraY*. To investgate whether or not MMK2628 was an *mraY* mutant, a clone containing only the *mraY* ORF was constructed. A 2675 bp *Eco*RV fragment was cloned into *Sma*I cut pUC18 to create pUCY1 (size 5.4 kb) where *mraY* is cloned such that it is expressed from P*lac* upstream (see Figure 4.3.2.). The clone also contains the C-terminal coding region of *murF* and the N-terminal coding region of *murD*. This did not complement MMK2628 at 42°C. The mutation

therefore lay in either *murE* or *murF*. A *murE* clone in pT7-4 was made by cutting pDEFY4 (see 4.2.2.) with *EcoRI/EcoRV* and then filling the recessed *EcoRI* site by using the Klenow fragment (see Chapter 2). This was then re-ligated to create pDBE4 (size ~4.2 kb), which now contains a 1813 bp fragment from the *KpnI* site in *ftsI* extending through *murE* to the *EcoRV* site in *murF* (see Figure 4.3.2.). MMK2628/pDBE4 transformants grew at 42°C on NB Tet/Amp agar plates. It was therefore concluded that MMK2628 was a *murE^ts* mutant.

The three ts mutants with an OV2 background (KH2157, 2219 and 2690) all had a filamentous phenotype at 42°C and it was concluded that all were therefore *ftsI^ts* alleles because they were not complemented by pDEG1. Transformants carrying pDIEFY4 were able to grow at 42°C. Subsequent transformation of the mutant strains with pT7-4I and complementation testing at 42°C showed that only KH2157 and KH2690 could grow. As seen previously with MMK129, 441 and 704 the complementation of KH2157 and KH2690 containing pT7-4I was not complete as there were many filmentous cells present at 42°C. KH2219/pT7-4I did not grow at 42°C. As it formed filaments at 42°C but was not complemented by *ftsI* it was thought to be an *mraY^ts* as the reported phenotypes of *murE* and *murF* are not filamentous (Maruyama *et al.*, 1988). Transformation of KH2219 with pUCY1 however, did not suppress the lethal filamentous phenotype at 42°C. The possibility of a double mutation was then considered and the clone pDIE4 was constructed by cutting pDIEFY4 with *EcoRV/EcoRI* to remove *mraY* and the C terminal region of *murF* (see Figure 4.3.2.). The *EcoRI* site was then filled using the Klenow fragment and the blunt ends re-ligated to create pDIE4, 6.1 kb in size. When KH2219 was transformed with pDIE4 it was able to grow on NB Tet/Amp at 42°C. The cells still formed filaments at 42°C and so complementation of the ts alleles was incomplete. Therefore KH2219 is a filamentous mutant of *murE*.

Therefore this method of producing an *mraY^ts* mutant did not work. Although many ts mutants were isolated, the screening of these for an *mraY^ts* allele proved fruitless. It is interesting that so many of the mutant alleles identified were for *ftsI*.

4.3.3. Construction of an *mraY* null strain.

The failure to isolate an *mraY*[ts] mutant led to an attempt to disrupt the chromosomal copy of *mraY*. *Xmn*I is a blunt cutting restriction enzyme which cuts once in *mraY* at 400 bp. pDEG1 was cut with *Xmn*I and the *Sma*I cut Ω fragment (Prentki and Kirsch, 1986) cloned into this site. This created pDYΩ1, 13.1 kb in size. A second *mraY* null construct was made by cloning the 0.9 kb CAT fragment, from pCM4 (Close and Rodriguez, 1982). This was restricted with *Bam*HI and "filled in " with the Klenow fragment: the blunt ended CAT fragment was then ligated into pDEG1 partially restricted with *Xmn*I . This made pDYC1, 12 kb, from which CAT was expressed in the same orientation as the other genes cloned in this plasmid.

Insertion into the chromosome via plasmid recombination. Certain methods allow the insertion of circular DNA into the chromosome by transforming mutant strains in which recombination with the chromosome is selected for (Chapter 3.2.2.). The same approach was applied to this work where the *polA*[ts] strain JC411 was transformed with pDYΩ and pDYC1. The transformants were cultured under the conditions described before in Chapter 3.2.2. Both transformants were cultured in LB Amp with Spc selection for pDYΩ1 and Cmp for pDYC1. After the temperature shifts, single colonies were isolated on selective agar and the plasmid DNA screened by restriction analysis for the wild type *mraY*. In all instances the disrupted gene was retained on the plasmid and not transformed to the chromosome. Attempts were made to isolate the chromosomal disruption by lysogenizing temperature shift cultures with P1 bacteriophage. A haploid strain , C600T was transformed with pDEG1. This was used as a host for P1 transduction using P1 lysates grown on both JC411/pDYΩ1 and pDYC1 during and after the shift of incubation from 42°C to 30°C. The selection was for *leu*[+] transductants grown on fully supplemented minus leucine VB salts agar Amp. *leu*[+] transductants were then screened for growth on LB Amp/Spc for the pDYΩ1 lysate and LB Amp/Cmp for the pDYC1 lysate. This method did not produce any Spc[R] or Cmp[R] transductants. Therefore the use of a *polA*[ts] mutant to insert a gene disruption onto the chromosome had once again failed. This might be due to the insertion of a plasmid into *mraY* being lethal, suggesting that expression of genes downstream of *mraY* requires the DNA upstream from *mraY*.

Temperature sensitive plasmids. The use of plasmids which are temperature sensitive for replication was employed to insert the *mraY* disruptions into the chromosome. The process is analogous to that using a *polA^{ts}* strain. Hamilton *et al.* (1986) developed a vector with a ts replicon, pMAK705, for inserting gene disruptions into the chromosome (Figure 4.3.3.). Plasmid replication is terminated at the non-permissive temperature. Selection for plasmid markers at restrictive temperature identifies the cells in which the plasmid has integrated into the chromosome. At the permissive temperature the plasmid can excise from the chromosome without affecting the host cells. For integration to occur there has to be sufficient homology with the region where integration is desired. Chromosomal disruptions which are lethal are complemented by virtue of expression of the wt allele on the plasmid unless they are polar. The disrupted allele inserted into the chromosome is complemented by the wild-type allele located on the plasmid. P*lac* upstream of the polylinker in pMAK705 allows expression of the wild-type insert. A further advantage of this method is that wild-type cells may be used as the host. The phenotype of cells where chromosomal disruption is successful can then be observed by culturing the mutant at the restrictive temperature, where the complementing plasmid will be lost from the majority of cells. In this laboratory S. Addinall has successfully introduced chromosomal disruptions using pMAK705.
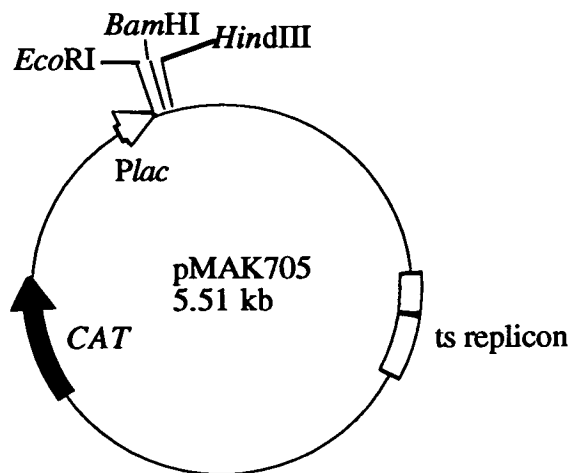

Figure 4.3.3. The temperature sensitive plasmid pMAK705 .

The 4.7 kb *EcoRV* fragment from pDYΩ1 was cloned into *SmaI* cut pMAK705 to create pMAKYΩ1, where *mraY*::Ω was cloned in the same orientation as the *lac* promoter in pMAK705. The 3.6 kb EcoRV *mraY*::CAT

fragment was cloned in the same manner as pMAKYΩ to make pMAKYC1. The host strain transformed with either pMAKYΩ1 or pMAKYC1 was C600T.

Method used to disrupt mraY. A single colony from each transformant was cultured overnight in LB Cmp at 30°C. Aliquots of 10^{-7} dilutions were plated onto LB Cmp agar and incubated at 30°C. 10^{-5} dilutions from the same cultures were plated on LB Cmp agar and incubated overnight a 42°C to select for single colonies. These are the cointegrant clones (see Figure 4.3.4.). The 30°C plate was used as a control. The cointegrants were purified by streaking onto LB Cmp agar and incubating at 42°C overnight. This was repeated. Single colonies from ten separate cointegrants were pooled in a flask of LB Cmp and incubated with shaking overnight at 30°C. This stage allowed the excision of the plasmid from the cointegrants. This was repeated twice and aliquots of 10^{-7} dilutions plated on LB Cmp agar and incubated overnight at 30°C. Single colonies were then selected and the plasmid DNA examined for the presence of the gene disruption by restriction analysis.
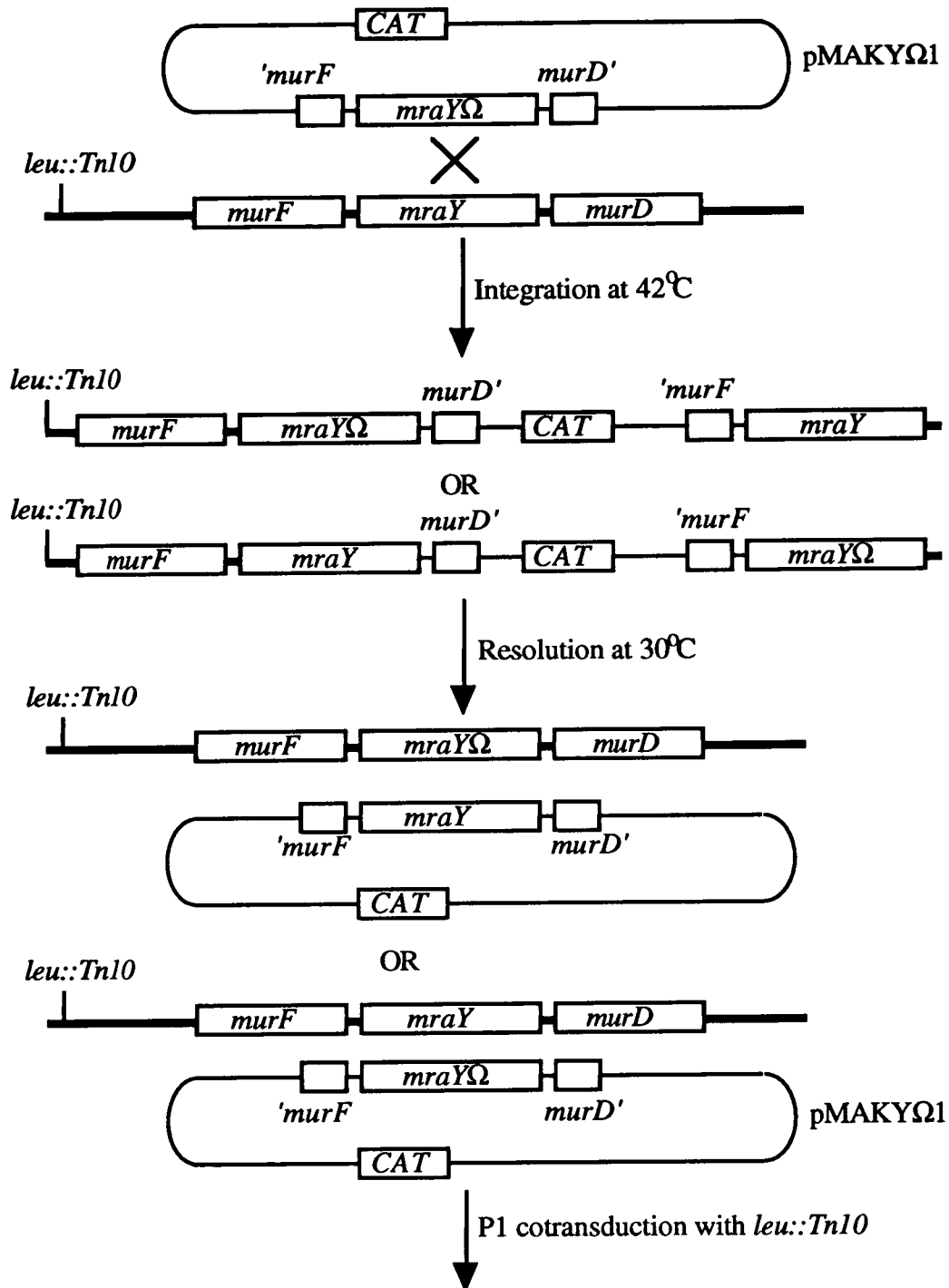
Figure 4.3.4. The integration of pMAKYΩ1 into the chromomsome at 42°C and subsequent resolution at 30°C. After resolution one of two events may happen. The null mutation can replace the wild-type chromosomal copy with subsequent complementation by the wild-type allele expressed from the plasmid or the null allele can remain *in situ* on the plasmid.

Failure of delivery. Small scale preparations of plasmid DNA from 40 isolates of both pMAKYΩ1 and pMAKYC1 were prepared, cut with *HindIII* and examined after agarose gel electrophoresis. All of the pMAKYC1 cells selected retained the CAT fragment on the plasmid. The same screening for pMAKYΩ1 isolates was performed and once again the Ω cassette was present in the plasmid of every isolate screened. Transformants of both pMAKYΩ1 and pMAKYC1 were grown at 42°C and screened for plasmid DNA by restriction and transformation. The plasmid was not recovered, and therefore must have successfully integrated onto the chromosome. The failure of the disruption to remain on the chromosome may be due to the homology either side of the cassettes used to disrupt *mraY*. Insufficient homology with the *mraY* region may result in pMAK integrating at another site on the chromosome. If this occurred then *mraY* would not be disrupted. Upstream of the *XmnI* site in *mraY* there is 1689 bp of homology and 970 bp downstream. The Ω fragment (2 kb) is larger than either of these and therefore the homology of this clone may be insufficient for insertion in *mraY*. The CAT fragment is only 0.9 kb, although the results would indicate that here too there was insufficient flanking DNA for homology with the chromosomal copy of *mraY*. Or, that neither *mraY+* plasmids can complement the null mutation on the chromosome.

Insertion of null alleles by linear transformation. Two *recD-* strains were chosen as recipients of linearised plasmid DNA. The strains were DL307 and D301. Both were initially transformed with pACEFY: a clone thought likely to complement *mraY*. pACEFY was constructed by restricting pACYC177 with *HindIII/BamHI* and cloning into these sites the 4.4 kb *HindIII/BglII* fragment from pDEG2. Linear DNA was prepared by restricting pDYΩ1 and pDYC1 with *KpnI* and recovering the purified fragments from the gel. *KpnI* restriction of these plasmids gave fragments of 10.4 kb for pDYΩ1 and 9.3 kb for pDYCAT1. The cells were transformed with 5 μg of each DNA using the electroporation method of transformation. The transformed cells were plated out with appropriate selection for the marker on the cassette.

The mutagenesis of *mraY* by this technique did not work. All of the potential linear transformants screened, contained the uncut plasmid DNA. Attempts were made to prevent this by restricting the gel-purified fragments again with *KpnI* and once more separating the fragment by agarose gel electrophoresis. These attempts reduced the number of transformants isolated

but again, all of the transformants recovered contained the plasmid DNA and not the chromosomal exchange.

Linear transformation with the "partial diploid", DSB1. This method was used successfully to introduce *ftsW* null alleles onto the chromosome (Chapter 3.2.). Both pDYΩ1 and pDBYC1 were restricted with *Kpn*I. The 10.4 kb and 9.3 kb *Kpn*I fragments from pDYΩ1 and pDYC1, respectively, were purified from agarose gels and used to transform DSB1 by electroporation. The YΩ transformants were selected for on LB Spc/Tet/Kan and the YC transformants were selected for on LB Cmp/Tet/Kan. Using YΩ, 64 Spc^R transformants were recovered. For YC, 107 were isolated. Screening for Amp^S revealed that 94% of YΩ and 98% of YC had the insert on the chromosome. This was on the assumption that the cells would be Amp^R if the unrestricted plasmid had been transformed. Four clones of each were chosen and the cells examined for the presence of plasmid DNA. None of the transformants selected contained plasmid DNA.

Confirmation of the YΩ disruption by P1 transduction. The location of the *mraY* disruptions could be analysed by measuring the cotransduction frequency with which the Ω cassette in *mraY* was replaced by the wild-type *mraY* allele linked to a separate selectable chromosomal marker. The marker used was *leu*::Tn9. The partial diploid containing *mraY*::Ω, DBYΩ1, was transduced with a P1 lysate prepared on W3110 *leu*::Tn9. The transductants were grown on LB Cmp. The Cmp^R isolates were then patched onto LB Spc and screened for Spc^R. When DBYΩ1 is transduced with selection for *leu*::Tn9, the marker can insert in either of the *mra* regions (Figure 4.3.5.). The cotransduction frequency between *leu* and *mraY* is ~75% in a haploid strain. Therefore the *mraY*::Ω null allele will be replaced by *mraY*$^+$ when transduced with a *leu*::Tn9/*mraY*$^+$ P1 lysate with a frequency of 75%. Taking the dipoidy of DBYΩ1 into account means that in 50% of cases the *leu*::Tn9 *mraY*$^+$ P1 lysate will replace the wild type genes in the other duplicated region, that is *leu*$^+$ *mraY*$^+$ (Figure 4.3.5.). Therefore the allele in the other, mutagenized, *mra* region will be replaced by *leu*::Tn9 *mraY*$^+$ with a cotransduction frequency of ~38%.

The screening of the Cmp^R *leu*::Tn9 transductants for Spc^R revealed that 36% of the Cmp^R population were now Spc^R, that is a cotransduction frequency of 38% between *leu*::Tn9 and *mraY* in the diploid strain. Correcting this for a cotransduction frequency in a haploid strain is 72%. This was judged to be

evidence that the $mraY::\Omega$ allele was inserted at the correct position on the chromosome. To confirm this assumption, a P1 lysate was prepared using DBYΩ1 and used to transduce a partial diploid containing a $leu::Tn9$ allele (DSB2). This is the reciprocal transduction where either of the host chromosomal $leu::Tn9$ $mraY^+$ alleles will be replaced with the leu^+ $mraY::\Omega$ introduced by P1 (leu^+ $mraY::\Omega$) transduction. When this occurs, the leu^+ allele is cotransduced with the $mraY::\Omega$ allele at a frequency of ~75% if the strain was haploid. As DSB2 is also diploid for the 2 minute region, the ~75% cotransduction frequency between leu and $mraY$ is halved to ~38%. After transduction with P1 (leu^+ $mraY::\Omega$) the cells were plated on LB Spc agar. The Spc^R isolates were then scored for growth on LB Cmp agar. This showed 37% of the isolates were Spc^R/Cmp^S. This indicates that $mraY::\Omega$ was cotransduced with leu^+ with a frequency of 37%. This is in a diploid strain and by correcting this for a haploid strain, the cotransduction frequency of leu with $mraY::\Omega$ is 74%. Therefore this provided evidence that $mraY::\Omega$ had inserted in the correct position in one of two mra duplications in DSB1.

Figure 4.3.5. The linear transformation of DSB1 with the 10.4 kb *mraY*::Ω *KpnI* fragment, from pDYΩ1, to produce DBYΩ1. The heavier lines represent the chromosomal duplication. The linear DNA can recombine with either of the duplicate *mra* regions (i.). ii. shows the diploid strain DBYΩ1 which contains a wild-type *mraY* allele and the mutant *mraY*::Ω allele. iii.-v. show the three events which may occur upon transduction with a P1 (*leu*::Tn9 *mraY*⁺) lysate. On the right of each diagram is the predicted transduction frequency (P) and the actual transduction frequency (A). In iii., both *leu*::Tn9 and *mraY* are cotransduced into DBYΩ1 at a frequency of 36%. This is only 1.5% less than the predicted cotransduction frequency of 37.5%. iv. and v. show the other events that may happen upon transduction of DBYΩ1 with P1 (*leu*::Tn9 *mraY*⁺). The calculated transduction frequencies are shown although the actual results could not be determined as both results give the same phenotype, that is Spc^R/Cmp^R.
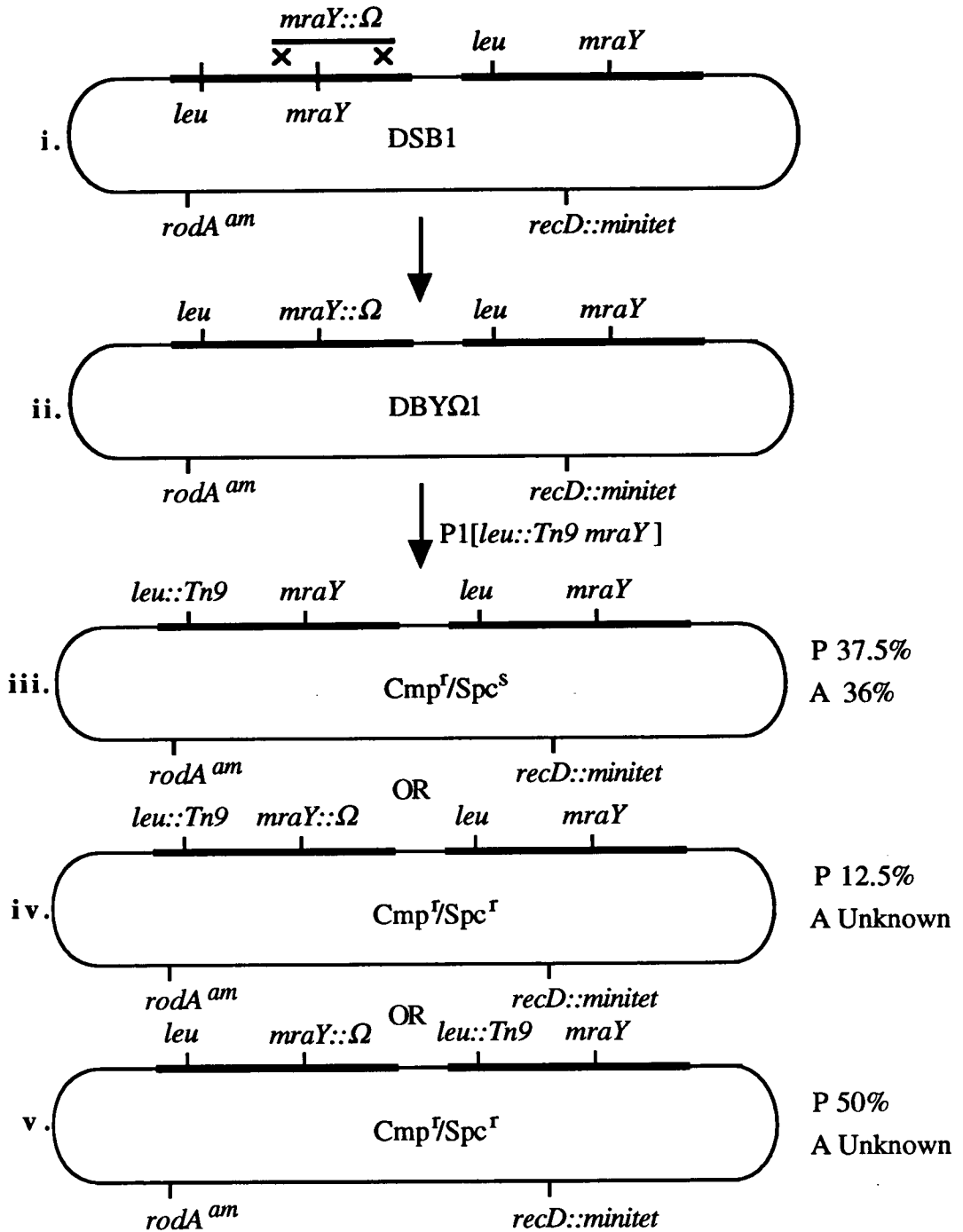
Figure 4.3.5. A schematic diagram depicting the linear transformation of the partial diploid strain DSB1 with *mraY*::Ω DNA and the resultant linear transformant, DBYΩ1. Also shown are the events upon transduction with a P1 (*leu*::Tn9 *mraY*+) lysate.

Confirmation of the mraY::CAT disruption by P1 transduction. The location of the mraY::CAT allele was also determined by the cotransduction frequency of *mraY* with another selectable marker using P1 transduction. However in this instance cotransduction between *leu* and *mraY* could not be used. This is because there was no appropriate selectable marker for *leu*. DBYC1 is already Tet^R due to the *rec*D::minitet marker and therefore *leu*::Tn10 could not be used. The use of *leu*::Tn9 was also inappropriate as the strain was already Cmp^R due to the CAT cassette used to disrupt *mraY*. Therefore another selectable marker was chosen. This was the *fts*W::Ω null mutation made in the previous chapter. The cotransduction frequency of *mraY* with *fts*W is nearly 100% in a haploid strain. This is since both genes are separated by only 1300 bp in the *mra* region. Therefore in the partial diploid DBYC1, *mraY*$^+$ will be cotransduced with *fts*W::Ω with a frequency of ~49% (Figure 4.3.6.). DBYC1 was transduced with a P1 lysate grown on DDWΩ1. The transductants were selected for on LB Spc agar. The Spc^R colonies were then screened for Cmp^R on LB Cmp agar. The screening revealed 48% of the Spc^R cells were now Cmp^S. Therefore the cotransduction frequency between *fts*W::Ω and *mraY*$^+$ was 48% for the partial diploid strain. Correcting this for a haploid strain, the cotransduction frequency becomes 96%.

The reciprocal transduction was performed with a P1 lysate prepared from a culture of DBYC1. The partial diploid *fts*W::Ω mutant, DDWΩ1, was transduced with P1 (*mraY*::CAT *fts*W$^+$) lysate. In this instance transductants were selected initially for Cmp^R on LB Cmp agar. These were then screened for the presence of the *fts*W::Ω allele, that is Spc^R. The percentage of Cmp^R cells which were also Spc^S was 49%. Again, correcting the cotransduction frequency for a haploid strain, gives a frequency of 98%. On the basis of these results it was concluded that the *mraY*::CAT allele had also replaced a wild-type *mraY* allele in the partial diploid strain.

Figure 4.3.6. Figure **i.** depicts the recombination between the 9.3 kb *KpnI mraY*::CAT fragment from pDYC1 and an *mra* region of the partial diploid, DSB1. The heavier lines represent the chromosomal duplication. **ii.** shows both the *mra* regions which contain the wild-type *mraY* allele in one and the *mraY*::CAT allele in the other. **iii.-v.** shows the possible events which can occur upon transduction with a P1 (*mraY*$^+$ *ftsW*::Ω) lysate and the predicted (P) and the actual (A) frequencies at which these events occur. **iii.** shows the actual cotransduction frequency between *mraY* and *ftsW*::Ω, this was found to be 48%, only 1% less than the predicted value. The predicted transductions for the events shown in **iv.** and **v.** are shown but the actual frequencies of these events could not be determined as both have the same selectable phenotype, namely Cmp^R/Spc^R.
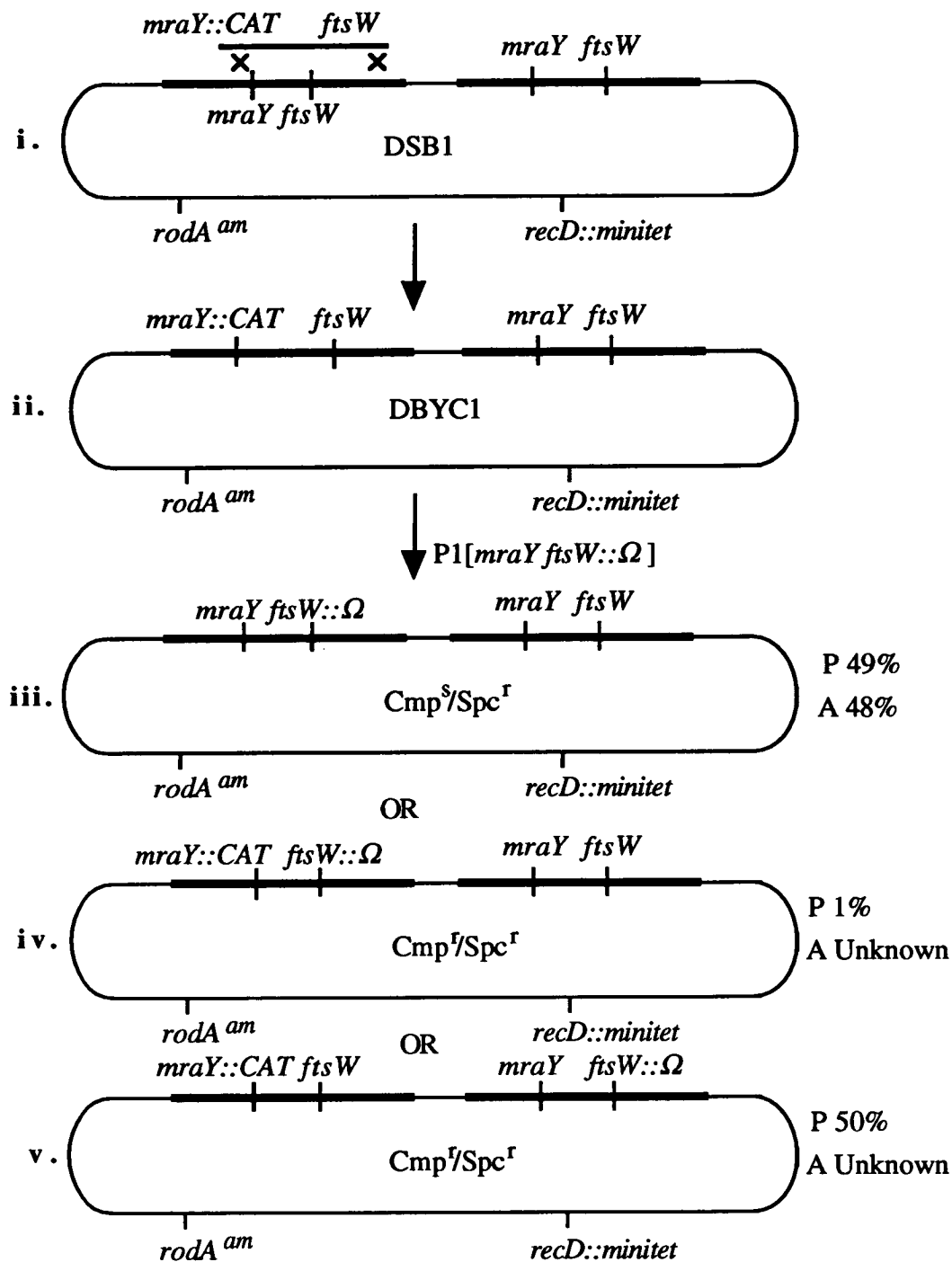
Figure 4.3.6. A series of schematic diagrams depicting the insertion of the 9.3 kb *mraY*::CAT *Kpn*I fragment from pDYC1 into the partial diploid DSB1. The other diagrams depict the *mraY* mutant DBYC1 and the phenotypes after transduction with a P1 (*mraY*+ *ftsW*::Ω) lysate.

Southern blotting of DBYC1. To provide direct evidence of the insertion of *mra*Y::CAT into the partial diploid, large scale genomic DNA purification (see Chapter 2) was used to extract DNA from DSB1 and DBYC1. The aim was to determine whether the CAT fragment was on the chromosome and if it was positioned in *mra*Y. As controls, pDEG1 and pDYC1 were also used. The DNAs were restricted twice with *Eco*RV. For the plasmid DNA controls 50 ng of DNA was used and 7.5 µg of genomic DNA. *Eco*RV cleaves a 2.7 kb fragment containing the wild-type *mra*Y. *Eco*RI restriction of DNA containing the CAT disruption produces a fragment of 3.6 kb. The restrictions, Southern blotting, preparation and labelling of probes, hybridization and filter stripping were as described in Chapter 2. The blot was probed with the ^{32}P labelled 2.7 kb *Eco*RV wt *mra*Y fragment and subsequently exposed (Figure 4.3.7.). The filter was then stripped of the 2.7 kb probe and hybridized with a labelled 0.9 kb CAT probe, the DNA being prepared from *Bam*HI cut pCM4.

The results in Figures 4.3.7 and 4.3.8. show that the *mra*Y::CAT disruption is on the chromosome. When probing with *mra*Y there are two bands lit in the lane containing the DBYC1 DNA. One band is at 2.7 kb and the other at 3.6 kb (Figure 4.3.7.). These are the *mra*Y+ and *mra*Y::CAT alleles, repectively. They are of equal density. This indicates that the wild-type and null alleles are present in equal concentration, that is diploid for *mra*Y. The solitary 2.7 kb *mra*Y+ fragment lit from DSB1, probed with the *Eco*RV fragment, is denser as it contains double the wild-type *mra*Y DNA of DBYC1 (Figure 4.3.7.). When probing for CAT only, DBYC1 and the control pDYC1, had bands at 3.6 kb which were lit (Figure 4.3.8.). The lighter bands at 2.7 kb were not hybridized by the CAT probe.

Figure 4.3.7.
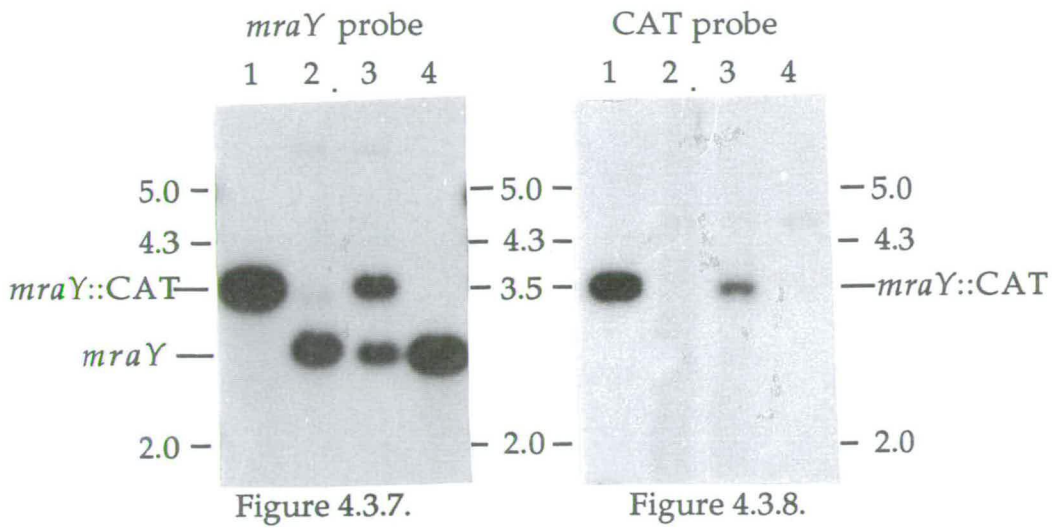
Figure 4.3.8.

Key: 1, pDYC1; 2, DSB1; 3, DBYC1 and 4, pDEG1.

Figures 4.3.7. and 8. The DNAs used for the Southern blot were all restricted with *EcoRV*. The blot on the left (Figure 4.3.7.) was hybridized with a wt *mraY* probe. The presence of two bands in lane 3 shows the restricted DNA from the partial diploid strain, DBYC1, with copies of both the wt DNA (2.7 kb) and the null *mraY* allele containing the CAT cassette at 3.6 kb. The *mraY*::CAT control DNA, pDYC1 has a single band lit which is also 3.6 kb. The host strain for the linear transformation, DSB1, only has the smaller 2.7 kb *EcoRV* fragment. This is the wt *mraY*. The wt control, pDEG1, reflects this in lane 4 as this too is 2.7 kb. Therefore only DBYC1 has both a wt allele and a null allele of *mraY*. When the same blot was hybridized with an *mraY*::CAT probe (Figure 4.3.8.), only the fragments containing *mraY*::CAT were lit. In this case only the control, pDYC1, in lane 1 and the mutant DBYC1, in lane 3 hybridized the *mraY*::CAT probe. Therefore DBYC1 contains both the null allele and the wt alleles of *mraY*.

4.3.4. Introduction of *mraY*::CAT into a haploid strain.

Further use of the *mraY*::Ω allele was discontinued upon the discovery that the T4 transcriptional terminator in the Ω cassette can effect the expression of other downstream genes, notably *ftsZ* (see Chapter 3.3.). Therefore the *mraY*::CAT disruption in DBYC1 was chosen for insertion into a haploid strain as the CAT fragment contains no terminator or promoter sequences which may affect the expression of the downstream genes (Close *et al.*, 1982). The haploid strain chosen to be mutated was C600T. This was first transformed with a series of plasmids thought to complement *mraY*. These were pDBY4, pDEFY4 and pDIEFY4 (see Figure 4.2.2.). It was not known if pDBY4 would complement the null *mraY* allele, although it contains 1.2 kb of upstream DNA (from the proposed start of the *mraY* ORF) which may contain a promoter for *mraY*. The clone pDEFY4 contains *mraY* and upstream DNA to the *Kpn*I restriction site in *ftsI*. This fragment was shown to complement both *murE*^{ts} and *murF*^{ts} mutant alleles when cloned in a high copy number plasmid (Mengin-Lecreulx *et al.*, 1989). Therefore it would seem likely that a *mraY* null allele would also be complemented by this vector. The clone pDIEFY4 (Figure 4.2.2.) also contains the intact *ftsI* gene, and can complement the *ftsI*^{ts} allele from TOE23 (this work, data not shown). This clone was also used as more DNA upstream from the *Kpn*I site in *ftsI* may contain promoters which may facilitate better expression of *mraY*.

The three species of C600T transformants were transduced with the P1 (*leu*$^+$ *mraY*::CAT) lysate. *leu*$^+$ transductants were selected for on fully supplemented minus leucine VB salts agar/Amp. The *leu*$^+$ isolates were then patched on LB Amp/Cmp and incubated at 37°C overnight. The only transformants which were Cmp^R contained pDIEFY4. The plasmid DNA from four Cmp^R isolates was screened for the presence of the CAT fragment in the plasmid by restricting the plasmid DNA with *Hind*III. This produces a linear band of 8.5 kb. If *mraY*::CAT had inserted in the plasmid then the size of pDIEFY4 restricted with *Hind*III would be 9.4 kb. The size of pDIEFY4 was 8.5 kb in all four isolates. Therefore *mraY*::CAT was located on the chromosome. The Cmp^R isolate chosen for further work was known as DBHY1. The location of the *mraY*::CAT allele was determined by P1 transduction using P1 (*leu*::Tn*10* *mraY*$^+$). DBHY1/pDIEFY4 was transduced with this lysate and transductants selected for on LB Amp/Tet agar. The Tet^R transductants were then screened for Cmp^R on LB Cmp agar. The cotransduction frequency between *leu*::Tn*10* and

mraY+ was 78% (Table 4.3.3.). Therefore *mraY*::CAT appeared to be located correctly in the *mra* region.

Sample no.	Tet^r	Cmp^r	*leu* $^+$	Cotransduction frequency (%)
100	100	22	0	78

Table 4.3.3. The cotransduction frequency between *leu*::Tn10 and *mraY*+, when transduced by P1 into DBHY1/pDEFY4.

As only pDIEFY4 appeared to complement *mraY*::CAT it was assumed that the promoter for the expression of *mraY* was located upstream of the *KpnI* site in *ftsI*. A clone where *mraY* was expressed by P*lac* was chosen. This was pUCY1, 5.4 kb in size, a clone made previously when screening ts mutant isolates (see 4.3.2.). It contains the 2.7 kb *EcoRV* fragment (consisting of '*murF mraY murD*') cloned into the *SmaI* site of pUC18. This was cloned in the same orientation as P*lac*. The basal expression from P*lac* should be sufficient express *mraY* to permit complementation of the *mraY*::CAT allele. C600T/pUCY1 was transduced with P1 (*leu*+ *mraY*::CAT) with selection for *leu*+ transductants on fully supplemented minus leucine VB salts Amp agar. The *leu*+ isolates were plated on LB Cmp/Amp agar. None were Cmp^R. Therefore the *mraY*::CAT allele was not complemented by pUCY1. The isolates were screened for Tet^R on LB Tet. This was to ensure that the *leu*::Tn10 had been replaced with *leu* + by the P1 transduction. None of the isolates were Tet^R; therefore *leu*::Tn10 had been replaced with *leu*+. The same experiment was repeated with the addition of IPTG to the selective media. This induces expression from P*lac* and should therefore produce greater expression of *mraY*. Once again all the *leu*+ isolates recovered were Cmp^S/Tet^S. The expression from P*lac* should be sufficient for pUCY1 to complement the of *mraY*::CAT allele. C600 *leu*::Tn10/pDEG1 was transduced with the same lysate and the same selection for *leu*+ applied. Once again no Cmp^R isolates were recovered from the *leu*+ transductants.

Therefore only pDIEFY4 could complement the *mraY*::CAT disruption. The notable difference between this and the other clones is the entire *ftsI* sequence contained in pDIEFY4. The expression of *mraY* from P*lac* in both pUCY1 and pDEG1 should be sufficient to complement *mraY*::CAT. Further examination of the ploidy of the partial diploid revealed that it appeared to be

haploid for *ftsI* (S. Addinall, pers comm.). The TOE23 (*ftsI^ts*) allele was cotransduced into SHA6 with *leu*::Tn9. The transductants were screened for growth at 42°C. Of these, ~45% of the Cmp^R transductants were sensitive to growth at 42°C. Therefore the partial diploid contains a null *ftsI* allele.

A C600T lysogen was made using λ*sep*$^+$ 82, a λ phage which complements *ftsI* mutants (Fletcher *et al.*, 1978). The lysogen was transformed with either pDEFY4, pDBY4 or pUCY1. The transformants were then transduced with a P1 (*leu*$^+$ *mraY*::CAT) lysate, prepared on DBYC1, with selection for *leu*$^+$ transductants on fully supplemented minus leucine VB salts agar/Amp. The *leu*$^+$ isolates were then plated on LB Cmp/Amp. The pDEFY4 and pUCY1 transformants produced some *leu*$^+$ isolates which were Cmp^R. C600 (λ82)/pDBY4 *leu*$^+$ transductants were all Cmp^S. This data suggests that the presence of a complementing *ftsI* allele based in the λ*sep*$^+$82 phage allows the transfer of *mraY*::CAT providing complementary copies of *mraY* are expressed.

The inability of pDBY4 to complement the *mraY*::CAT allele may be explained by the lack of a suitable promoter in the upstream DNA from the start of *mraY* until the *Eco*RV site in *murF* (Figure 4.2.2.). The vector used to construct pDBY4 was pT7-4; this does not contain a suitable promoter for the expression of cloned inserts when transformed in C600. pUCY1 contains the same 2.7 *Eco*RV fragment but there is basal expression from P*lac* upstream of the polylinker. The ability of pDEFY4 to complement the *mraY*::CAT allele suggests that the promoter for *mraY* may lie in the region of DNA spanning from the *Kpn*I restriction site in *ftsI* to the *Eco*RV restriction site in *murF* (Figure 4.2.2.). The vector used to construct pDEFY4 is also pT7-4 and therefore the transcription of *mraY* is limited to the upstream DNA sequence (Figure 4.2.2.). Because pDEFY4 can complement *murE^ts*, the promoter is probably located between the *Kpn*I site and the start of *murE*. (i.e. within *ftsI*).

A P1 lysate was prepared on C600 (λ82$^+$) *mraY*::CAT /pDEFY4 (DBHY2). This was used to transduce C600/pDEFY4. The aim of this experiment was to isolate an *mraY*::CAT allele which is not linked to the *ftsI* null allele. The clone pDEFY4 was chosen to provide complementation for any *mraY*::CAT mutants which were isolated, as it had been previously shown to complement the *mraY*::CAT allele when transducing C600 (λ82$^+$)/pDEFY4. The selection for transductants was on LB Cmp/Amp agar. Three Cmp^R transductants were isolated. It was presumed that these cells contained only the *mraY* null allele and not the *ftsI* null allele. One *mraY*::CAT isolate was chosen and a P1 lysate was prepared on this strain.

C600T was then transformed with a variety of plasmids to determine if the *ftsI* null allele was present. The plasmids used were pDEFY4, pDBY4 and pUCY1. None of these can complement an *ftsI* mutant allele. The previous transfer of the *mraY*::CAT allele in the $\lambda82^+$ lysogen had shown that both pDEFY4 and pUCY1 could complement whereas pDBY4 could not. The C600T transformants were transduced with the P1 (*leu*$^+$ *mraY*::CAT) lysate and transductants were selected for growth on fully supplemented minus leucine VB salts/Amp agar. *leu*$^+$ transductants were recovered and patched on LB Tet and LB Cmp agars. Once more some of the pDEFY4 and pUCY1 *leu*$^+$ transductants were transduced to Cmp^R whilst all *leu*$^+$ pDBY4 isolates remained Cmp^S (Table 4.3.4.). All the *leu*$^+$ isolates were Tet^S, suggesting the successful replacement of the *leu*::Tn10 allele in the three species of transformants transduced with the P1 (*leu*$^+$ *mraY*::CAT) lysate.

Plasmid	Sample no.	*leu*$^+$	Cmp^r	*leu*$^+$*mraY::CAT* cotransduction frequency (%).
pDBY4	100	100	0	0
pDEFY4	100	100	69	69
pUCY1	100	100	73	73

Table 4.3.4. The complementation of the *mraY*::CAT allele and the cotransduction frequency between *leu*$^+$ and *mraY*::CAT. Cotransduction frequency approximate sampling error $+/-8\%(\sqrt{N})$.

4.3.5. Studies on the complementation of *mraY*::CAT.

The inability of the partial diploid to simultaneously contain both Δ*mraY* alleles shows that *mraY* is an essential gene for growth in *E.coli*. The aim of this work was to test whether the 1080 bp *mraY* ORF, identified by Ikeda *et al.* (1990), can complement the null mutation. In addition, the location of the *mraY* promoter region could be identified by cloning *mraY* with longer sequences of the upstream DNA. The clones pDEG1 (Figure 3.2.2.), pDEG2, pDEFY4, pDBY4 (Figure 4.2.2.) and pUCY1 and 2 (Figure 4.3.2.) have been described earlier in this work. These were some of the plasmids used for the study of the complementation of the *mraY*::CAT allele.

Other clones were constructed in various plasmid backgrounds. The vector pJF118EH (Furst *et al.*, 1986) was restricted with *Sma*I and the 2.7 kb *Eco*RV fragment cloned into this. The exogenous promoter located upstream of the polylinker in pJF118EH is P*tac*. The *Eco*RV fragment containing *mra*Y was cloned in both orientations relative to P*tac*. These clones were pJFY1 and pJFY2, both 7.8 kb in size. The *mra*Y sequence located within the *Eco*RV fragment was expressed from P*tac* in pJFY1. In pJFY2 the orientation of *mra*Y was the reverse to the P*tac* promoter. The DNA from the *Pvu*II restriction site in *mur*E to the *Bgl*II restriction site in *mur*D was cloned in pT7-4. This was achieved by cutting pDEFY4 with *Hind*III and filling the recessed 3' ends with the Klenow enzyme. A 6.8 kb linear DNA fragment was then partially restricted with *Pvu*II. The 6.1 kb fragment was isolated by agarose gel electrophoresis and purified and ligated. This construct was pDFY4 which contains '*mur*E *mur*F *mra*Y *mur*D'.(Figure 4.3.9).
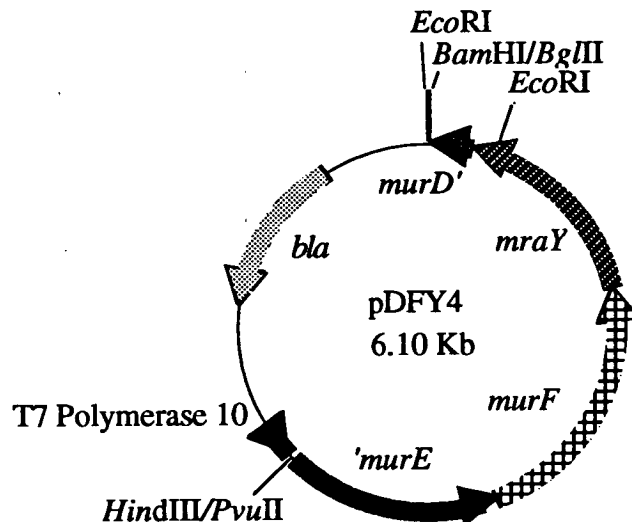

Figure 4.3.9. The vector pDFY4 used in complementation studies of *mra*Y::CAT.

The gene 10 rbs/*mra*Y fusion was subcloned from pETY3c into pJF118EH. pJFY3c was also tested for the ability to complement *mra*Y::CAT. This was a construct made for the *in vitro* labelling of the MraY peptide in Chapter 4.2.9.. This contains a fusion between the *mra*Y ORF and the gene 10 rbs. A clone containing an incomplete ORF of *mra*Y was constructed in pT7-4. pDEFY4 was cut with with *Eco*RI and the 6.4 kb restriction fragment purified and religated. This produced pDEF4, a vector of 6.4 kb (Figure 4.3.10.). The 4 kb DNA spans from the *Kpn*I restriction site in *fts*I to the *Eco*RI site in *mra*Y. The nucleotide

sequence for *mraY* is truncated to 860 bp. The same 4 kb fragment, when cloned in pUC18, was found to complement both the *murE*[ts] and the *murF*[ts] mutations (Mengin-Lecreulx *et al.*, 1989). The host strain transformed with the plasmids was C600. The list of plasmids used and the genes cloned within are listed in Table 4.3.5. As controls pUC18, pT7-4 and pJF118EH were also transformed into C600.
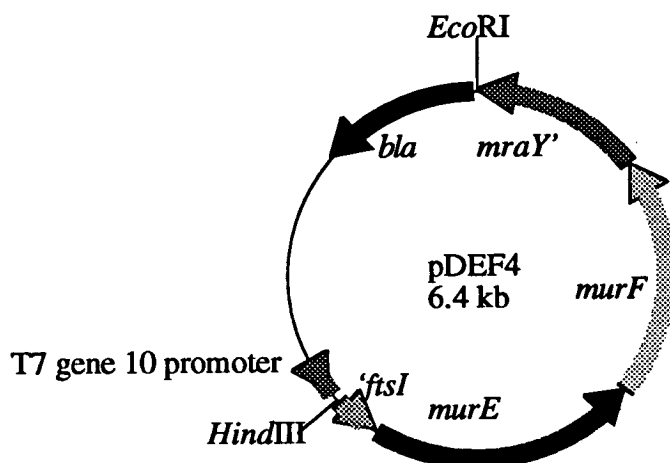

Figure 4.3.10. The vector pDEF4 which contains a truncated copy of *mraY* where the terminal 220 bp of the C terminal coding region of *mraY* have been removed.

The transformants were transduced with a P1 (*leu*::Tn10/*mraY*::CAT) lysate derived from the haploid strain, DBYC2/pDEFY4. The transduction mixtures were plated on LB Tet/Amp plates. The selection of Tet[R] cells before screening for Cmp[R] was a control to ensure that each type of transformant had been transduced to *leu*⁻. The Tet[R] colonies isolated were then patched onto LB Cmp/Amp plates. The clones which can complement *mraY*::CAT were screened for by this method (Table 4.3.6.)

Plasmid	Genes cloned	Vector	Promoter/ Expression	Size (kb)
pDEG1	*'ftsI to murC'*	pUC18	P*lac*/Yes	11.1
pDEG2	*'ftsI to murC'*	pUC18	P*lac*/ No	11.1
pDEFY4	*'ftsI murE murF mraY murD'*	pT7-4	T7/No	6.8
pDFY4	*'murE murF mraY murD'*	pT7-4	T7/No	6.1
pDBY4	*'murF mraY murD'*	pT7-4	T7/No	5.1
pJFY1	*'murF mraY murD'*	pJF118EH	P*tac*/Yes	7.8
pJFY2	*'murF mraY murD'*	pJF118EH	P*tac*/ No	7.8
pJFY3c	*gene10/mraY murD'*	pJF118EH	P*tac*/Yes	6.7
pUCY1	*'murF mraY murD'*	pUC18	P*lac*/Yes	5.4
pUCY2	*'murF mraY murD'*	pUC18	P*lac*/ No	5.4
pDEF4	*'ftsI murE murF mraY'*	pT7-4	T7/No	6.4

Table 4.3.5. The plasmid clones used to complement the *mraY*::CAT allele. Also shown are the genes cloned within, the plasmid background, promoter, expression of *mraY* from the promoter and the size of the construct.

Plasmid	Complementation of *mraY*::CAT	Plasmid	Complementation of *mraY*::CAT
pDEG1	+	pJFY3c	+
pDEG2	+	pJF118EH	−
pUCY1	+	pDEFY4	+
pUCY2	−	pDEF4	−
pUC18	−	pDBY4	−
pJFY1	+	pDFY4	−
pJFY2	−	pT7-4	−

Table 4.3.6. Plasmid constructs used to screen for the complementation of *mraY*::CAT. The types of promoter and their ability to express *mraY* in the host strain, C600, are listed in Table 4.3.5. above.

The complementation of the *mraY*::CAT allele occurred with six of the fourteen clones transformed in C600. The smaller cloned fragments, containing *mraY*, were unable to complement *mraY*::CAT; unless expressed from an external plasmid based promoter. The clones pJFY1, pJFY3c and pUCY1 are examples of this where basal expression from P*tac* or P*lac*, respectively, gave complementation of the null allele. In addition, when the 2.7 kb fragment is cloned in the opposing orientation to P*lac* or P*tac*, as in pUCY2 and pJFY2 respectively, the *mraY*::CAT allele also was not complemented. Therefore the nucleotide sequence upstream from the start of *mraY* until the *Eco*RV site in *murF*, does not contain a promoter capable of expressing *mraY*. This is also shown by pDBY4 which did not complement the *mraY*::CAT allele either. Another pT7-4 clone, pDFY4 (Table 4.3.5.), also does not complement the *mraY*::CAT allele. This contains an extra 1.7 kb of the upstream sequence from the *Eco*RVsite in *murF*, until the *Pvu*II restriction site in *murE*. That is a total of 2751 bp of the DNA upstream from the start codon for *mraY*. As this clone is constructed in pT7-4, the insert is not expressed from an exogenous promoter. Therefore to complement *mraY*::CAT, the promoter region for *mraY* would have to be present in the DNA sequence further upstream from the *Pvu*II restriction site in *murE*.

The clones pDEG1, pDEG2 and pDEFY4 all contain a further 638 bp of upstream DNA from the *Pvu*II restriction site in *murE* to the *Kpn*I site located in *ftsI*. Significantly all three clones complemented the *mraY*::CAT allele. pDEG1 was expected to complement as the insert (including *mraY*) is cloned such that it is expressed from P*lac*. pDEG2 contains *mraY* cloned in the opposite orientation to the P*lac* promoter. Therefore for pDEG2 to complement *mraY*::CAT, expression of *mraY* must come from a promoter located upstream of *mraY* within the cloned sequence. pDEFY4 does not contain an exogenous bacterial promoter. Therefore the expression of the cloned genes must start from within the cloned sequence. These clones all contain an extra 638 bp of DNA upstream from the *Pvu*II site in *murE* until the *Kpn*I site in *ftsI*. Therefore the 638 bp *Kpn*I/*Pvu*II region must contain the promoter for *mraY* (Figure 4.3.11.).
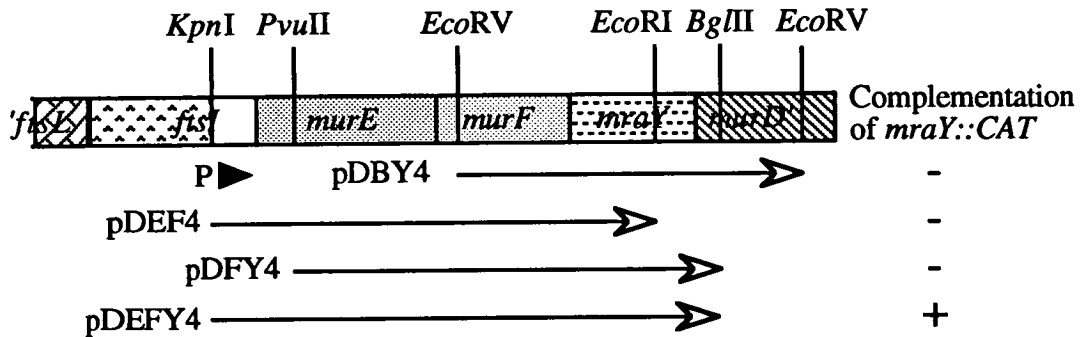
Figure 4.3.11. A schematic diagram showing the regions of DNA cloned in pT7-4 used to study complementation of *mraY*::CAT. pDEFY4 is the smallest fragment to achieve this. Shown in the diagram is the potential *mraY* promoter (filled arrow head) and its location in *ftsI* between *Kpn*I and the start of *murE*.

The ability of pJFY3c to complement the *mraY*::CAT disruption shows that the sequence of *mraY* was not altered during the PCR amplification reaction to facilitate the cloning of the *mraY* coding sequence into the fusion vector pET3c (see Chapter 4.2.3.). The clone pDEF4 was not able to complement *mraY*::CAT. Therefore the truncated *mraY* peptide produced from the 860 bp sequence is unable to rescue the cell from the depletion of MraY. If the entire *mraY* ORF was present in this clone, the *mraY*::CAT allele can be complemented, that is pDEFY4. Therefore the 1080 bp sequence proposed by Ikeda *et al.* (1989) to be the *mraY* ORF does complement the *mraY*::CAT allele and may be considered to be correct.

4.3.6. Suppression of *mraY* expression prevents cell growth in the *mraY*::CAT mutant

The preliminary experiments on the disrupted *mraY* allelle show that it is an essential gene and that the ORF of 1080 bp can complement this. A promoter for *mraY* is located in a 638 bp region, spanning from the *Kpn*I site in *ftsI* to the *Pvu*II site in *murE* which is 2751-3389 bp upstream from the start codon of *mraY*. The 2.7 kb *Eco*RV fragment was cloned into pBAD18 (Guzman *et al.*, 1995) in the same orientation as the P$_{BAD}$ to make pBAY1, a construct 7.1 kb in size (Figure. 4.3.12). The regulation of the P$_{BAD}$ promoter by arabinose and glucose, respectively, is discussed in Chapter 3.4. A pBAD18 clone containing the upstream nucleotide sequence until *Kpn*I in *ftsI* was constructed by restricting pBAD18 with *Eco*RI and *Bam*HI which restrict in the pBAD18 polylinker. A 4.4 kb fragment was restricted from pDEG1 using *Bgl*II and restricting partially with

*Eco*RI. The 4.4 kb *Eco*RI/*Bgl*II fragment was ligated into the linearised pBAD18 DNA to create pBEFY, 8.8 kb (Figure 4.3.13.).
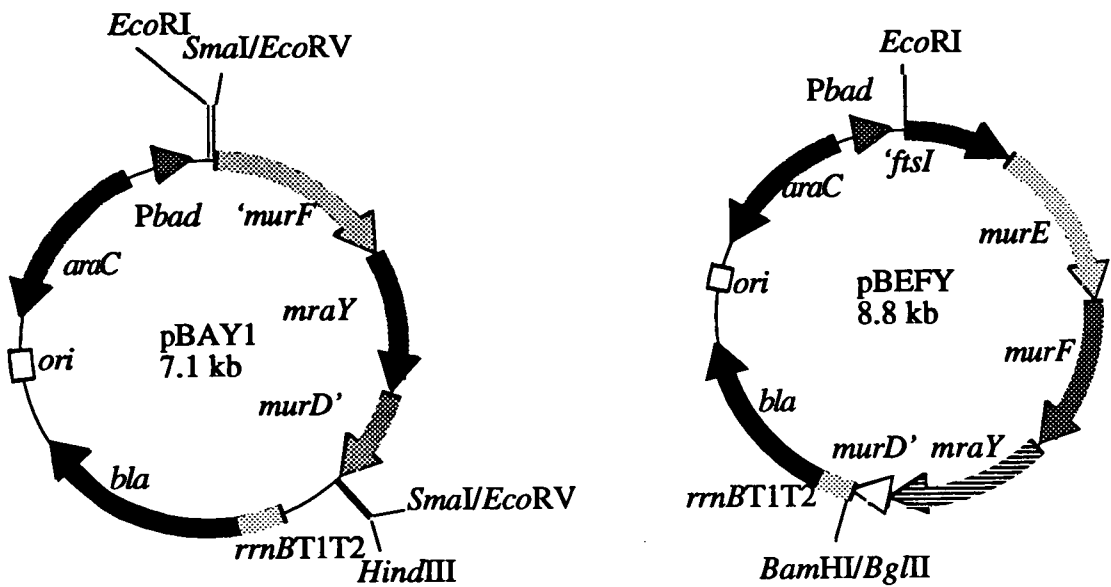
Figures 4.3.12. and 13. The two pBAD18 *mraY* clones, pBAY1 and pBEFY, used to complement *mraY*::CAT and study the effects of Δ*mraY* on cell growth.

C600 was transformed with the pBAD18 *mraY* clones, pBAY1 and pBEFY, and transduced with a P1 (*leu*::Tn10 *mraY*::CAT) lysate. The transductants were selected for on LB Tet/Amp plates supplemented with 1% arabinose. The Tet^R colonies isolated were then patched onto LB Cmp/Amp plates supplemented with 1% arabinose. The isolates which were Cmp^R were then streaked on LB Cmp/Amp agar supplemented with either 1% arabinose or 1% glucose. The Cmp^R/pBEFY transductants grew irrespective of the presence of either arabinose or glucose in the media. The C600/pBAY1 transformants transduced to Cmp^R grew only on plates supplemented with arabinose. These results confirm that *mraY* is an essential gene. The abilty of pBEFY to complement *mraY*::CAT when P$_{BAD}$ expression is repressed also shows that the independent expression of *mraY* comes from a promoter located more than 2.7 kb upstream from the proposed start codon for *mraY*. Microscopic examination of the *mraY*::CAT/pBAY1 cells cultured on glucose revealed that the MraY null phenotype affected cell growth, rather than exclusively cell division. That is, cells were both short and long rods which were often misshapen. Many of the cells had lysed, extruding spheroplasts of cytoplasm presumably through ruptures in the cell wall.

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4.3.7. Characterization of the ΔmraY phenotype.

The previous work to create an *mraY* null mutant revealed that *mraY* is an essential gene. Depletion of MraY in cells leads to a cessation in growth and ultimately to cell lysis. The aims of this experiment were to monitor closely the effects of depletion of MraY in a culture of growing cells with respect to culture density, cell size and cell number. An *mraY*::CAT *leu*::Tn10 mutant, DBYC2/pBAY1, was chosen. An overnight culture of DBYC2/pBAY1 was used to inoculate filtered LB Tet/Cmp supplemented with 1% arabinose with shaking at 37°C. An *mraY*+ control, C600T/pBAY1, was cultured under the same conditions, except for the presence of Cmp in the media. The growth of the cultures, supplemented with 1% arabinose, was followed by measuring the OD_{540}. At each sampling for measurement of the OD_{540}, 0.5 ml of culture was fixed in sample buffer and stored at 4°C for subsequent analysis of cell size and cell numbers using a Coulter Channelyser and Counter.

When the cultures had attained a steady growth rate, 1 ml aliquots from each culture were harvested. The cells in the aliquots were pelleted by centrifugation and washed in 1 ml of LB containing either 1% arabinose or glucose, prewarmed to 37°C. This step was then repeated. The washed cells were then used as inocula for 25 ml volumes of prewarmed (37°C) and filtered LB Tet/Cmp containing either 1% glucose or arabinose. The C600T control cultures used similar media without Cmp. The growth of the cultures was then followed by measuring the OD_{540} and cells fixed from the sample volumes. Cultures were diluted into fresh prewarmed, filtered LB media (1% arabinose or glucose) when the OD_{540} increased above 0.250.

The data shown in Figure 4.3.14. shows that the growth rate of DBYC2/pBAY1 is initially similar to that of the culture grown in LB supplemented with 1% glucose. The mass doubling time was calcluated by plotting the OD_{540} against time on semilog graph paper. This was calculated to be ~33 minutes per mass doubling of the culture when grown in LB with 1% arabinose.

Initially the optical density of the culture grown in LB 1% glucose (that is expression of *mraY* from P_{BAD} is repressed) increased at a similar rate to that of the induced arabinose culture (Figure 4.3.14.). However after ~90 minutes of culturing in LB 1% glucose media the mutant quickly slowed in growth until stasis was reached after 160 minutes. The culture densities of the *mraY*+ controls are shown in Figure 4.3.15. The presence of either arabinose or glucose in the

medium did not affect the growth rates of either culture. The mass doubling time was calculated to be ~31 minutes. This is almost identical to the mutant, DBYC2/pBAY1, cultured in LB 1% arabinose media. The growth rates of the wild-type and mutant strains grown in LB 1% arabinose were plotted together in Figure 4.3.16. The growth of the same strains in LB 1% glucose was also measured (Figure 4.3.17.). Cells from both mutant cultures were compared microscopically, to observe the effects of MraY depletion in actively growing cells. When the mutant, DBYC2/pBAY1, was grown in LB 1% arabinose the cells grew as predominantly as rods with some occasional filamentous cells and some cells which had lysed (Figure 4.3.18.). After 3 hours of growth the LB 1% glucose culture, however, consisted of a mixture of filaments and squat cells which appeared ovoid, that is, tapering from one pole to the other (Figure 4.3.19.). In addition to these many other cells had lysed.

Figure 4.3.14. Growth curves of the DBHY2/pBAY1 where expression of *mraY* from P$_{BAD}$ was either induced or repressed . When *mraY* is expressed in arabinose based media, the culture quickly reaches log growth from stationary phase and sustains this throughout the experiment until the growth slowed with the entrance of the culture into late log growth phase. The culture mass doubling time for this culture was estimated to be ~33 minutes. Upon repression with glucose, the growth rate was initially unaffected. However after 90 minutes of growth the generation time started to slow rapidly. The culture then stopped growing entirely and remained in this state for the remainder of the experiment. Cell shape at this point was small rods which were often misshapen. That is they were often tapered. Towards the end of the experiment the cells had started to lyse extruding sheroplasts. The morphology of the induced culture was normal, that is predominantly rods with occasional filaments.

Figure 4.3.15. Growth rates of the C600T (wild-type) strain containing pBAY1. The growth rates of both cultures are the same, irrespective of the arabinose or glucose supplements. The doubling times were estimated to be ~31 minutes for both cultures. These remain the same throughout the entire experiment. These data show that the induction or repression of *mraY* expression from pBAY1 does not affect the growth rates of wild-type cells.
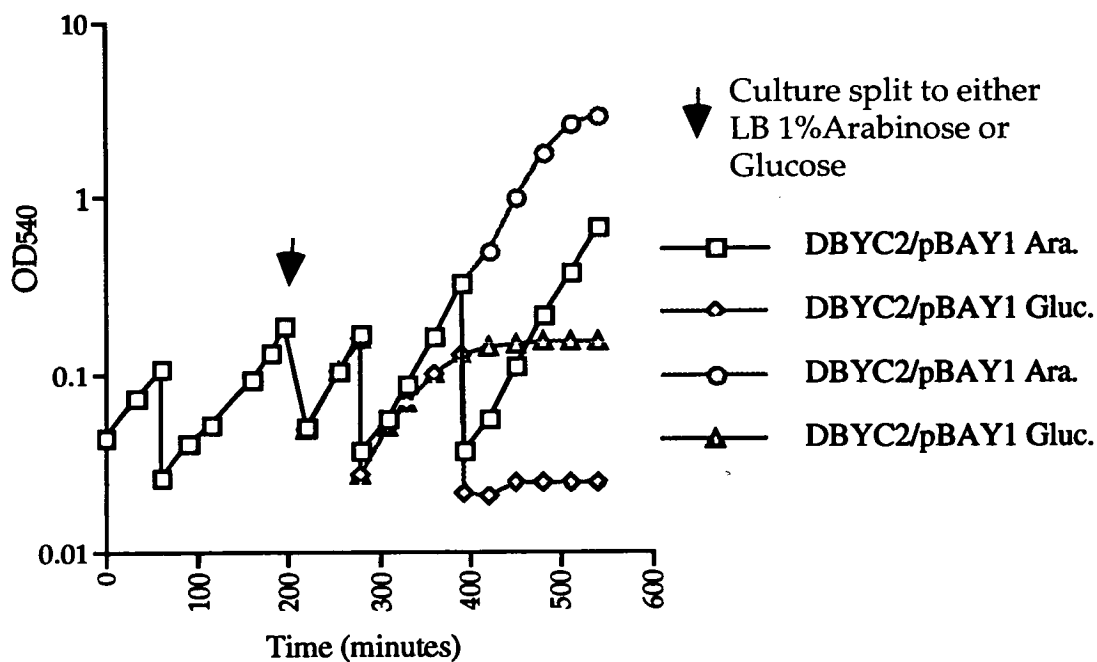
Figure 4.3.14. The culturing of DBYC2/pBAY1 in LB Tet/Cmp supplemented with either 1% arabinose or 1% glucose.
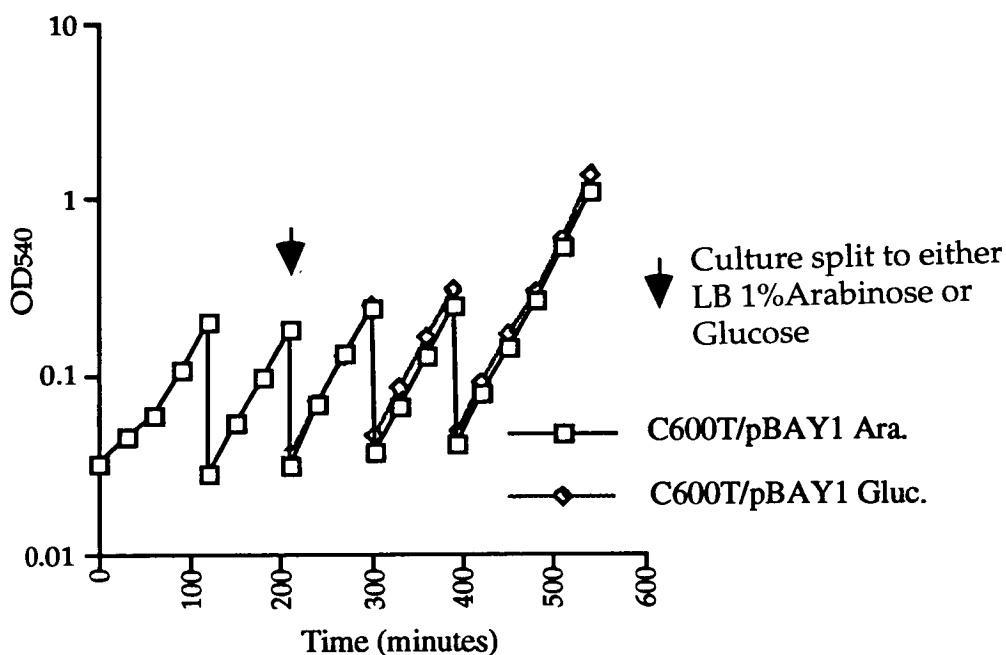

Figure 4.3.15. The growth of C600/pBAY1 in LB Tet supplemented with either 1% arabinose or 1% glucose

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Figure 4.3.16. The graphs show that there is little difference between the culture mass doubling times for the mutant and the wild-type strains when *mraY* is expressed from pBAY1 cultured with LB 1% arabinose. This indicates that the *mraY*::CAT allele is fully complemented when related to the growth of the culture. That is pBAY1 can complement *mraY*::CAT when expression from P$_{BAD}$ is induced.

Figure 4.3.17. The data presented here clearly shows the effects of the repression of *mraY* expression from pBAY1. The wild-type strain is unhindered by the addition of glucose, as previously shown in Figure 4.3.14. The effect of represion of *mraY* expression from P$_{BAD}$ can be seen to occur after 120 minutes of culturing in LB 1% glucose.

Figure 4.3.16. A comparison of the growth rates of the *mraY*::CAT mutant and the *mraY* wild-type strains grown cultured in the presence of arabinose.

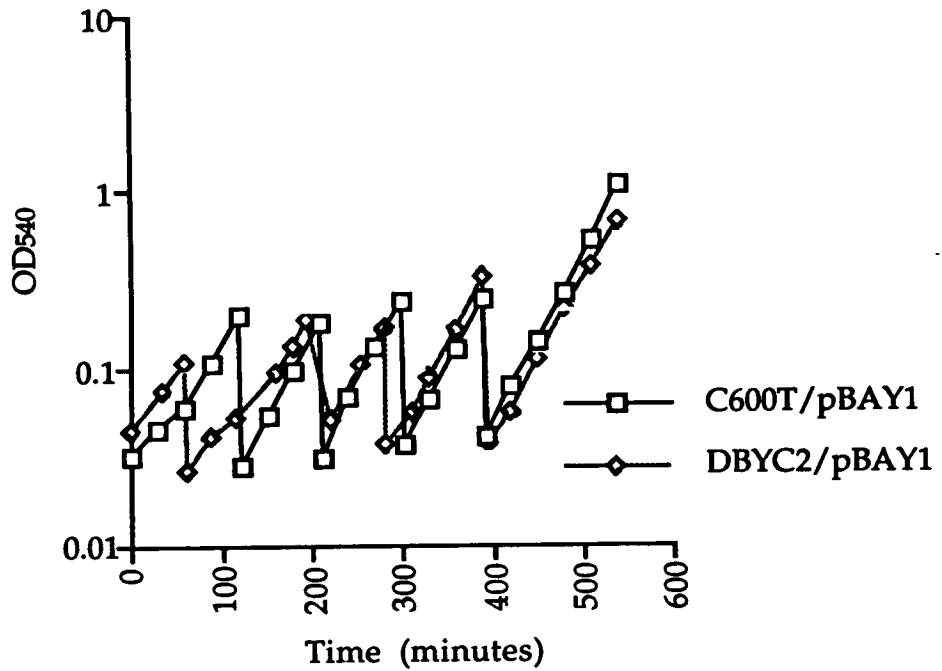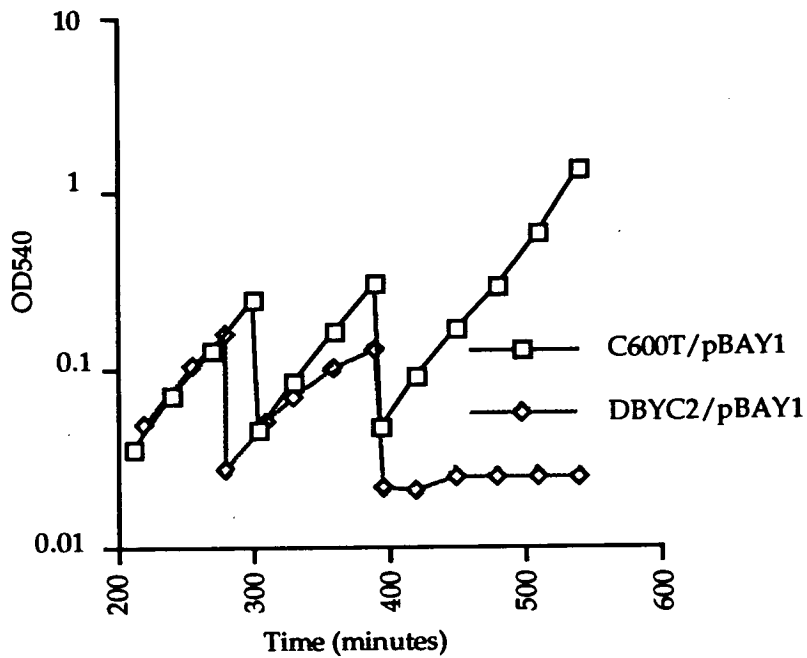**Figure 4.3.17.** A comparison of the growth rates of the *mraY*::CAT mutant and the *mraY* wild-type strains grown cultured in the presence of glucose.

Figure 4.3.18. DBYC2/pBAY1 after 180 minutes of culturing in LB Tet/Cmp/ 1% ara at 37°C. Bar is equal to 10 μm.
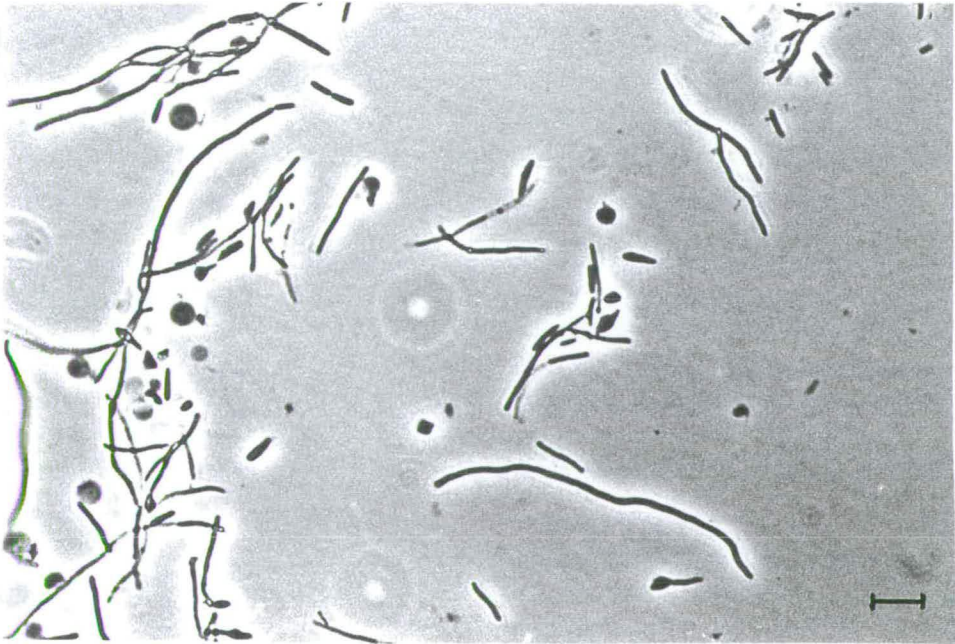

Figure 4.3.19. DBYC2/pBAY1 after 180 minutes of culturing in LB Tet/Cmp/ 1% gluc at 37°C. Bar is equal to 10 μm.

Aliquots of the fixed cell samples were analysed for cell size and cell numbers using a Coulter Channelyser (Model C-1000) and Coulter Counter (Model ZB). The data for the median cell size of DBYC2/pBAY1 cultured in LB supplemented with either 1% arabinose or 1% glucose is shown in Figure 4.3.20. The data for the LB arabinose culture show an increase in cell size, in terms of median cell volume, from 12 units to 40 units during the first 60 minutes of growth. This reflects the increase in cell size as cells in the culture leave stationary phase and prepare to enter log phase growth. The cells introduced to the LB glucose media initially remain at ~40 units before starting to decrease in size after 60 minutes. The cell size decreases to ~20 units before briefly stabilizing for 60 minutes at 20-25 units before decreasing yet again in size. At this point the sizing of cells from the LB glucose culture then proved impossible because detritus in the culture prevented the accurate measurement of cell size data. As the LB arabinose culture increased in density (that is approaching stationary phase) the median cell size once more started to decrease as the growth rate started to decrease.

The data presented for the median cell volume of the wild-type strain, C600T, when grown in LB 1% glucose remains very similar to the 1% arabinose culture throughout the experiment. This indicates that the suppression or induction of *mraY* expression (that is the wild-type sequence) has no effect on the cell volume. The median cell volume of the wild-type cultures was the same as for the mutant grown in LB arabinose (Figure 4.3.21.). Therefore the induction or repression of expression of *mraY* does not affect the size of cells. The similarity in median cell volume of these cultures reveals that pBAY1 can complement the *mraY* null allele to such a degree that the median of the mutant cells is the same as the wild-type cells (Figure 4.3.22.). Therefore the median cell size data of the mutant and wild-type cultures reflects the culture density data, that is the OD_{540} and cell size data are similar for both the wild-type and the mutant when cultured in LB arabinose.

Figure 4.3.20. The plot reveals the median cell volume of the LB arabinose culture throughout the experiment and the effects of MraY depletion due to repression of P_{BAD} by the addition of glucose to the culture medium. The initial increase in cell size can be seen for the LB arabinose culture as the cells aproach log phase growth. Whilst in log phase growth the median cell volume settles at ~40 units in size. This then starts to decrease as the cells reach late log phase growth. The switch from LB arabinose to LB glucose media does not immediately affect the size of the cells. A change occurs after 60 minutes of growth when the median cell volume then decreases over the next 100 minutes until no further readings could be taken.

Figure 4.3.21. The median cell volume of the wild-type cells initially increases from 15 units to 40 units as the cells prepare to enter log growth phase. The cell size remains stable throughout log growth and then falls as the cells are in late log growth. There is no difference in the median cell volume between the two cultures. Therefore the expression or repression of *mraY* has no effect on the median cell size of the wild-type strain.
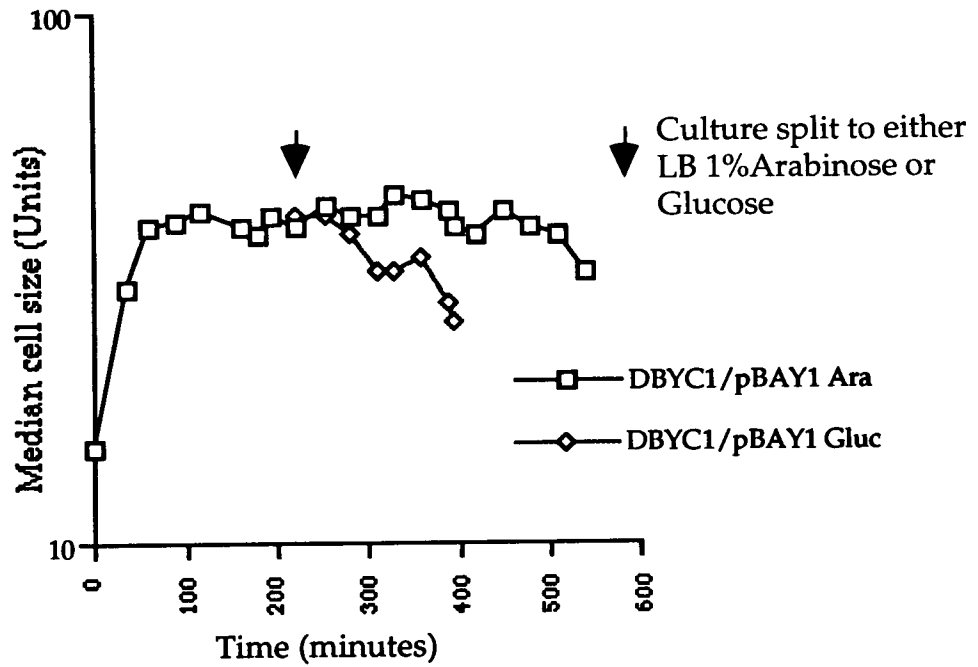
Figure 4.3.20. A comparison of the median cell volume of DBYC2/pBAY1 cultured in either 1% arabinose or 1% glucose.
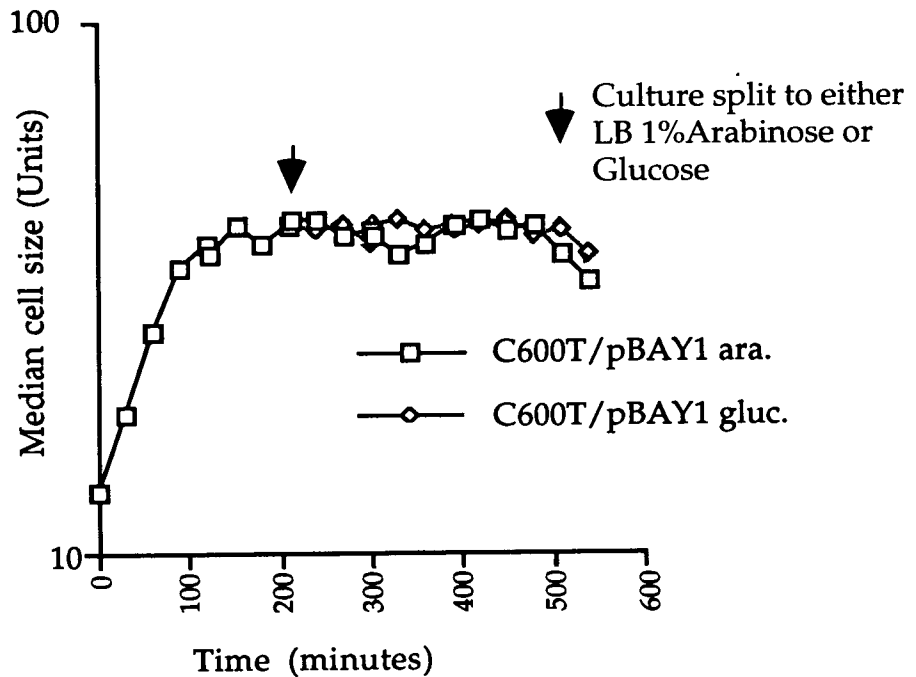

Figure 4.3.21. The median cell sizes of C600T/pBAY1 cultured in either LB Tet/1% arabinose or 1% glucose.
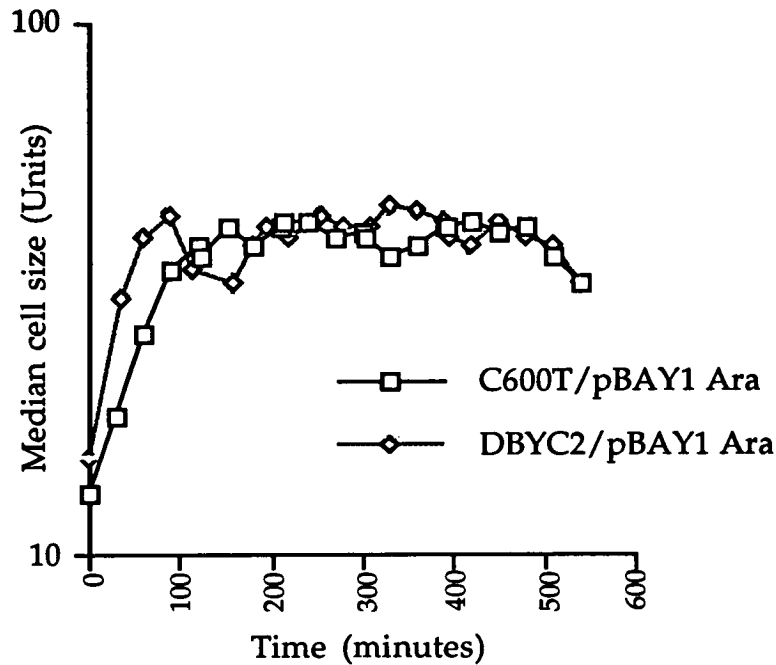
Figure 4.3.22. The median cell size of both the mutant and wild-type *mraY* strains grown in LB 1% arabinose. Both strains exhibit similar characteristics when grown in media supplemented with arabinose. The mutant culture may have a more rapid increase in cell size from leaving stationary phase. In log growth both cultures are the same. This is the same for late log growth when the median cell size of both cultures starts to decrease.

The effect of MraY depletion on the number of cells per ml of culture was measured using a Coulter Counter Model ZB. The data for DBYC2/pBAY1 cultured in LB supplemented with either 1% arabinose or 1% glucose can be seen in Figure 4.3.23. The number of cells in the LB arabinose culture is typical of cells entering log growth, that is the cell number remains relatively stable whilst the cell size increases prior to growth and division. This can be seen by the rise in OD540 and in the median cell size (Figures 4.3.12 and 19.). After 90 minutes growth the cells start to divide regularly. This continues throughout the remainder of the experiment. The cells which are transferred from arabinose based media to glucose based LB media are initially unaffected . That is, the number of cells per ml of LB glucose culture is similar to the numbers of cells per ml for the arabinose culture. These data correlates with the OD540 and median cell size data which also remains similar to the data collected for the LB arabinose culture.

However unlike the OD540 and median cell size data from the LB glucose culture, the number of cells per ml does not stop rising: although the increase in

cell number per ml for the LB glucose culture is less than that in the LB arabinose culture. The increase in the cell numbers is consistent with the OD540 whilst the median cell size decreases. For this to occur there have to be more and smaller cells, in the culture. Part of the count will also be due to cellular debris from lysed cells. Thus the depletion of MraY from cells has an effect much the same as entry into stationary phase. That is cell size decreases while the culture density remains constant.

The numbers of cells per ml in the wild-type cultures were very similar (Figure 4.3.23.). This was to be expected, given that the OD540 and median cell size of the cultures were nearly identical (Figures 4.4.14. and 4.3.21.). The cells per ml of the wild-type and mutant cultures grown in LB arabinose are again similar. These data are also consistent with earlier comparisons of the OD540 and the median cell size (Figures 4.3.18. and 4.3.25.).

Therefore this analysis of the *mraY*::CAT mutant has revealed that provided MraY is supplied from a plasmid based vector, the null disruption has no noticeable effect on the growth rate or the number of cells per ml of culture. It does appear to have a lethal effect on a small proportion of cells within the culture (Figure 4.3.17.). The median cell size, however, does not reflect the presence of a significant part of the cell population being filaments. The depletion of MraY from the *mraY*::CAT background has a lethal effect. The growth of the culture is inhibited, the median cell size is rapidly reduced and the cells assume aberrant morphology. The presence of the filaments observed in Figure 4.3.19. is not reflected by the data produced by the Coulter Counter.

Figure 4.3.23. The number of cells per ml of the *mraY* mutant cultured in LB supplemented with either arabinose or glucose. Initally the number of cells per ml for the LB arabinose culture rises only slowly. This results in an increase in cell size prior to division in the log phase growth . The transfer of part of this culture to a glucose based medium does not affect cell division initially. After 90 minutes the number of cells per ml in the LB glucose culture decreases in relation to the arabinose culture., This effect is also seen with respect to the OD540 measurements and the median cell size. This is the effect of the depletion of MraY from the cells.

Figure 4.3.24. The comparison of cell numbers per ml of the wild-type cultures grown in LB supplemented with either arabinose or glucose. At the onset of growth the cells number in the LB arabinose culture does not significantly increase. This is expected before the cells enter log phase growth. When the LB glucose culture is inoculated, there is a slight drop in cell number when compared to the LB arabinose culture. This may be due to the change in medium composition. There are no other differences, therefore the expression of MraY from pBAY1 has no effect on the number of cells per ml in a wild-type strain.
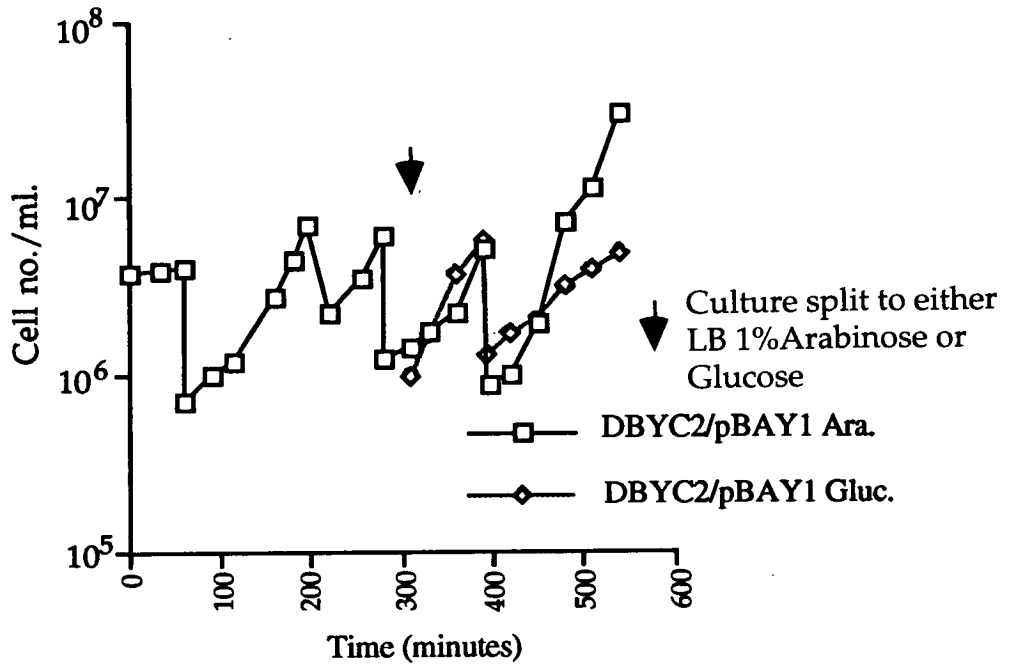
Figure 4.3.23. Cell number per ml for DBYC2/pBAY1 when cultured in LB Cmp/Tet with either 1% arabinose or glucose.
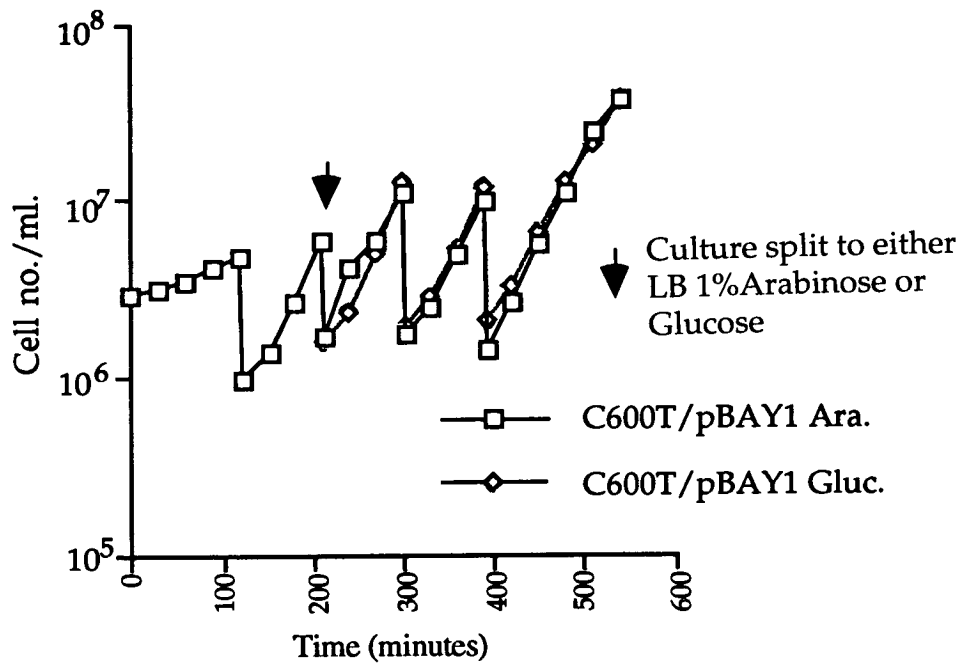
Figure 4.3.24. Cell number per ml of the wild-type strain cultured in LB Tet with either 1% arabinose or glucose.

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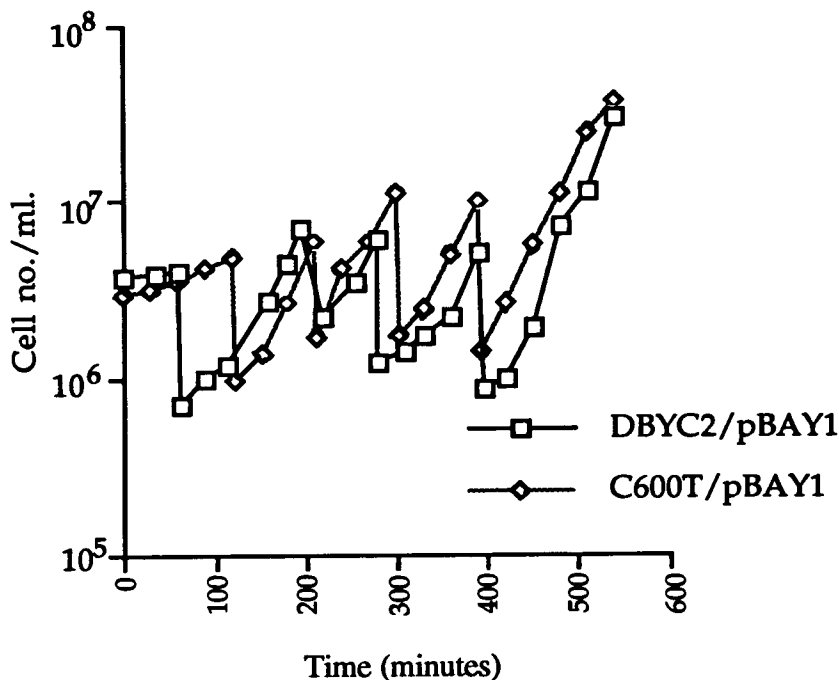
Figure 4.3.25. Cell number per ml of the wild-type control, C600T/pBAY1, cultured in LB Tet plus either 1% arabinose or glucose. The numbers of cells per ml from either the culture are very similar, especially with respect to the OD540 and the median cell sizes of the cultures, which were measured previously.

4.3.8. The effect of MraY depletion on filamentous cells.

The cell wall ruptures reported in the MraY mutants were sometimes observed at the midpoint of the lysed cell. This led to speculation that MraY may be directly involved in the division process. To test this hypothesis, an experiment was designed to reduce the levels of MraY in filamenting cells and monitor the effect on the growth rate and cell morphology. DBYC2/pBAY1 was induced to filament by the addition of nalidixic acid (Nal) to the culture medium. Nalidixic acid causes the filamentation of cells by inducing the SOS response (see Chapter1.3.4.). The presence of Nal in the cell inhibits DNA gyrase and therefore prevents DNA replication (Sugino et al, 1977). The inhibition of DNA replication is an effector of the SOS response. The function of FtsZ is affected by the SfiA (SulA) inactivating (reversibly) the FtsZ protein when the SOS response is induced thus preventing the cells from division. Thus, cells grow as filaments.

An inoculum from an overnight culture of DBYC2/pBAY1 was grown in LB Cmp/Tet supplemented with 1% arabinose and incubated at 37°C with shaking. The growth rate of the culture was monitored by measuring the OD_{540}. Once the cells had reached a steady state, four 1ml aliquots were removed and the cells pelleted and washed in LB Cmp/Amp, supplemented with either 1% arabinose or glucose. This step was repeated. The washed cells were then used as inocula for LB Cmp/Tet with 1% arabinose or 1% glucose, supplemented with Nal. As controls, cells were also cultured in the same media without Nal. The cultures were incubated at 37°C with shaking. Regular sampling to measure the density of the cultures at OD_{540} was performed. These data were plotted in Figure 4.3.26.
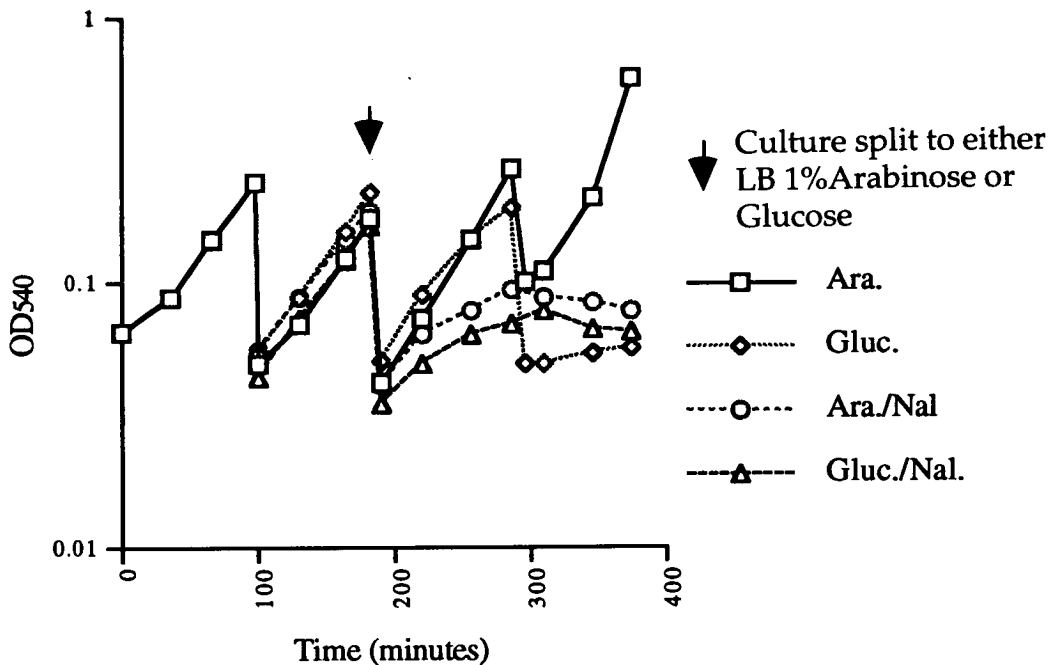
Figure 4.3.26. A comparison of the growth of DBYC2/pBAY1 grown in LB 1% arabinose or 1% glucose, with or without Naldixic acid.

After 90 minutes of growth the OD_{540} of both the LB Cmp/Tet/Nal arabinose and the glucose cultured cells started to slow when compared to the control cultures. The control cultures without Nal were unaffected at this stage. After three hours the cells grown in LB Cmp/Tet 1% glucose also slowed in growth. Microscopic examination of the cells showed that the presence of Nal induced filamentation in both the arabinose and glucose cultures. The cells from the 1% glucose culture were examined after 200 minutes of growth. At this point the effect of MraY depletion could be seen on the growth rate of the control

culture DBYC2/pBAY1 grown in 1% glucose. The morphology of the cells from the DBYC2/pBAY1 culture grown in LB glucose was similar to that observed before, that is short squat cells which were swollen and slightly rounded with lysing cells. By comparison, the Nal treated cultures contained filamentous cells, some of which had lysed. A comparison between the arabinose and glucose cultures treated with Nal revealed that although both were composed entirely of filamentous cells, the rate of cell lysis appeared to be greater in the glucose culture. The arabinose culture without Nal grew constantly throughout the experiment (Figures 4.3.26 and 4.3.27.) and microscopic examination of the culture revealed it to comprise mainly of rod-shaped cells with occasional filaments .

A comparison of the growth rates of DBYC2/pBAY1 cultured in arabinose or glucose in the presence of Nal reveals that the presence or absence of MraY has little effect on the cell growth (Figure 4.3.28.). Both cultures grew at a similar rate and only microscopic examination of the cells revealed a higher degree of lysis in the 1% glucose culture. The absence of MraY did not produce any obvious morphological defects in the filamenting cells other than increased lysis. In addition, the leaking of cytoplasm from cell wall ruptures in the mutants did not appear to be localised at potential division sites, that is the location of ruptures in the cell wall seemed to be random, occurring at any point on the lateral cell wall.

Figure 4.3.27. The growth rate of DBYC2/pBAY1 cultured in LB Cmp/Tet supplemented with either 1% arabinose or 1% glucose. The mass doubling time for the arabinose culture was ~35 minutes. Initially the growth rate of the glucose culture was unaffected. After ~90 minutes of exponential growth, the glucose culture stopped and did not increase for the duration of the experiment.

Figure 4.3.28. The growth rates of both cultures was soon affected after introduction to Nal supplemented media. The cultures were equally affected. That is the arabinose or glucose supplements made no difference to the growth rates. Microscopic examination of the cultures revealed both to be composed of filamentous cells after 100 minutes of growth in LB Nal. In the latter stages, the filaments of both cultures had started to lyse. Filamentation was more commonplace in the glucose grown culture, presumably due to the additional effect of MraY depletion. The lysis of the cells was not limited to the potential division sites.
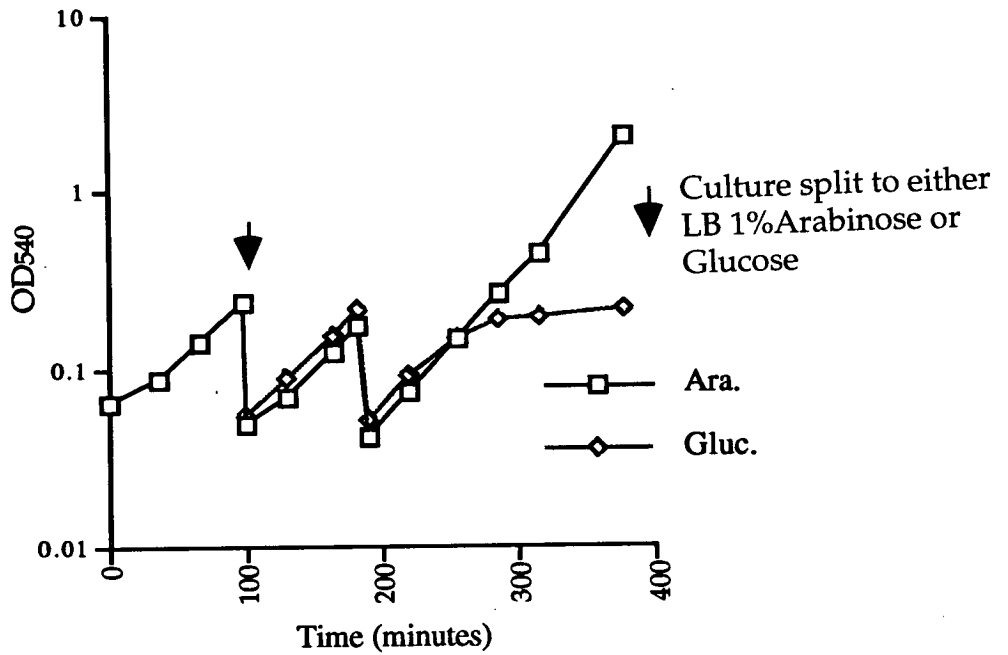
Figure 4.3.27. The growth curves of the *mraY*::CAT mutant cultured in either LB 1% arabinose or 1% glucose.
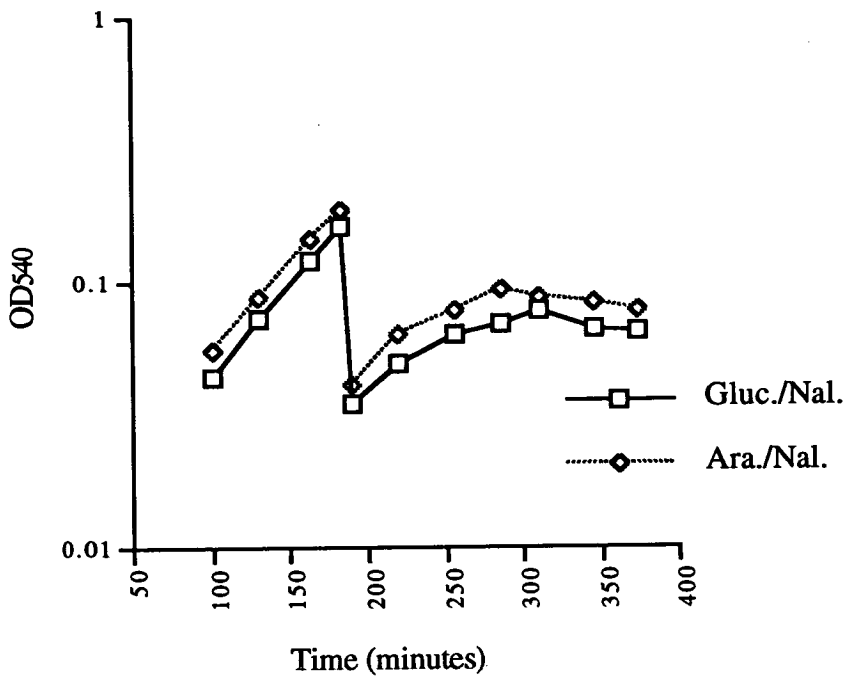

Figure 4.3.28. The growth of the DBYC2/pBAY in LB Nal supplemented with either 1% arabinose or 1% glucose.

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4.3.9. The promoter activity expressed from a potential *mraY* promoter region.

The creation of an *mraY* null allele made it possible to identify a region of DNA upstream from *mraY* necessary to allow complementation in the absence of an exogenous promoter. The use of pT7-4 clones facilitated the screening for this region (Figure 4.3.10.). The clones pDBY4 and pDFY4 could not complement the *mraY*::CAT allele. The clone pDFY4 contains 2751 bp of upstream DNA from the start codon of *mraY* but this was unable to complement the null allele. Therefore the *mraY* promoter region lies upstream from this. The clone pDEFY4 could complement the *mraY*::CAT allele. pDEFY4 is identical to pDFY4 with the exception that pDEFY4 contains a further 638 bp of the upstream DNA from the *Kpn*I site in *ftsI* up to the*Pvu*II site in *murE*. Therefore this fragment contains a promoter for *mraY*. There is also evidence for the promoters for *murE* and *murF* being located in this fragment. This was shown by Mengin-Lecreulx *et al.* (1989), who could not complement a *murF*ᵗˢ mutant unless the DNA from the *Kpn*I site in *ftsI* was present upstream of the *murF* ORF. They also revealed that a promoter for *murE* is located after the *Kpn*I site in *ftsI* immediately upstream from *murE*. It may be that the same promoter is responsible for the expression of *murE murF* and *mraY*.

From this work it has been shown that smaller fragments of DNA containing the entire *mraY* ORF can complement the *mraY*::CAT null allele, providing an external promoter is present to express *mraY*. Examples of this are pUCY1, pJFY1 and pBAY1. The latter is an excellent example as induction of P$_{BAD}$ in pBAY1 allows complementation, whilst repression of expression of P$_{BAD}$ causes cell death. Therefore the presence of upstream DNA is not essential. This removes the possibility that either of the two upstream genes, *murE* and *murF*, or *murD* located downstream may also have been mutated. That is pUCY1, pJFY1 and pBAY1 only contain the complete ORF for *mraY* and therefore can only complement this.

Promoter activity from the 628 bp region, was determined by measuring the β-galactosidase activity as described by Miller (1972). The reporter system used was the single copy phage "operon" *lacZ* vector as described by Simons *et al.* (1987; see Chapter 3. Figures 3.3.43-44.). The 628 bp fragment was first subcloned into pUC18 to create a clone where the 628 bp fragment would be flanked by an *Eco*RI restriction site upstream and a

*Bam*HI restriction site downstream. This would allow a unidirectional cloning into pRS551, where *lacZ* in pRS551 would be expressed from the putative promoter site in the 628 bp fragment.

The 628 bp fragment was removed from pDEFY4 by restriction with *Sma*I and *Pvu*II. This produced a fragment of 630 bp in size. This was then cloned into pUC18 restricted with *Sma*I. The fragment was cloned in both orientations to create pBSD5 and pBSD6. These were both 3.3 kb in size. The clone pBSD5 was chosen as the 628 bp fragment could be easily introduced to the promoter vector pRS551 by an *Eco*RI/*Bam*HI restriction of both vectors and the subsequent ligation of the 630 bp fragment into the restricted pRS551. This made pBSD40 where *lacZ* was expressed from the 628 bp fragment which facilitates the expression of *murE*, *murF* and *mraY* (Figure 4.3.29).
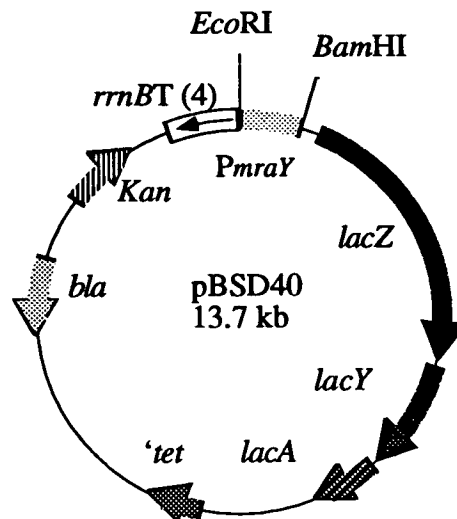
Figure 4.3.29. The clone pBSD40 which was used to transfer the putative *mraY* promoter region into a single copy λ prophage.

A prophage containing the promoter *lacZ* fusion from pBSD40 was then constructed by culturing TP8503 (λRS45)/pBSD40 and inducing lysis by UV. Prophage into which pBSD40 has recombined will confer Kan^R on a host cell. The lysate was used to infect TP8503 and Kan^R lysogens selected on LB Kan agar. A Kan^R lysogen was recovered and this was used to make a high titre phage stock by again using UV induction of a λ prophage. The lysate (λBSD40) was used to infect TP8503 and Kan^R lysogens selected on LB Kan agar. One was chosen and streaked on LB Kan/X-Gal. A promoterless

phage control (λBSD0, see Chapter 3.3.) was also used. The λBSD40 lysogen produced blue colonies whilst the λBSD0 control produced colorless colonies. As a simple test for promoter activity this showed that *lacZ* was being expressed. However in the previous chapter a similar prophage *lacZ* fusion produced blue colonies when cultured under the same conditions, but upon more detailed analysis the β-galactosidase activity was minimal. Simons *et al.* (1987) state that the cloning of any DNA sequence into the single copy reporter system will produce low levels of β-galactosidase activity.

The promoter activity from TP8503(λBSD40), was estimated by measuring β-galactosidase activity in LB Kan at 37°C. A promoterless phage control, TP8503 (λBSD0), was also used where the prophage contains no insert upstream from *lacZ*. The cultures were grown from stationary phase and maintained in log phase growth by diluting when appropriate. The cultures were then allowed to enter stationary phase. Samples were taken at each OD$_{600}$ measurement and assayed for β-galactosidase activity using the method described by Miller (1972, see Chapter 2.). Therefore the β-galactosidase activity produced from the proposed *mraY* promoter region was measured throughout the growth cycle of the culture (Figures 4.3.30. and 4.3.31). It was presumed that the promoter activity would be highest when the cells were in log phase growth as *mraY* is involved in production of precursor, lipid I (Ikeda *et al.*, 1991; Mengin-Lecreulx *et al.*, 1991; van Heijenoort *et al.*, 1992), for the construction of the murein sacculus during cell growth.
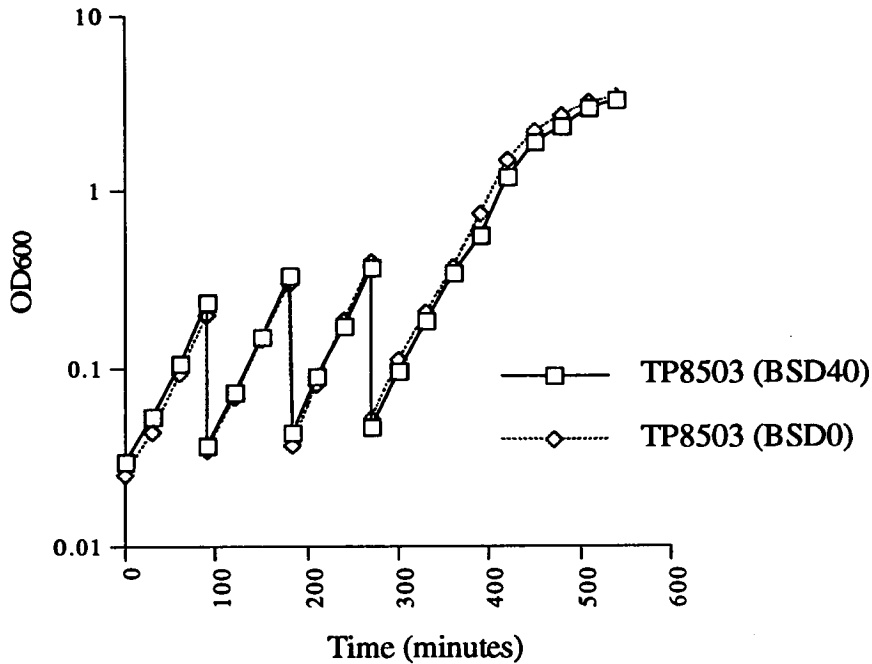
Figure 4.3.30. The growth curves of the reporter assay vector lysogen TP8503 (λBSD40) and the promoterless control lysogen TP8503 (λBSD0).

The data presented in Figure 4.3.31 suggests that there is promoter activity produced by the 628 bp fragment. However when compared to data collected from other promoter regions in *E.coli* such as for *ftsZ*, this activity is very low (R. Smith, PhD thesis, 1995). Higher promoter activity has also been reported for the promoter of *pcnB* (N. Binns, pers comm.) using the same prophage reporter system. The high levels of activity of the *ftsZ* promoters may be due to the high levels of FtsZ required by the cell (5000-≤20000 molecules per cell [Bi and Lutkenhaus, 1991]). The cellular levels of MurE and MraY in the cell are not known, although earlier work in this thesis shows that the MraY peptide is not easily overexpressed. The actual level of MurF in the cell is also unknown but deRoubin *et al.* (1992) showed that the catalytic activity of MurF within cells can vary slightly depending on the composition of the culture medium in which the cells are grown.
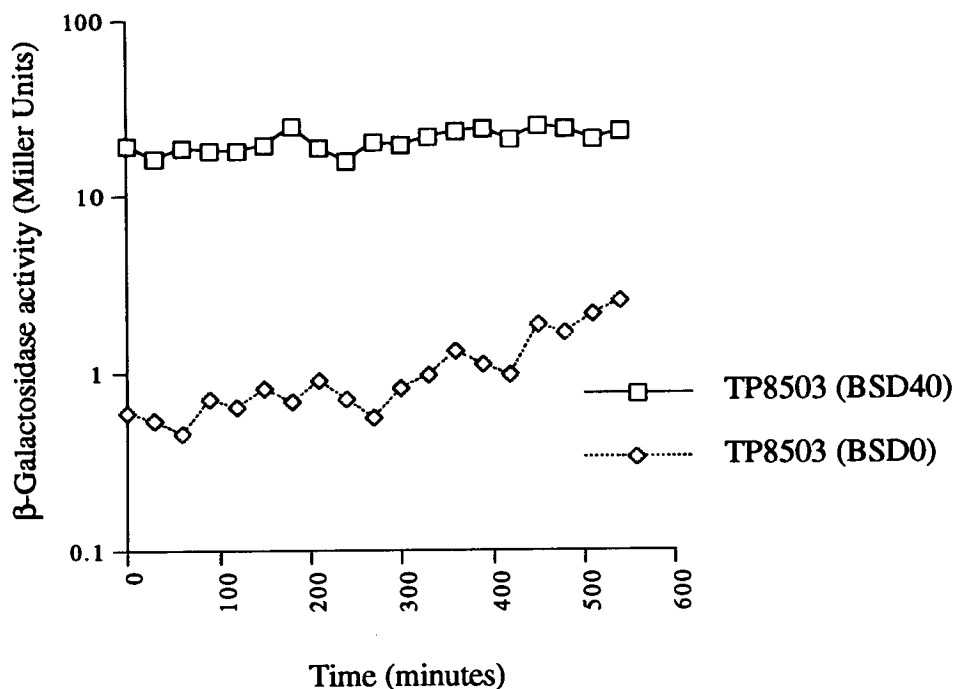
Figure 4.3.31. The β-galactosidase activity expressed from λBSD40 and the promoterless control λBSD0 cultures (Figure 4.3.30.). The activity expressed from the λ clone is approximately ten times greater than the control. The β-galactosidase activity does not vary throughout the experiment. This would suggest that the expression of *mraY* and indeed *murE* and *murF* is constant throughout the growth cycle. That is the promoter activity is the same whether the cells are in stationary phase or in log phase.

The nucleotide sequence of the 628 *KpnI/PvuII* fragment was examined for the presence of consensus sequences for σ factors. The consensus sequence for a σ^{70} promoter was found in the 628 bp fragment (Helmann and Chamberlain, 1988). The -10 region had homology with 5 out of the six bases for the σ^{70} consensus sequence. These are shown in Figure 4.3.31. There were three bases which matched the consensus sequence for the -35 region. These were 17 bp upstream from the -10 site (Figure 4.3.32.)

*Kpn*I   17 bp

GGTACCGCGAAAAAGGTCGGGC<u>CGGAC</u>GGTCGCTACATCAATAAA<u>T</u>
<u>ATATT</u>GCTTATACCGCAGGCGTTGCGCCTGCGAGTCAGCCGCGCTTC
GCGCTGGTTGTTGTTATCAACGATCCGCAGGCGGGTAAATACTACGG
CGGCGCCGTTTCCGCGCCGGTCTTTGGTGCCATCATGGGCGGCGTATT
GCGTACCATGAACATCGAGCCGGATGCGCTGACAACGGGCGATAAA
AATGAATTTGTGATTAATCAAGGCGAGGGGACAGG**TGG**CAGATCGT
AATTTGCGCGACCTTCTTGCTCCGTGGGTGCCAGACGCACCTTCGCGA
GCACTGCGAGAGATGACACTCGACAGCCGTGTGGCTGCGGCGGGCG
ATCTCTTTGTAGCTGTAGTAGGTCATCAGGCGGACGGGCGTCGATAT
ATCCCGCAGGCGATAGCGCAAGGTGTGGCTGCCATTATTGCAGAGGC
GAAAGATGAGGCGACCGATGGTGAAATCCGTGAAATGCACGGCGTA
CCGGTCATCTATCTCAGCCAGCTCAACGAGCGTTTATCTGCACTGGCG
GGCCGCTTTTACCATGAACCCTCTGACAATTTACGTCTCGTGGGCGTA
ACGGGCACCAACGGCAAAACCACGACTACCCAGCTG

Figure 4.3.32. The nucleotide sequence of the 628 bp *Kpn*I/*Pvu*II fragment. Underlined are the potential σ^{70} promoter -10 and -35 regions. The underlined bases in bold are those which exactly match the σ^{70} consensus sequence, that is TATAAT (-10) and TTGACA (-35). Also shown in bold is the start codon for *murE* (TGG) and the terminating codon of *ftsI* (TAA).

Therefore there is some homology to a σ^{70} promoter consensus sequence located upstream from the start of *murE* in *ftsI*. This is significant as Mengin-Lecreulx *et al.* (1989) showed that a promoter is located between the *Kpn*I site in *ftsI* and the start of *murE*, this being essential for the expression of *murE* and *murF*. This was shown by studies on the complementation of ts mutants by cloning the region, from *Kpn*I in *ftsI* until *Eco*RI in *mra*Y, in the opposite orientation to the P*lac* promoter in pUC18. This clone could complement both *murE* and *murF* ts mutants strains when cultured at 42°C. As there was no complementation of the *mra*Y::CAT mutant allele with clones containing upstream DNA until the 628 bp fragment located in *ftsI-murE*; it was assumed that *mra*Y also shares the same promoter as *murE* and *murF*. As these genes produce enzymes whose functions are involved in the synthesis of the dissacharide pentapeptide precusor for murein synthesis it is possible that they rely on the same promoter for their mutual expression. The levels of protein produced may

be regulated by the stability of the mRNA transcript or of the nascent peptides produced. Previous work by Mengin-Lecreulx *et al.* (1989) and in this study have shown that both the MurE and MurF peptides can be over-expressed when cloned together. The isolation of MraY peptide has so far proved elusive . This has led to speculation that its translation may be tightly regulated (this work).

Discussion.

Attempts to show a peptide *in vivo* from the putative *mraY* ORF failed. Expression of *mraY* fusions to either the *gene 10* rbs or the *gene 10* polyHis tag did not produce a peptide. The SDS-PAGE analysis of *in vitro* synthesis of the protein did produce a product of the predicted molecular weight. However, the plasmid used for this experiment also contained the ORF for *lacI*. LacI is similar in size to MraY and it may be argued that LacI and not MraY was produced by the *in vitro* labelling study. To resolve this, the cloned DNA could be transferred to an alternative expression vector which contains no ORFs which encode peptides of a similar size to MraY.

The failure to detect the MraY peptide *in vivo* using common methods for gene overexpression shows that the expression of *mraY* by the cell may be highly regulated. Factors which may contribute to this include the rapid degradation of the *mraY* mRNA or perhaps proteolytic degradation of the MraY peptide. Some evidence of proteolytic activity is provided by the size of the MurFpolyHisMraY chimera peptides which migrated during SDS PAGE analysis as peptides 20 kDa smaller than their actual calculated molecular weight (see Figures 4.2.27.-28.). One observation contrary to the hypothesis that proteases are responsible for the degradation of MraY is that basal expression of *mraY* from pETY3c causes a lag in the growth rate. This was shown by the increase in the doubling time of BL21 (λDE3)/pETY3c when compared with BL21 (λDE3)/pET3c (see Figure 4.2.18.). It seems surprising that a small increase in the basal level of MraY is such that the regulatory action of the proteases is diluted. In addition, none of the autoradiograms from SDS PAGE analysis of wild type MraY expression show smaller peptides which may correspond to degraded forms of MraY. The construction of a clone to produce a polyHisMraY fusion peptide (pETHY1) also did not produce visible peptide. The growth rate of the host strain [BL21 (λDE3)] however was similar to the control hosting pET16b.

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MraY was not detected *in vivo* it is possible that the low levels produced could be identified immunologically, although this was not attempted.

The construction of an *mraY* null mutant allele allowed investigation of the mutant phenotype of *mraY*. The effects of MraY depletion in cells was to prevent growth and division, alter the shape of the cells and cause cell lysis. Cell size was affected by the mutation with the median cell volume rapidly decreasing after suppression of *mraY* expression. However, microscopy showed that there are also filaments formed upon the repression of *mraY* expression. As some cells grew as short filaments it cast doubts on whether MraY is involved solely with cell wall biosynthesis as filamentation obviously requires the biosynthesis of peptidoglycan for extending the cell wall. Why this should happen is unknown. Ikeda *et al.* (1991) have shown *in vitro* that the levels of the lipid I peptidoglycan precursor increased when cytoplasmic extracts from cells containing an *mraY* clone were incubated with labelled substrates (MurNAc-pentapeptide containing [^{3}H] DAP and bactoprenol). When compared to the filamentous phenotype produced by a division mutant grown under similar conditions (eg *ftsW*::CAT, Figure 3.3.22.), the filaments of the *mraY*::CAT mutant were smaller. The production of a mixed phenotype may indicate that the filamentation induced was not the direct effect of MraY depletion in the cell but instead an artefact whereby division was inhibited and the gradual reduction of cell wall precursor material resulted in cell death before the cell could form long filaments. An explanation for this may be that decreasing levels of MraY somehow induce the SOS response to inhibit division or that lower levels of Lipid I affect enzymes or complexes involved in cell division.

The data produced from the Coulter Counter did not reflect the presence of the filaments seen in the medium. The debris from lysing cells may have masked the presence of filaments in the culture. Another observation was that some of the mutant cells grown in arabinose had also lysed. Expression of *mraY* in wild-type strains was not lethal. As the media used for the growth experiments did not contain β-lactamase it is possible that the lysed cells had lost the complementing plasmid. Without MraY, the coordinated processes of peptidoglyan strand synthesis and saccular hydrolysis would be disrupted, resulting in cell death.

The description of the *murG*^{ts} mutant by Mengin-Lecreulx *et al.* (1991) showed that the morphology of the cells changed from rods to become misshapen and swollen. These did not appear to increase in size unlike the

*mra*Y::CAT mutant. The *mur*G peptide was shown to catalyse binding of UDP-GlucNAc to Lipid I (Mengin-Lecreulx *et al.*, 1991; Mengin-Lecreulx *et al.*, 1993). That is the undecaprenyl bound NAc-mur pentapeptide. The resultant molecule is undecaprenyl NAc-Glu NAc-Mur pentapeptide (Lipid II). This is the disaccharide pentapeptide precursor for murein strand synthesis, bound to the undecaprenol carrier molecule (Mengin-Lecreulx *et al.*, 1991). Therefore mutations in either *mra*Y or *mur*G should have a similar effect on the cell as both are involved in the same pathway. The difference in the reported morphologies may be due to the nature of the mutations. The *mur*G mutant (Salmond *et al.*, 1983) is an amber mutation in a *supF*^{ts} strain. The *mra*Y allele is a null mutation but extra plasmid based copies of *mra*Y supplied under the control of an inducible promoter are used to complement. The construction of a similar null allele for *mur*G and plasmid based complementing copies of *mur*G may reveal similar morphologies for both *mra*Y and *mur*G.

To more accurately study the phenotype produced by the *mra*Y::CAT mutant better control of *mra*Y expression is required. One method would be to clone *ara*C, P$_{BAD}$ and *mra*Y from pBEFY1 into the vector pRS551 and to then screen for the *in vivo* transfer of the clone into λRS45, the λ phage reporter (See Chapter 3, Figures 3.3.43.). This would produce a clone of *mra*Y in single copy with the expression of *mra*Y under the control of P$_{BAD}$. This approach has been used successfully for the study of a *gro*EL mutant (N. Maclennan, pers. comm.). The *mra*Y::CAT mutant under the control of the phage based *mra*Y clone would not be affected by plasmid loss, a possible cause of cell lysis in the arabinose induced DBYC2/pBAY1 culture. In addition, a single copy may allow greater control over the cellular levels of MraY ensuring a more uniform mutant phenotype when *mra*Y expression is repressed.

A putative promoter for *mra*Y was identified more than 2.7 kb upstream in *fts*I. It is proposed that this promoter also controls *mur*F and *mur*E expression, the two genes located immediately upstream from *mra*Y. That this must be the *mra*Y promoter is concluded from the observation that the null mutant was complemented only by the *mra*Y clones which contained this upstream region if no exogenous promoter were provided (eg P*lac* in pUC18). This discovery is consistent with a pattern found throughout the *mra* region, where the promoters for essential genes are often located far upstream from their ORF. Examples of this are *fts*I (Hara and Park, 1993),

murF (Mengin-Lecreulx *et al.*, 1989), *fts*W (this work) and *fts*Z (Dai and Lutkenhaus, 1991). Therefore the expression of more than one gene may be facilitated by the expression from one promoter. Thus although the hypothesis of the 'mini operon' within the *mra* region may be putatively correct it is not completely understood. Tsui *et al.* (1994) found evidence for a 'superoperon' in *E. coli* at 94.8 minutes. The genes which comprise this operon are expressed from several promoters and there is some evidence for posttranscriptional modification of the mRNAs as a control for gene expression (Tsui and Winkler, 1994). The *mra* region may be similar to this consisting of a series of small independent operons or one large operon (from *mraZ* to *envA*) with subsequent posttranscriptional modification to provide the necessary balance of growth and division peptides required for normal vegetative growth of *E.coli*. An example of this would be *fts*Z which produces the most common cell division protein in *E.coli* (Bi and Lutkenhaus, 1991). *fts*Z has three known promoters located at varying distances upstream from the ORF. Another unidentified *fts*Z promoter is located upstream of *fts*W. This is 7 kb upstream from the start of *fts*Z. The *fts*Z transcripts are also posttranscriptionally modified by RNase E cleavage at a site in *fts*A, the upstream gene (Cam *et al.*, 1994). The striking similarity between the organization of the *mra* regions of other bacterial species may lend credence to the *mra* region being composed of small operons. The sequencing of the *H. influenzae* genome has revealed that the organisation of the *mra* region is identical to that of *E.coli* (Fleichmann *et al.*, 1995). In *B.subtilis*, a Gram positive bacterium, homologues of many of the genes found in the *mra* region are also arranged in the same order (Daniels and Errington, 1993). However, there is a terminator between *ftsI* and *murE* in *B. subtilis*, and nothing is known about promoter locations or other terminators. The expression of *mraY* in *B. subtilis* may also differ from *E. coli* as the *murF* ORF is not present in the *mra* region of the former organism.

The *lacZ* promoter assays on the putative *mraY* promoter in *murE* produced very low LacZ activity (R. Smith, pers. comm.). This contradicts the complementation studies of the null mutant where the upstream DNA to the *Kpn*I site in *fts*I is mandatory for complementation if an external promoter is not supplied. An alternative method to monitor the expression of *mraY* is by assaying the CAT activity from either the partial diploid mutant, DBYC1, or from the haploid strain DBYC2. In both strains *mraY* is disrupted by the promoterless CAT gene, cloned in the same orientation as

mraY and therefore expression of *mraY* will result in the production of CAT. In addition the overproduction of MraY from pBEFY1 within these strains may also show if MraY autoregulates its expression.

The proposed role for MraY as a UDP-N-acetylmuramoyl-pentapeptide: undecaprenyl-phosphate phospho-N-acetylmuramoyl-pentapeptide transferase by Ikeda *et al.* (1991) could be studied *in vivo* by studying the biochemistry of the null mutant. One of two approaches could be followed. Firstly the difference in the ratios of lipid I and Lipid II could be compared in the mutant strain DBYC2/pBEFY1 grown in both arabinose and glucose based media. If the hypothesis of Ikeda *et al.* (1991) is correct then levels of both Lipid I and Lipid II would become zero in the glucose culture as compared to a ratio of 1:3 which was calculated for the wild-type by van Heijenoort *et al.* (1992). Alternatively the amount of incorporation of [^{3}H] DAP in the sacculi isolated from the two cultures could be compared. The sacculi isolated from the glucose based culture would be expected to show decreasing levels of incorporation of the [^{3}H] label as Lipid I is no longer produced in the absence of MraY.

Chapter 5.

A comparison of the translational efficiencies of genes from *ftsI* to *murC*.

Chapter 5. A comparison of the translational efficiencies of genes from *ftsI* to *murC*.

5.1. Introduction

The genes of the *mra* region are contiguous, being transcribed in the same direction (Donachie, 1993). This study and others (Dai and Lutkenhaus, 1991; Mengin-Lecreulx *et al.*, 1989) have shown that genes in the *mra* region are often expressed from common promoters such that their expression may be controlled as a series of small operons. Some of the proteins translated from the same mRNA transcript have been shown to be translated at different levels. For example FtsQ and FtsA are differentially translated from the same transcript at a ratio of 1:75 respectively (Mukherjee and Donachie, 1990). The difference in the densities of [^{35}S]-methionine labelled FtsI, MurE and MurF proteins after SDS-PAGE analysis in Chapter 4.2.1. is a further example of translational control of gene expression. Since these three proteins were translated from the same transcript, mechanisms to control the efficiency of translation must exist. In addition, the translational efficiencies of the proteins may influenced by the post-transcriptional processing of the mRNAs produced. To date there has been scant research into the stabilities of mRNAs produced from the *mra* region in particular upstream from *ddlB*.. Cam *et al.* (1994) showed that the *ftsZ* transcript from P1$_{ftsZ}$ is processed by RNaseE.

The T7 system was employed to examine the efficiency of translation of genes of the *mra* cluster (Studier and Moffat, 1986). Earlier studies on the translational efficiencies of FtsQ, FtsA and FtsZ used the same method to measure the expression of these proteins *in vivo* (Mukherjee and Donachie, 1990). In their study, Mukherjee and Donachie (1990) compared the translational efficiencies of FtsQ and FtsA, relative to β-lactamase. This was achieved by cloning these genes into pT7-4 where *bla* is cotranscribed with the cloned insert from the Ø10 promoter. Therefore both the cloned gene and *bla* are translated from the same mRNA transcript originating from Ø10 (Figure 5.1.1.). A measure of translation of the cloned gene(s) can be made by comparing the amount of label incorporated into the corresponding proteins.

In the course of attempting to identify the proteins made from *ftsW* and *mraY* in this work, many clones of the genes in the *mra* region have

already been made in pT7 vectors. Therefore it was decided to create several more clones such that each gene from *ftsI* to *murC* was cloned individually into either pT7-4 or pT7-3 (Tabor and Richardson, 1986). The aim was to compare the translation of each gene to *bla* . By assuming a standard value of 1.0 for the efficiency of translation of the *bla* gene, the levels of translation of cloned genes can be compared to that of *bla*. Levels of translation were thus measured as the amount of radiolabelled ^{35}S methionine which was incorporated into the over-produced peptides. Such values were also corrected for the number of methionyl residues in each of the proteins. Furthermore, the use of the T7 RNA polymerase system inhibits promoter activity from the host promoters which may be present in the clone. Therefore the only proteins produced are those translated from the mRNA transcript originating from the Ø10 promoter.
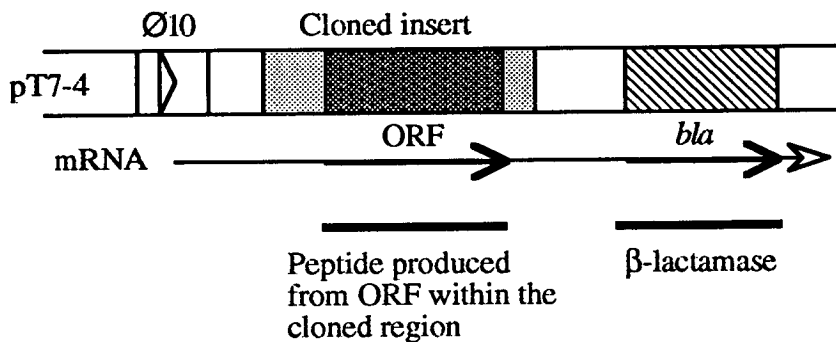
Figure 5.1.1. A schematic diagram showing the transcription of a cloned insert and *bla* from the Ø10 promoter. Also shown is the subsequent translation of the ORF (dark shading) from the cloned insert and β-lactamase (diagonal shading).

In their work, Mukherjee and Donachie (1990) compared the levels of translation between FtsQ and FtsA, when cloned individually and together, to β-lactamase. They found that the poor translation of FtsQ and FtsA is due their ribosome binding sequences and that there was no translational coupling between *ftsQ* and *ftsA* since the presence of *ftsQ* upstream from *ftsA* did not enhance translation of the latter.

The aim of this work was to over-express and label each gene from *ftsI* to *murC* in either pT7-4 or pT7-3 (this has the reverse polylinker to pT7-4). SDS-PAGE was used to separate the proteins and using densitometry the ratio of radiolabelled protein to radiolabelled β-lactamase could be

established for each clone. Since each ratio is based on the amount of β-lactamase produced it is possible to compare the levels of over-expressed proteins. It was hoped that this could be used to gain an estimate of the translational efficiency of the proteins from *ftsI* to *murC*.

5.2.1. Construction of *mra* clones in pT7-4 and pT7-3.

Several clones used for this research had been constructed already. These include pDBE4, pDBY4, pDBD3, pDEFY4, pDIE4 and pDIEFY4 constructed in Chapter 4.3.2. and 4.3.3. The construct pDBW4 was described in Chapter 3.2.2. A clone of *ddlB* was not included since the DdlB peptide is 32840 Da and therefore would be masked by β-lactamase when the samples are analysed by 10% SDS-PAGE.

The clones used to express individual genes from *ftsI* to *murC* in pT7-4 and pT7-3 are shown in Figure 5.2.1. pT7-4I was a gift from T. Ogura. The *murE* clone, pDBE4, was constructed such that it contained a 1810 bp fragment from the *Kpn*I site in *ftsI* to the *Eco*RV site in *murF*. The *murF* clone, pDBF4, contained a 2938 bp *Pvu*II fragment cloned into the *Sma*I site in pT7-4. pDBD3 contained a 1958 bp fragment from the *Eco*RI site in *mraY* to the *Cla*I site in *ftsW*, in pT7-3. The clone pDBG4 was constructed by cloning a 1710 bp *Sal*I fragment from pDEG1 into pT7-4. Finally, the *murC* clone, pDBC3, was constructed by restricting an '*ftsW murG murC ddlB*' pT7-3 clone, pTGC3, with *Sma*I. This removes the N-terminal region of *murG*. The 2787 bp fragment thus contained '*murG murC* and *ddlB*' in pT7-3.
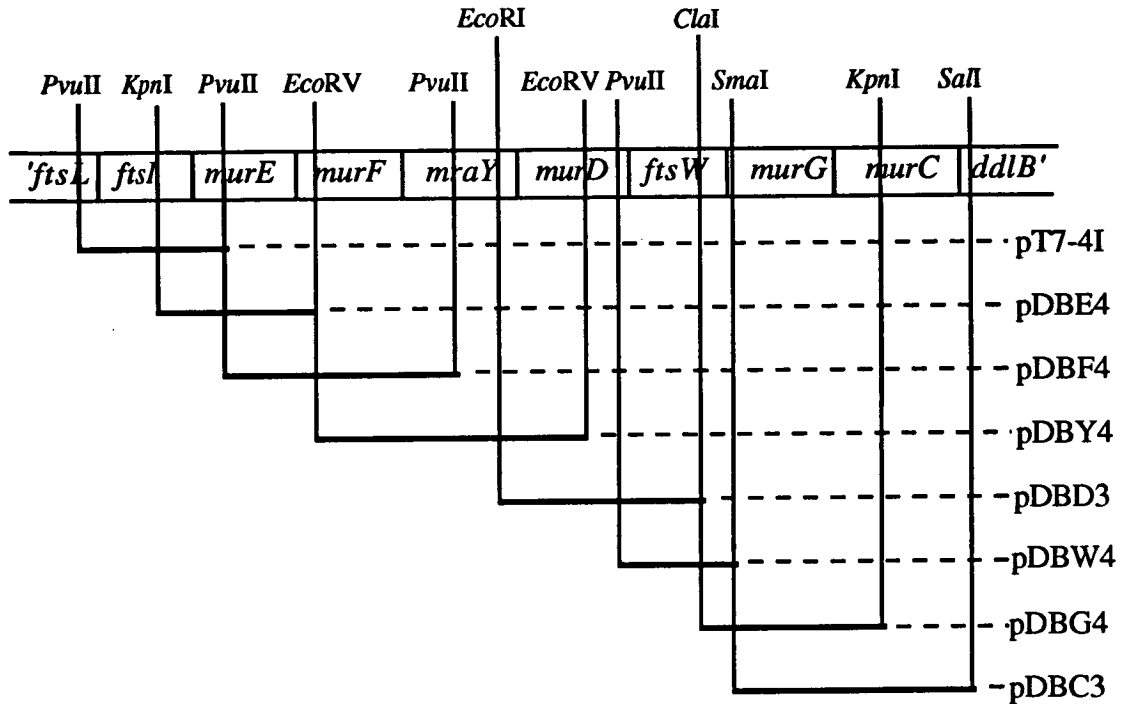
Figure 5.2.1. The clones of single genes from *ftsI* to *murC* in either pT7-4 (4) or pT7-3 (3).

For the investigation of possible translational coupling, different clones were made spanning from *ftsI* to *mraY* (Figure 5.2.2.) and from *murD* to *murC* (Figure 5.2.3.) This choice was made on the basis that expression of *murE*, *murF* and *mraY* is dependant on a promoter sequence located in *ftsI*. The expression of *murD* and *ftsW* is dependant on a promoter located in *mraY* (Mengin-Lecreulx *et al.*, 1989; this work Chapters 3 and 4). Therefore it was thought suitable to measure the translational efficiency with clones starting from the *Eco*RI site in *mraY*. A promoter for the expression of *murG* lies in the C-terminus of *ftsW* close to the *murG* start codon (Mengin-Lecreulx *et al.*, 1989). This may also be the promoter for the expression of *murC*.

The clones used to investigate translational coupling from *ftsI* until *mraY* were derived from pDIEFY4 (Figure 5.2.3.). To construct pDIEFY4, a 4.4 kb *Hind*III/*Bgl*II fragment from pDEG2 was initially cloned into *Hind*III/*Bam*HI restricted pT7-4 to create pDEFY4 (previously described in Chapter 4.2.1.) was also included in the study. pDEFY4 contains the complete ORFs for *murE*, *murF* and *mraY* (see Chapter 4., Figure 4.2.2.). The entire *ftsI* ORF was introduced into pDEFY4 by subcloning a 1.9 kb

*Hind*III/*Kpn*I restriction fragment from pT7-4I. An *Eco*RI restriction of pDIEFY4 cleaved the C-terminal portion of *mraY*. Subsequent religation of the 6 kb fragment produced pDIEF4. pDIE4 is described in Chapter 4.3.1. and was constructed by restricting pDIEFY4 with *Eco*RI/*Eco*RV, followed by blunt ending by Klenow and religation. In this clone the only entire ORFs are those for *ftsI* and *murE*..
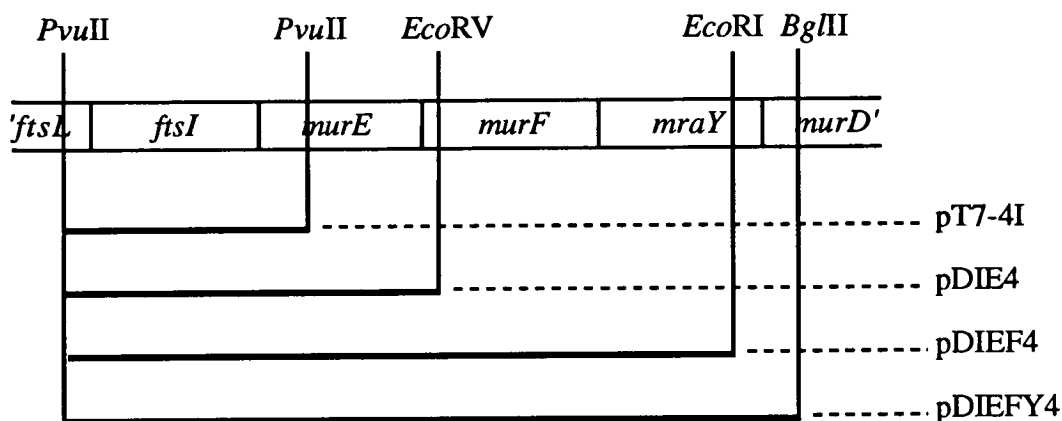
Figure 5.2.2. The series of clones constructed to determine possible translational coupling between *ftsI* and *mraY*.

The *murD* to *murC* clones were constructed by cloning a 4.4 kb *Eco*RI fragment from pDEG2 into *Eco*RI restricted pT7-3 (Figure 5.2.3.). This was pTDG3, 6.8 kb in size. pTDC3 was constructed by cloning the 2787 bp *Sal*I fragment from pDDC1 into *Sal*I restricted pTDG3. This clone contained the intact ORFs for *murD ftsW murG* and *murC*. A clone of *murG murC* was made by cloning the 2787 bp *Sal*I fragment from pDDC1 into *Sal*I restricted pT7-3, such that both *murG* and *murC* were transcribed from the Ø10 promoter. This was pTGC3.
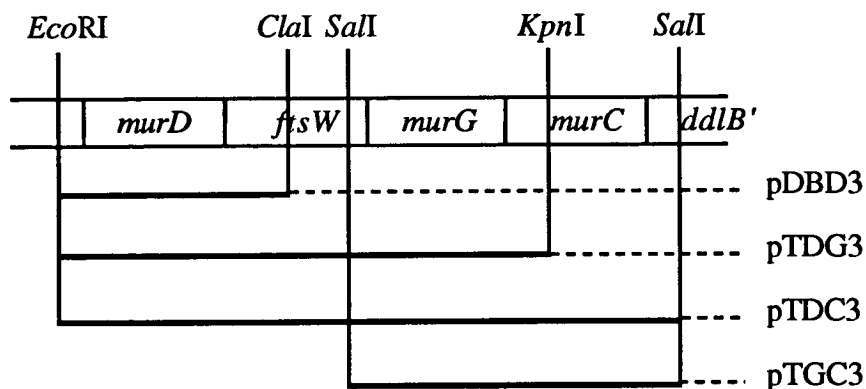
Figure 5.2.3. The clones used to measure translational coupling from *murD* to *murC*.

5.3.1. Expression, labelling and densitometry of the proteins.

The host strain for the *in vivo* expression and labelling of the pT7 clones was BL21(λDE3)/pLysS. The transformants were cultured in Spizizens minimal broth and the cultures induced and labelled with L-[^{35}S]-Methionine as described in Chapter 2. The labelled samples were analysed using 10% SDS-PAGE. The results of the SDS-PAGE analysis of the individual clones, pT7-4I-pDIEFY4 and pTGC3-pTDC3, respectively, are shown in Figures 5.3.1., 2. and 3. The densities of each labelled band were measured by scanning the dried SDS-PAGE gels on a Phosphorimager, Molecular Dynamics Series 4000. Each SDS-PAGE gel sample was stored in a database and could be displayed as a digital image. A rectangular area within each sample lane of the gel was analysed by densitometry (Figure 5.3.4.). The size of the rectangle was constant for each lane scanned. The length of the rectangle was such that part of each labelled protein band was encompassed. The densitometry of labelled proteins within each rectangle was analysed using a computing densitometer, Molecular Dynamics Series 3000. The software package used to analyse the data was ImageQuant by Molecular Dynamics.
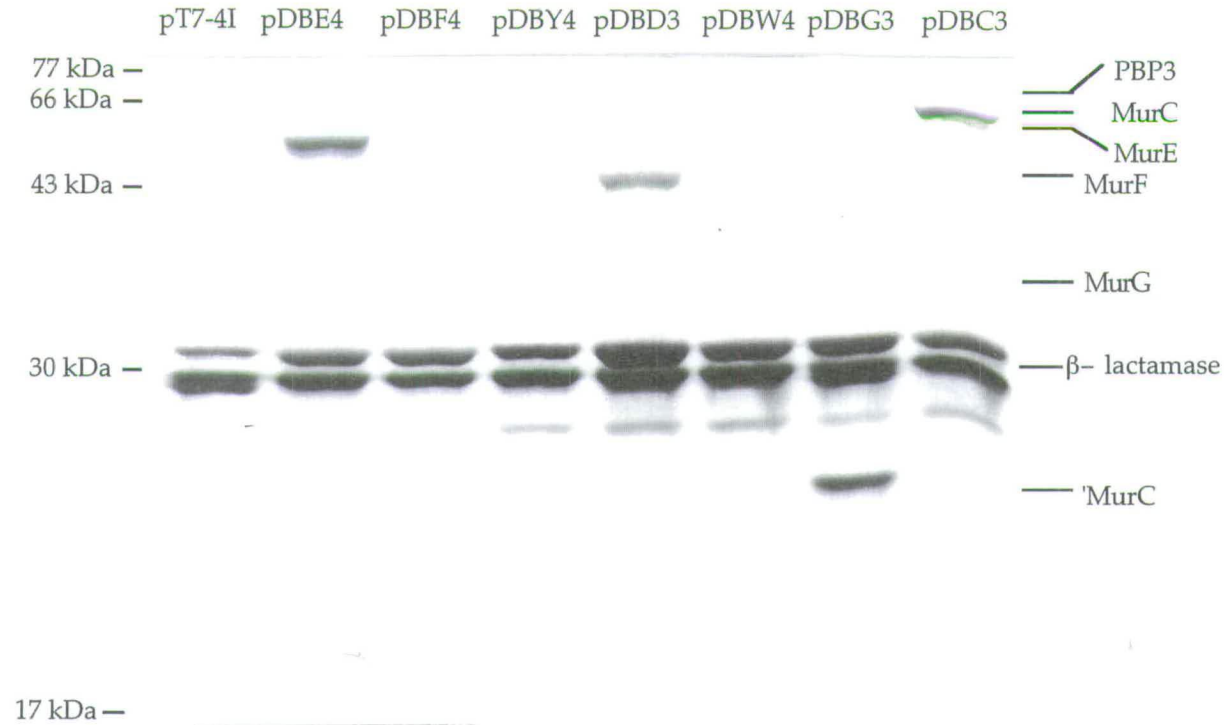
Figure 5.3.1. The over-expression and labelling of single clones from *ftsI* until *murC*. Each clone produced β-lactamase when expressed and therefore the transcription and translation *in vivo* was successful. The clones pDBF4, pDBY4 and pDBW4 (lanes 3, 4 and 5) did not produce their respective peptides. The other clones of *ftsI, murE, murD, murG* and *murC* produced labelled peptides of the predicted sizes. In lane 7 a band of ~24 kDa is present. This is the truncated form of MurC, produced from the *murG* clone, pDBG3.
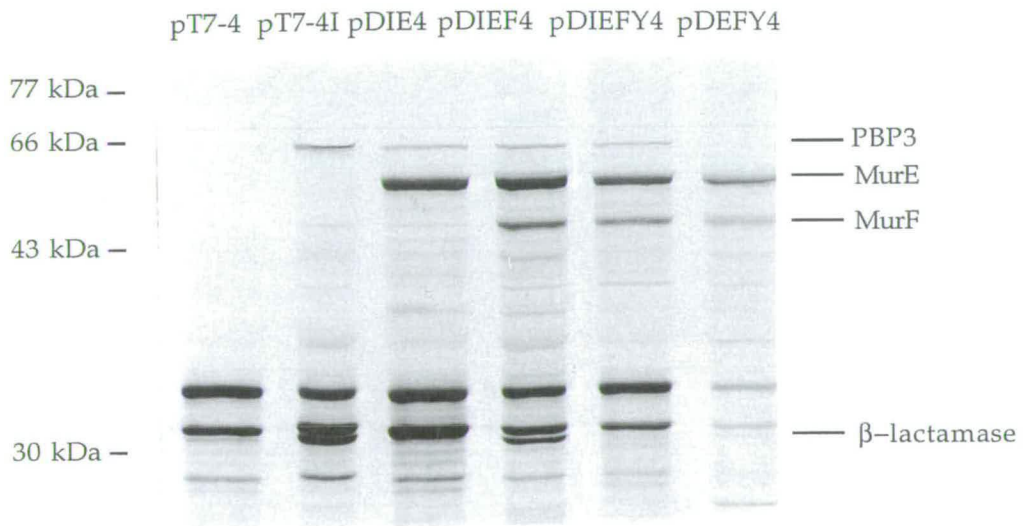
Figure 5.3.2. The over-expression of clones from *ftsI* to *ftsI-mraY* (lanes 2-5 respectively). Also included is a pT7-4 control (lane 1) and pDEFY4 (lane 6). The labelled proteins resolved after SDS-PAGE correspond to PBP3, MurE, MurF and β-lactamase respectively as they decrease in size.
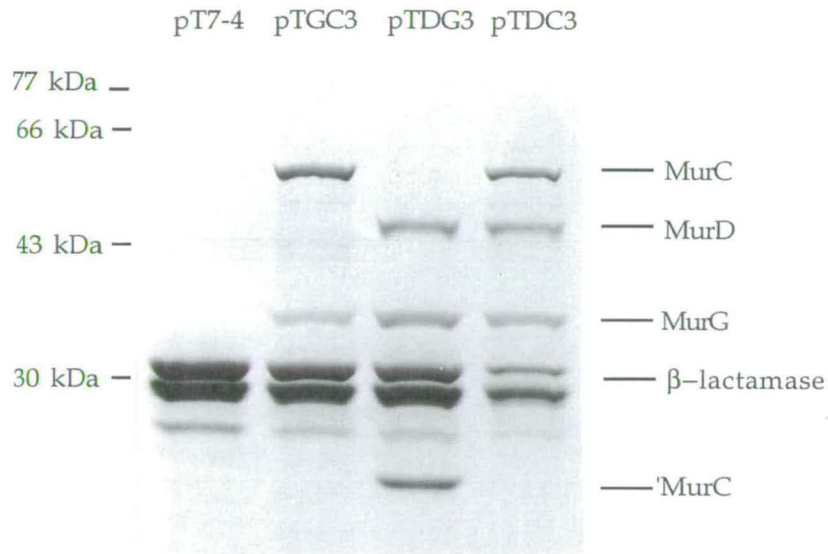
Figure 5.3.3. The over-expression of clones pTGC3, pTDG3 and pTDC3 vrespectively (lanes 2-4). pT7-4 is in lane 1. The proteins apparent are MurC, MurD, MurG and β-lactamase in order of decreasing molecular size. A truncated form of MurC was produced from pTDG3 in lane 3.
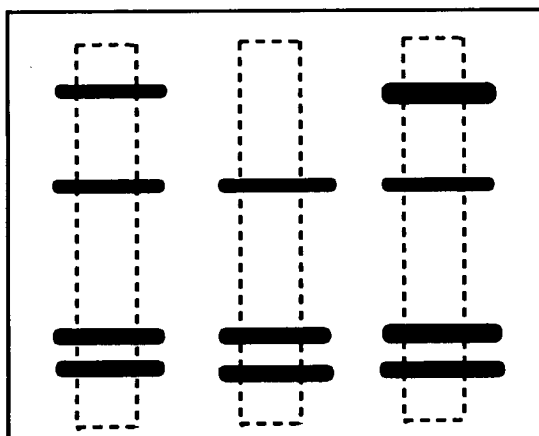
Figure 5.3.4. The method of sampling of lanes used for densitometry. The image from the phosphorimager of the gel was displayed on screen and a rectangle (dashed) drawn over the sample lane to be measured. The same size of rectangle was used to measure the densitometry of each sample lane. The dark bands represent labelled peptides after SDS-PAGE analysis.

The data from the densitometry measurements using the ImageQuant program was presented in two forms. One was graphically, as a cross section of each sample area; consisting of a series of peaks. Each peak corresponding to a labelled protein within the sample area (Figure 5.3.5.). The area of each peak was relative to the signal given from each labelled protein within the sample area (Figure 5.3.5.). This data was also expressed numerically. The data was presented as the total area of each peak and also the percentage area of each peak, relative to the total area within the sample. For example, the strongest signal will produce the highest area and this will therefore have the highest percentage peak compared to the other peaks in the sample (Figure 5.3.6.). Also presented were the X and Y coordinates for the pinnacle of each peak.
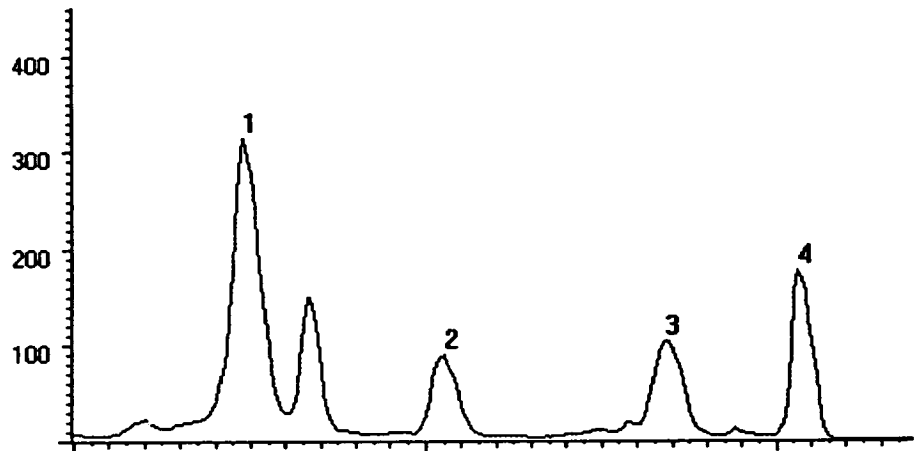
Figure 5.3.4. A representation of a graphic scan of pTDC3. The peaks 1-4 correspond to the β-lactamase, MurG, MurD and MurC proteins respectively.

Once each sample lane on the gels had been scanned and the densitometry performed, a series of figures relating to the percentage area for each labelled sample were produced. Before direct comparisons of percentage areas from each sample lane were made, each set of sample data was first standardized relative to the β-lactamase produced within each sample. Also included in the standardization was the number of methionyl residues present in each protein. The proteins studied contain different numbers of metionyl residues and therefore the signal produced from a labelled protein is dependant not only on the amount of protein produced but also the number of labelled residues incorporated in the protein.

PEAK AREA	% PEAK	X-POS PEAK	Y-POS PEAK	OBJECT NAME
1 5353	53.422	621	461	TDC
2 1195	11.927	621	519	TDC
3 1676	16.730	621	590	TDC
4 1796	17.921	621	631	TDC

Figure 5.3.6. An example of the numerical data supplied by the ImageQuant program used for densitometry. The data corresponds to the graphical data as displayed in Figure 5.3.5.

5.4.1. Results of densitometry and discussion.

The relative values for the levels of protein translation were calculated from the percentage peak area. Values for the over-expression of the pT7-4I-pDBC4 clones are shown in Table 1. The values have been corrected for the number of methionine residues per protein species. Each figure is relative to the β-lactamase percentage peak area produced by each clone. The value of each figure is given as a percentage of the calculated β-lactamase.

Clone/Protein	% Expression
pT7-4I/ PBP3	8.5
pDBE4/MurE	56
pDBD3/MurD	71
pDBG3/MurG	3
pDBC/MurC	25

Table 5.3.1. The expression values of proteins produced from the single pT7-4 (3) clones.

The value for the pDBW4 clone could not be calculated as the FtsW protein is masked by β-lactamase proteins (Khattar *et al.*, 1994). pDBY4 does not produce the MraY protein, only β-lactamase (Figure 5.2.1.; see Chapter 4.2.1.). Unusually, the over-expression of pDBF4 did not produce a protein of 47 kDa. Only β-lactamase was produced (Figure 5.2.1.). This was unexpected as the larger clones containing *murF* (namely pDEFY4, pDIEF4 and pDIEFY4) all produced a 47 kDa protein corresponding to the MurF protein (Figure 5.2.2.). The absence of translation of MurF from pDBF4 therefore suggests that the presence of *murE* may be required for the translation of *murF*. The translation of β-lactamase from the same construct signifies that transcription was directed from the Ø10 promoter. The clones containing both *murE* and *murF* produce the MurE and the MurF proteins when expressed and labelled (see Figure 5.3.2.). This may be an example of translational coupling between adjacent genes. The cloned insert was examined by restriction analysis to ensure that pDBF4 contained *murF* and that it was cloned in the correct orientation relative to the Ø10 promoter in pT7-4. The plasmid DNA was restricted with *Eco*RV and in tandem with either *Hind*III or *Eco*RI. Agarose gel electrophoresis of the restricted DNAs

showed that the linear DNA was the correct size for pDBF4 and that the fragment was cloned in the correct orientation relative to Ø10. pDBF4 was restricted with EcoRI/HindIII and the 2.7 kb murF fragment was cloned into the corresponding cloning sites in pUC19. This made pUF19, a plasmid 5.4 kb in size. The murF ts mutant, C600F, was transformed with pUF19 and tested for growth at 42°C. pUF19 complemented the murF ts allele and therefore the insert cloned originally in pDBF4 contained murF. Further studies are necessary to determine if translational coupling does exist between murE and murF, although it has been shown that both genes share the same promoter and therefore it may be possible that MurF translation is dependant on the prior translation of MurE. There are few suitable restriction sites in murE with which to make alternative clones of murF. If other clones of murF also did not produce the MurF protein then a strong argument for translational coupling of MurE and MurF could be presented. However, Nakamura et al. (1988) did label a peptide from a plasmid containing murF in vivo, although this clone utilized a PvuI restriction site 788 bp downstream from the PvuII restriction site used in the construction of pDBF4. Therefore this region may play a role in the regulation of expression of MurF. Mengin-Lecreulx et al. (1989) cloned the PvuII fragment into pUC18 and this too complemented the murF ts mutant but the clone was not used for any of the in vivo labelling experiments presented in their work.

From the values presented in Table 5.3.1. it can be seen that expression and translation of the proteins MurD, MurE and MurC were highest. These proteins are involved in the cytoplasmic synthesis of the disaccharide pentapeptide, (Mengin-Lecreulx et al., 1993). The data shows that they were more efficiently translated than either PBP3 or MurG. The cellular concentration of PBP3 is estimated to be 50 molecules per cell (Spratt, 1975). There is no data determining the cellular concentration of MurG although the kinetics of the MurG peptide in the biosynthesis of Lipid II has been studied by Mengin-Lecreulx et al. (1991).

The values calculated from the densitometry readings for the expression of the pT7-4I-pDIEFY4 and pDEFY4 clones are shown in Table 5.3.2.. The levels of translation for PBP3 when expressed from the clones were consistently low. The values for levels of MurE fluctuate by 100%. The average value is 44.5%. It is unclear if the presence of ftsI upstream from murE has an effect on it's translation. The clone pDEFY4 gives a value of 82.9% for MurE when compared to β-lactamase, the highest of all values in

this experiment. The *murE* only clone, pDBE4, however produces a lower value of 56%. If the presence of *ftsI* upstream from *murE* affects MurE translation then over-expression of pDBE4 would be expected to give a similarly high value. However the pDBE4 value is closer to the values recorded for pDIE4 and pDIEF4 over-expression. It would therefore appear that *ftsI* does not affect the expression or translation of *murE*. The levels of MurF translation are more constant, with a deviation of only 14 between all the calculated values. Comparing the values of MurE to MurF expression reveals that they are expressed in a ratio of ~2:1, respectively. No readings were recorded for MraY although this was expected (see Chapter 4.2.1.). The greater sensitivity of the phosphorimager compared to X-ray film further demonstrated that the MraY protein is not produced, or perhaps it is not isolated by the method used to prepare protein samples.

Protein	pT7-4I	pDIE4	pDIEF4	pDIEFY4	pDEFY4
PBP3	6.4	2.5	8.7	5.8	----------
MurE	----------	30	65	54	82.9
MurF	----------	----------	29	31	43
MraY	----------	----------	----------	----------	----------

Table 5.3.2. The percentage values for the translation of PBP3, MurE and MurF.

The over-expression of the *murD* reveals that once again, values are not consistent. Each clone of MurD is similar in that the upstream DNA from the start codon of *murD* in each of the three clones pDBD3, pTDG3 and pTDC3 extends to the *Eco*RI restriction site in *mraY*. Both of the larger clones, pTDG3 and pTDC3, produce almost identical levels of MurD, that is 46-47%. When *murD* is expressed with only *bla*, the ratio of MurD protein being produced is greater, being 71% when compared to β-lactamase. The decrease in *murD* translation in the larger clones may be due to increased competition for the label, ^{35}S methionine, since pTDG3 also expresses *murD*, *ftsW* and *bla* from the same transcript and pTDC3 also expresses these genes and in addition *murC*.

Protein	pTGC3	pTDG3	pTDC3
MurD	—————	46	47
FtsW	—————	—————	—————
MurG	11	11	24
MurC	27	—————	49

Table 5.3.3. The percentage values for the ratios between β-lactamase and MurD, MurG and MurC respectively.

The over-expression of murG produced less labelled protein than the other mur genes studied. The values vary from 3% to 24%. The presence of murD and ftsW upstream may affect the efficiency of translation of murG. The amount of MurG produced from the clone pTDG3 is similar to that from the murG and murC clone, pTGC3. The other clone containing upstream DNA, pTDC3, produced a greater value for MurG expression, being more than double that of either the pTGC3 of pTDG3 values. The single clone of murG, pDBG3, produced a very low value of only 3% (Table 5.3.2.). Therefore, the presence of upstream DNA from murG may facilitate greater translation of MurG when it is over-expressed.

The values presented for the over-expression of MurC are also ambiguous. Both the individual clone of murC, pDBC3, and the clone containing 'ftsW murG murC ddlB', pTGC3, gave similar values of ~25%. The largest clone, pTDC3, gave a value twice that of pTGC3 and pDBC3. It is again unclear whether this may be due to improved translation of MurC or decreased translation of bla due to competition for label by the four other proteins translated from the pTDC3 transcript. If the amount of labelled β-lactamase is reduced then this affects the ratio of β-lactamase to the other proteins produced. This could give a false indication of the over-expression of MurD-MurC giving each a relative higher value than the smaller clones. For both MurG and MurC the ratio of each protein to β-lactamase doubled. This is not reflected for MurD, which remained constant.

The expression and labelling of clones in this experiment does show that the levels of translation of ftsI, murE, murF, murD, murG and murC all vary with respect to each other. This suggests that like ftsQ, ftsA and ftsZ , the expression of these other mra genes is controlled, in part, at a translational level. This is reflected by comparing the values of the ftsI protein, PBP3, and MurC. Similarly, MurG is translated in comparatively

small amounts to MurC. The values for MurE and MurF appear to indicate that they are expressed in a ratio of ~2:1, respectively.

Further research needs to be carried out on the translation of these genes. In particular, the over-expression of MurF. This is to determine if *murE* is actually required for the efficient translation of MurF. The complementation of C600F with pUF19 would suggest that MurF can be expressed without *murE*. An approach similar to that employed by Mukherjee and Donachie (1990) whereby each gene is fused to the same rbs (for example the gene 10 rbs in the pET3 vectors) may promote better translation of certain genes. These would indicate the role of the rbs sequences in the efficient translation of the respective proteins. This would be expected to allow the production of similar levels of proteins. If the translation of particular proteins was low then the role of other factors in protein translation such as the codon usage and mRNA structure and stability could then be examined.

Chapter 6.

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