THE ROLE OF MUREIN HYDROLASES IN THE CELL DIVISION OF *Escherichia coli*.

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Declaration

I declare that this thesis was composed by myself, and that, except where stated otherwise, the work presented within it is my own.

David Hugh Edwards.
December 1993.

Dedication

To my Mum and Dad

"its not big and its not clever!"

Abstract

Escherichia coli is a Gram-negative rod shaped bacterium that grows by lateral elongation before forming a septum at its centre and dividing into two daughter cells. During growth and division the cell's shape and integrity is maintained by a rigid cell wall or murein sacculus. This sacculus is essentially, a monolayer constructed from repeated peptidoglycan sub-units. The synthesis and hydrolysis of the cell wall is mediated by a number of enzymes, including a class of penicillin-sensitive proteins, the PBPs. One of these, PBP3, is a septum specific peptidoglycan synthetase. The work in this thesis is concerned with suppression of a temperature sensitive mutation (*ftsI*23) in the gene for PBP3.

It is shown that *ftsI*23 can be suppressed by increased levels of the cell's major DD-carboxypeptidases, PBP5 and PBP6. A second known suppresser of *ftsI* mutations, *sufI*, is also shown to suppress *ftsI*23 by increasing the levels of PBP6 and membrane bound DD-carboxypeptidase activity. These results are proposed to indicate that PBP3 has a preference for 'tripeptide acceptors' as substrate. In conjunction with murein analysis of *ftsI*23 suppressed strains, it is further proposed that these acceptors are necessary for the formation of a set of critical but temporary 'tetrapeptide-tripeptide' cross-bridges. These cross-bridges are envisaged to be required for the incorporation of nascent peptidoglycan at the septum.

To investigate this further a strain lacking all known periplasmic DD-carboxypeptidases was constructed. The viability of this strain suggests that the DD-carboxypeptidase activity mediated by PBP4, PBP5 and PBP6, is not essential for cell growth and division. It is also demonstrated that deletion of the gene for the major DD-endopeptidase, *dacB* (PBP4) can suppress *ftsI*23 (PBP3) in the absence of salt. This is argued to result from decreasing the hydrolysis of the critical 'tetra-tri' cross-bridges.

In addition the approximate locations of the gene for the LD-carboxypeptidase and *dacC* (PBP6) mapped to 37 minutes and 19 minutes, respectively. It is also shown that strains completely lacking another peptidoglycan synthetase, RodA, grow as stable, mecillinam resistant spheres.

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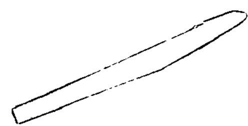
Finally I would like to come to my family. My sister Elaine has provided me with food, money, and lots of laughter. I thank Chris for many splendid bottles of whisky and wine, and my Aunt Margaret for all her help. Without my parents none of this would have been possible. They have provided me with all the love and security I needed, and anyone who knows me will realise this is a lot! This thesis is as much to do with Mary and Hugh Edwards as it is to do with their son, and as a geneticist I feel pretty secure in this point of view!

Abbreviations

A	Adenine
amp	Ampicillin
ADP	adenosine 5'–diphosphate
ATP	adenosine 5'–triphosphate
bp	base pairs
BSA	Bovine Serum Albumin
C	Cytosine
cAMP	3',5'-cyclic adenosine monophosphate
Cm	Chloramphenicol
CAPS	(3[Cyclohexylamino]-1-propane-sulfonic acid)
D-Ala	D-Alanine
dATP	deoxyadenosine-5'-triphosphate
dCTP	deoxycytidine-5'-triphosphate
DD-Cpase	DD-carboxypeptidase
	(also 'carboypeptidase 1A' or 'carbosypeptidase I')
D-Glu	D-Glutamic acid
dGTP	deoxyguanosine-5'-triphosphate
dH_2O	sterile distilled H_2O
ddH_2O	sterile double distilled H_2O
dTTP	deoxythmidine-5'-triphosphate
dNTP	deoxynucleoside-5'-triphosphate
DNA	Deoxyribonucleic acid
Δ	delete
DTT	Dithiothreitol
EDTA	Diaminoethanetetra-acetic acid
g	gram
G	Guanine
GDP	Guanosine triphosphate
GlcNAc	*N*-acetyl Glucosamine
GTP	Guanosine triphosphate
HMW	High Molecular Weight
HPLC	High Performance Liquid Chromatography
I	Inosine
kb	kilobase pairs
kD	KiloDalton

Klenow	Large fragment of DNA polymerase I
Km	Kanamycin
L-Ala	L-Alanine
LD-Cpase	LD-carboxypeptidase (also carboxypeptidase II)
λ	Bacteriophage lambda
mA	milliamp(s)
*m*DAP	mesoDiaminopimelic acid
MIC	Minimum Inhibitory Concentration
mins	Minutes
MOI	Multiplicity of Infection
MurNAc	*N*-acetyl Muramic acid
NMR	Nuclear Magnetic Resonance
OD	Optical density
ORF	Open Reading Frame
ori	Origin of replication.
PAL	Peptidoglycan Associated Lipoprotein
PBP	Penicillin-binding Protein
PEG	Polyethylene glycol
Penta	Pentapeptide side chain
PIPS	Penicillin Insensitive Peptidoglycan Synthesis
pmole	Picomoles
ppGpp	guanosine tetraphosphate
PVDF	Polyvinylidene diflouride
R	Resistance
RNA	Ribonucleic acid
RNases	Ribonuclease
SDS	Sodium dodececyl sulphate
SDS-PAGE	Sodium dodececyl sulphate Polyacrylamide Gel Electrophoresis
Sp	Spectinomycin
Sm	Streptomycin
σ	Sigma
T	Thymine
Tc	Tetracycline
TE	Tris-HCl EDTA
TEMED	N,N,N',N'-tetramethylethylenediamine
Tetra	Tetrapeptide side chain



Tri	Tripeptide side chain
Tn	Transposon
Tris	tris(hydroxymethyl)aminomethane
UDP	Uridine diphosphate
uv	ultraviolet
W:V	Weight to Volume
WT	Wild Type

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CHAPTER 1

INTRODUCTION

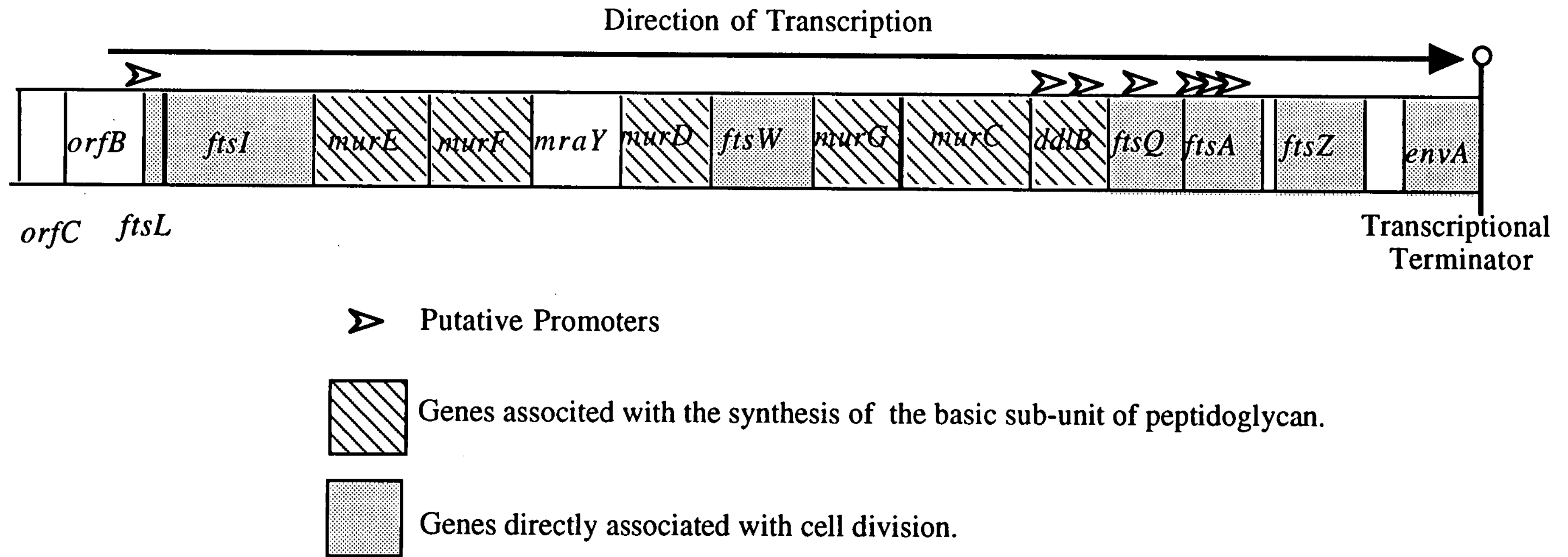
Escherichia coli is a Gram-negative rod shaped organism that grows by lateral elongation before forming a cross-wall, or septum, and dividing into two equally sized sister cells. During growth and prior to division the cell's DNA is replicated and partitioned on either side of the future division site. This process of replication, partition and division is very efficient and results in the production of very few DNA-less cells (Hiraga *et al.*, 1989; Jaffe *et al.*, 1989). The cell's integrity throughout this cell cycle is maintained against an internal osmotic pressure estimated to be between 2 and 5 atmospheres (Mitchell & Moyle, 1956) by a rigid cell wall. This cell wall; or sacculus, consists of a complex heteropolysaccharide called peptidoglycan or murein. Until 1991 the murein sacculus represented the only force-bearing structure identified in *E.coli.* It had been speculated the sacculus was responsible for the initial steps in cell division and even chromosomal segregation (Jacob *et al.*, 1963). At its simplest such an explanation considered the in-growth of the sacculus to push the cytoplasmic membrane in and pull the outer membrane behind it (Cook *et al.*, 1989). This explanation ignored the ability of bacterial L-forms, which lack a rigid cell wall, to divide (Onoda *et al.*, 1987). It was only with the identification of FtsZ as a division specific cytokinetic protein (Bi & Lutkenhaus, 1991) that the perception of the bacterial cell wall changed. It now appears that contraction of the cytoplasmic membrane could drive the constriction of the cell.

Study of the bacterial cell cycle has centred on the fundamental processes of DNA replication, nucleoid partition and cell division. These processes have been proposed to follow separate but co-ordinated cycles (Donachie *et al.*, 1984; Nordstrum *et al.*, 1991). They are separate cycles because division can continue when replication and partition is blocked, and replication and partition can continue when division is arrested. They are co-ordinated because blockage of replication or partition can arrest division.

1.1 The Division Proteins.

The study of division has been based upon the isolation of conditional mutants. These mutants became known by their 'filamentous temperature sensitive phenotype' and were called *fts*. Identification of the mutations responsible revealed the existence of several discrete proteins that appeared to act sequentially. The genes for six of these proteins are located in the *mra* operon located at 2.5 minutes on the *E.coli* chromosome (Figure 1.1).

Figure: 1.1. The *mra* operon of *Escherichia coli*.



1.1.1 FtsZ

The FtsZ protein is responsible for the earliest stage identified in division (Lutkenhaus *et al.*, 1980; Begg & Donachie, 1985). It is the most abundant cell division protein, estimated to be present in 5,000 to 20,000 copies per cell (Bi & Lutkenhaus, 1991). The protein aggregates on the cell membrane to form a circumferential ring in the cytoplasm prior to division, and remains localised at the leading edge of constriction during septation (Bi & Lutkenhaus, 1991; Lutkenhaus 1993). FtsZ is the target for the cell division inhibitor (SfiA protein) produced in response to DNA damage (Lutkenhaus, 1983; Jones & Holland, 1985; Bi & Lutkenhaus, 1990). During normal cell growth FtsZ protein can potentially localise at one of three division sites. The first is the cell centre, which represents the future division site, the other two are the cell poles which represent old division sites. The protein is normally prevented from assembling at the cell poles by the presence of a protein complex consisting of MinC, MinD and MinE. Disruption of this protein complex can lead to the septum forming randomly at any of the three potential division sites. If division occurs at the cell poles anucleate minicells are generated. Minicells may also be formed by the overproduction of FtsZ, a process which results in an increase in the number of division events per cell. FtsZ is the only protein identified which can increase the number of division events.

FtsZ has four regions of homology with eukaryotic tubulins (Lutkenhaus, 1993). One of these represents a highly conserved GTP/GDP binding site. In vivo the protein has been demonstrated to have a weak GTPase activity. This has led to the proposal that the FtsZ molecules self-assemble using GTP and form a contractile ring at the site of the future septum (Lutkenhaus, 1993). The existence of a cytokinetic process that can act independently from the invagination of the murein sacculus has been suggested by the ability of bacterial L-forms to divide (Onoda *et al.*, 1987).

An interaction between the FtsZ ring and the murein sacculus has been implied by alterations in the geometry of the FtsZ ring affecting cell pole morphology (Bi & Lutkenhaus, 1992). Such an interaction has been postulated to involve cytoplasmic domains of other division proteins including FtsQ and PBP3 (Lutkenhaus, 1993).

1.1.2 FtsA.

FtsA is a 46.4 kD protein whose gene is located directly upstream of FtsZ. The two genes appear to be co-transcribed but differentially translated (Mukherjee & Donachie, 1990). Only 150 molecules of FtsA are estimated to be present in the cell (Wang & Gayda, 1992). The ratio of FtsA to FtsZ has been established to be critical, and if disturbed will produce a block to division (Dai & Lutkenhaus, 1992; Dewar *et al.*, 1992). The protein localises to the cytoplasmic membrane but has no signal sequence or exceptional hydrophobicity, possibly indicating an interaction with FtsZ (Chan & Gayada, 1988; Pla *et al.*, 1990). An *ftsA* allele that affects penicillin-binding to PBP3 has been isolated (Tormo & Vicente, 1984). It has also been reported that temperature sensitive *ftsA* and *ftsI* (PBP3) alleles can be suppressed by overproduction of FtsN (Dai *et al.*, 1993). These two observations have been proposed to indicate that PBP3 and FtsA interact. However the characteristic phenotype of FtsA mutants is of long filamentous cells with regular, blunt constrictions. By contrast cells blocked by inactivation of PBP3 produce long straight filaments, which are suggested to be blocked before the development of blunt constrictions (Begg & Donachie, 1985). Consequently it would appear FtsA is not required for the initiation of PBP3 activity but possibly for its continuation.

1.1.3 FtsQ.

FtsQ is a 31 kD protein that is essentially periplasmic but includes a membrane spanning domain and short cytoplasmic region (Begg *et al.*, 1980; Yi & Lutkenhaus, 1985). It shares this structure and a non-cleavable sequence near its amino terminus with FtsL, PBP3 and FtsN (Dai *et al.*, 1993). The protein is estimated to be present in only 50-100 copies per cell (Carson *et al.*, 1991). The *ftsQ* gene is located upstream of *ftsA* and *ftsZ*, and all three are probably co-transcribed (Lutkenhaus & Wu, 1980.). The low levels of FtsQ produced appear to result from a very poor ribosomal binding site. Inactivation of this essential protein (Carson *et al.*, 1991) produces straight non-septate filaments, and it is suggested to block division between the activities of FtsZ and FtsA (Begg & Donachie, 1985).

1.1.4 FtsI (PBP3).

The *ftsI* gene encodes the septum specific murein synthetase PBP3 (Ishino & Matsuhashi, 1981; Spratt, 1977). The PBP3 protein is estimated to be present in 50 copies per cell (Spratt 1977) and is a transmembrane protein with a catalytic domain located in the periplasm (Bowler & Spratt, 1989). It has been demonstrated to catalyse a penicillin-sensitive peptidoglycan cross-linkage *in vitro* (Ishino & Matsuhashi, 1981), and has been proposed to form a functional complex with the FtsW protein (Matsuhashi *et al.*, 1990). Inactivation of either of these genes produces characteristic non-septate filaments (Begg & Donachie, 1985). The enzymes role and function will be discussed in more detail with the other PBPs.

1.1.5 FtsL.

The *ftsL* gene, also called *mra*R (Ueki *et al.*,1992), lies directly upstream of *ftsI* and is the site of the *ftsI*36 and *lts*36 mutations (Matsuhashi *et al.*,1990). The *ftsI*36 mutation in the gene blocks division to produce straight non-septate filaments. The 13.6 kD protein produced by *fts*L is present in 30 to 40 copies per cell. FtsL has a short cytoplasmic amino-terminus, a membrane spanning domain and periplasmic carboxyl-terminus (Guzman *et al.*, 1992). The phenotype of *fts*L mutants, the number of FtsL molecules, and the proteins' structure are all similar to PBP3 and FtsQ. Consequently it has been proposed these three proteins may form a 'stoichiometric complex' (Guzman *et al.*, 1992). It is also interesting to note the protein has a leucine zipper motif in its periplasmic domain which could be used in dimerisation (Guzman *et al* 1992).

1.1.6 EnvA.

EnvA is a 34 kD cytoplasmic protein encoded by the final gene in the *mra* operon (Beal & Lutkenhaus, 1987; Jones & Holland, 1985). Its deletion or overproduction is lethal (Beal & Lutkenhaus, 1987; Sullivan & Donachie, 1984). Mutations in *envA* produce characteristic chains of cells blocked in septum splitting (Wolf-Watz & Normark, 1976). Mutations also alter the outer membrane and make the cell hyperpermeable to antibiotics (Grunstrom *et al.*, 1980). The *envA* gene is located 100 bases downstream of *ftsZ* and is followed by a strong transcriptional terminator (Beal & Lutkenhaus, 1987).

Table: A. Cell Division Genes of *E.coli*.

Gene	Location (mins)	Name of Operon	Size in kD	Copies per Cell	Function
ftsZ	2.5	mra	40	5,000-20,000	Cytokinetic protein that functions in the initiation of cell division.
ftsA	2.5	mra	46.4	150	Membrane Protein that interacts with PBP3.
ftsQ	2.5	mra	31	50-100	Required for Septation.
ftsI (*pbpA*)	2.5	mra	64	50	Septum specific Peptidoglycan synthetase.
ftsL	2.5	mra	13.6	30-40	Required for Sepatation.
envA	2.5		34	?	Essential for cell separation
ftsN	88.5		36	50	Required for Sepatation Suppressor of *ftsI* and *ftsA* mutations
ftsY	69	*ftsYEX*	54	?	Shares homology to eukaryotic signal recognition particle
ftsE	69	*ftsYEX*	25	?	Shares homology to transport proteins.
ftsX	69	*ftsYEX*	38	?	Required for cell division
ftsW	2.5	mra	46	?	Proposed to interact with PBP3for the synthesis of Septal Peptidoglycan.

1.1.7 FtsN.

FtsN was isolated as a multi-copy suppresser of *ftsA* mutants, and was subsequently shown to also suppress *ftsI* mutations (Dai *et al.*, 1993). Inactivation of FtsN produces long aseptate filaments that resemble FtsQ, PBP3, and FtsL division blocks. The protein is estimated to be present in 50 copies per cell and has a similar hydropathy profile to FtsQ, PBP3 and FtsL (Dai *et al.*, 1993). Thus it has been proposed that this protein may also form a functional complex with FtsQ, FtsL, and PBP3. The gene encoding this 36 kD protein is located at 88.5 minutes on the *E.coli* chromosome.

1.1.8 FtsY, FtsE, FtsX.

The genes for these three proteins form an operon at 69 minutes (Gill *et al.*, 1986). FtsE has homology to nucleotide binding proteins involved in protein transport. Filamentation by *ftsE* mutants is growth rate dependent (Taschner *et al.*, 1988). FtsY is homologous to a eukaryotic secretory protein (Lutkenhaus, 1993b), while the product of *ftsX* has no significant homology to known proteins. All three proteins lack a signal sequence (Gill *et al.*, 1986) and are associated with the cytoplasmic membrane (Gill *et al.*, 1987). It has been proposed they interrupt division by interfering with the transport of other division proteins.

It is not known exactly how many of these division proteins interact directly with peptidoglycan but by blocking division they can all be said to interfere with the normal construction of a hemisperical cell wall at the septum.

1.2 Murein Sacculus.

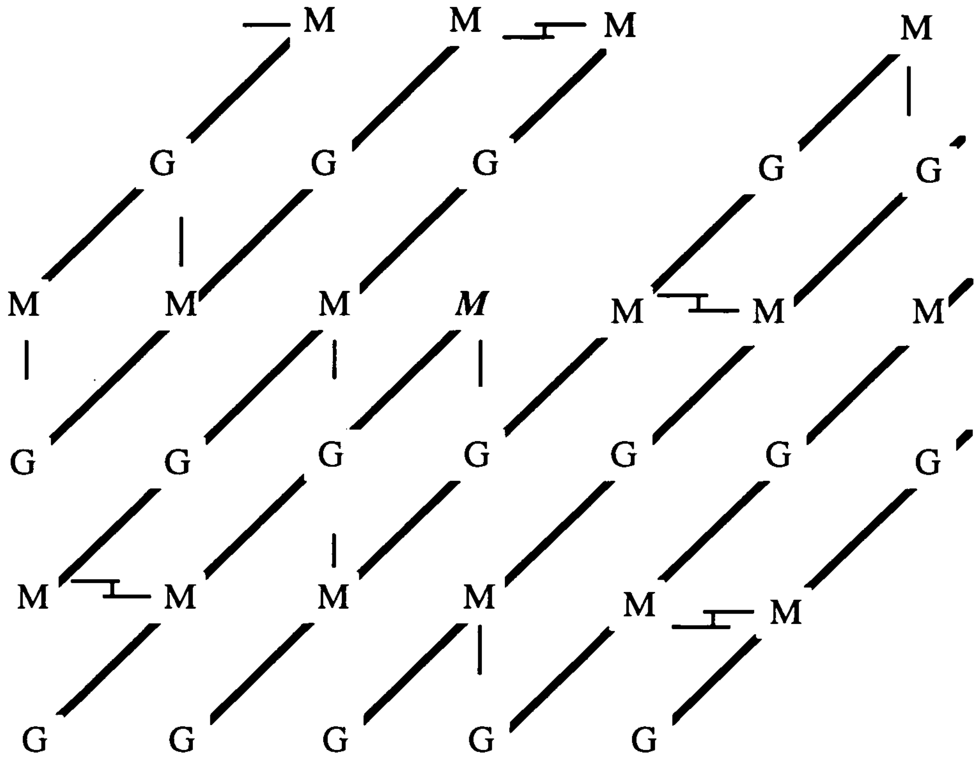
The murein sacculus is located in the periplasm. It is attached to the outer membrane by cross-linked lipoprotein (Braun & Holtje, 1974) and to the inner membrane by nascent peptidoglycan. The latest studies with NMR and Fast Atom Bombardment, suggest that 70–80% of the cell wall is a monolayer of peptidoglycan, and 20–30% is triple-layered (Labischinski *et al.*,1991). This coresponds well to the monolayer predicted from *m*DAP quantification (Braun *et al.*, 1983). It has also been confirmed by infrared spectroscopy that the sacculus is a flexible non-crystalline structure (Naumann *et al.*, 1982).

The sacculus is constructed from repeated peptidoglycan sub-units (Figure:1.2). These sub-units are extended to form chains which are cross-linked together to form a highly flexible net, capable of expanding and contracting under osmotic pressure (Labischinski *et al.*, 1983) (Figure:1.3). The fundamental unit from which high moleular weight murein is constructed is the disaccharide pentapeptide. This consists of a disaccharide backbone of *N*-acetyl-Muramic acid (MurNAc) and *N*-acetyl-Glucosamine (GlucNAc) joined by a β-1-4 glycosidic bond. The muramic acid residue carries a five-membered amino acid side chain, or pentapeptide. The pentapeptide is constructed by the sequential addition of L-alanine, D-Glutamic acid, *m*DAP, and a pre-synthesised D-alanine-D-alanine dipeptide. The enzymes responsible for each step have been elucidated and are represented in (Figure:1.4.) Substrate analogues which interfere with the cytoplasmic assembly of the pentapeptide side chain have been described (Neuhaus & Hommes, 1981). These include the D-alanine analogue D-cycloserine, which inhibits the conversion of L-alanine to D-alanine (Cassidy & Kahan, 1973) and the construction of the D-alanyl-D-alanine dipeptide (Pisabarro *et al*., 1986).

The majority of genes encoding the enzymes for the final stages of peptidoglycan biosynthesis are located in the *mra* operon located at 2.5 minutes on the *E.coli.* chromosome (Figure: 1.1). These include *murC*, *murD*, *murE*, *ddlB* *murF*, *mraY*, and *murG.* A second D-ala-D-ala ligase (*ddlA*) and alanine racemase have been identified outside the two minute region (Zawadzke *et al.*, 1991).

N-acetyl-muramyl-pentapeptide and *N*-acetylglucosamine are synthesised as UDP derivatives in the cytoplasm. Once this is achieved these saccharides have to be glycosylated together and transported across the cytoplasmic membrane. To overcome the lipophilic barrier posed by the cytoplasmic membrane these sub-units are attached to a hydrophilic carrier, C55-isoprenylphosphate (undecaprenol phosphate or bactoprenol) (Umbreit & Strominger, 1972). The muramyl-pentapeptide is transferred to the lipid carrier by a specific transferase MraY (Geiss & Plapp, 1978; Ikeda *et al.*, 1991) and UMP is released. A second translocase MurG (Bupp & van Heijenoort, 1993) catalyses transglycosylation of UDP-GlcNAc to undecaprenol-P-P-acetylmuramylpentapeptide, to form the undecaprenol linked disaccharide pentapeptide (Reisinger *et al.*, 1980). The bactoprenol carrier molecules are present in low numbers, consequently recycling of these carriers is essential (Holtje, 1985). The lipid carrier molecule can also transport tripeptide disaccharide (van Heijenoort *et al.*, 1992). However from *in vitro* studies

Figure; 1.3. Arrangement of Glycan Chains and peptide side chains in the Murein Sacculus.



Notes; M, *N*-acetylmuramic acid
G, *N*-acetylglucosamine
M, 1-6-anhydro-*N*-acetylmuramic acid

it would appear tripeptides are transported three to four times less efficiently than disaccharide pentapeptides (van Heijenoort *et al.*, 1993).

The lipid linked disaccharide pentapeptide represents the direct precursor of high molecular weight murein. Once transported across the cytoplasmic membrane these sub-units are assembled into chains of disaccharides cross-linked together by their peptide side chains. The energy for chain extension; transglycosylation, is provided by hydrolysis of the lipid attachment. The energy for cross-linkage; transpeptidation, is supplied by the hydrolysis of the terminal D-alanine of a pentapeptide side chain. The peptide side chains also participate in cross-linking the sacculus to the outer membrane via the peptidoglycan associated lipoprotein (PAL).

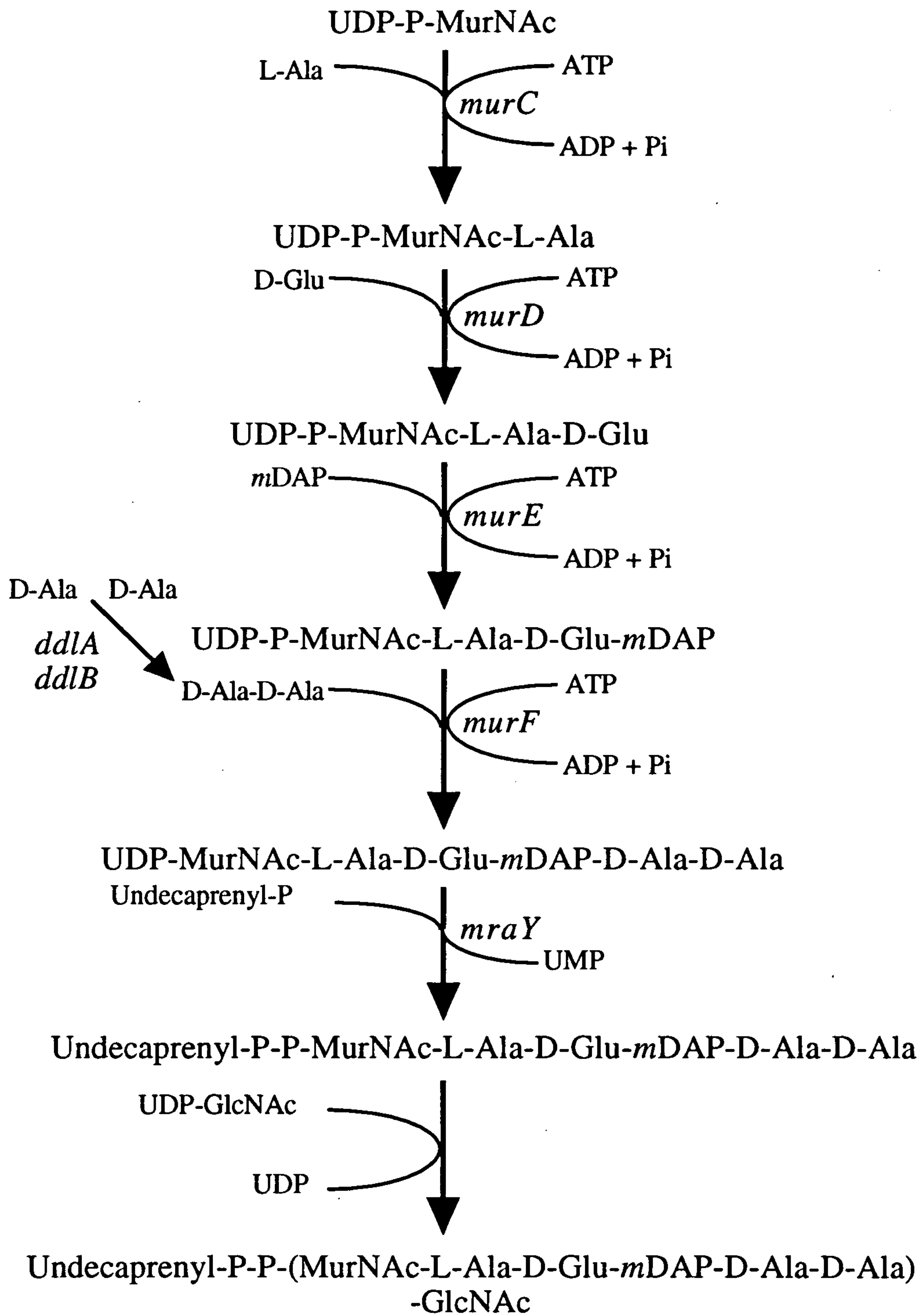
1.2.1 Structure.

Electron microscopy of sacculi that have had their peptide bridges partially broken, either enzymically (Verwer *et al.,* 1978.) or mechanically (Verwer *et al.*, 1980), has indicated that the glycan strands are arranged perpendicular to the long axis of the cell. This fits the observation of sharp vertical cuts observed in sacculi isolated from penicillin treated cells (Schwarz *et al.,* 1969). X-ray data has suggested that these glycan strands are twisted into a helix. This helix completes one 360° rotation every 4 to 4.5 disaccharide units (Labischinski *et al.*, 1985). It is also apparent from HPLC analysis of murein sub-units that the average chain length for the sacculus is 30 disaccharides. Assuming an average circumference of 2 nm for *E.coli* it would require 30 such chains in tandem orientation to span the sacculus once (Holtje & Schwarz, 1985). Though the average length of glycan chains in the sacculus is 30 disaccharide units the mode is estimated to be 10 disaccharides. Therefore it would appear that a few long chains and numerous short chains are arranged in tandem to span the circumference of the cell (Holtje & Glauner, 1990).

1.2.2 Cross-Linkage Patern of the Sacculus.

Digestion of isolated sacculi with muramidase and the separation and identification of the products by HPLC has enabled the structure of the murein sacculus in vivo to be examined (Glauner, 1986; Glauner *et al.*, 1988). This technique accompanied by pulse-labelling of murein precursors with H^3*m*DAP has also produced the first outline of saccular growth and maturation in vivo.

Figure: 1.4. The Synthesis of Peptidoglycan precursors.



Notes; MurNAc, *N*-acetylmuramic acid; Pi, inorganic phosphate; *m*DAP, Diaminopimelic Acid; GlcNAc, N-acetyl-glucosamine.

New peptidoglycan sub-units are anchored to the membrane and energised by their pyrophosphate linkage to the lipid carrier molecule. The sugar backbone is then extended by transglycosylation to form chains of up to 50 disaccharides in length (Glauner & Holtje, 1990). These chains are then shortened to an average length of 30 disaccharide units. This process is performed by lytic transglycosylases and results in the sugar chain terminating in a 1-6 anhydromuramic acid residue (Glauner & Holtje, 1990). At the same time as nascent murein is being extended the peptide side chains are being subjected to the action of transpeptidases and DD-Carboxypeptidases. Transpeptidases cross-link adjacent peptide side chains by a reaction that derives energy from hydrolysis a terminal D-alanine. The predominant cross-bridge constructed by transpeptidation involves cross-linking of a terminal D-alanine from one side chain to the D-centre of *m*DAP on the other (Figure:1.5). This structure is referred to as a DD-cross-bridge. The peptide side chain with the D-alanine participating in the cross-link is referred to as the 'donor', and the chain with the participating *m*DAP the 'acceptor'.

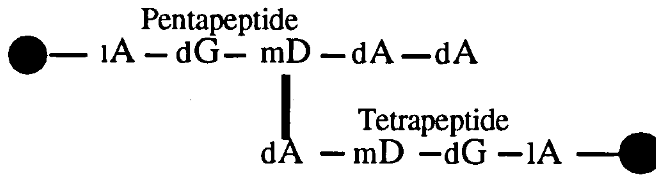
The D-centre of one *m*DAP can also be cross-linked to the L-centre of another *m*DAP to form an LD-cross-bridge (Glauner *et al.*, 1988) (Figure:1.6). These cross-links increase in the presence of penicillin, when autolysins are over-produced, or if cells are subjected to amino-acid starvation (Dreihuis, 1989; Holtje & Glauner, 1990). Consequently it has been proposed that they represent a rescue mechanism to maintain saccular integrity (Holtje & Glauner, 1990).

Cross-linkage by DD-transpeptidase and LD-transpeptidase can result in a variety of cross-bridges (Figures:1.5, 1.6). The simplest involve two adjacent chains with one acting as a donor and undergoing hydrolysis of its terminal D-alanine to enable cross-linkage to the D-centre of the acceptor side chain. The resulting dimer can consist of penta-tetra, tetra-tetra, or tetra-tri side chains. LD-transpeptidation enables the construction of tetra-tri, or tri-tri cross bridges.

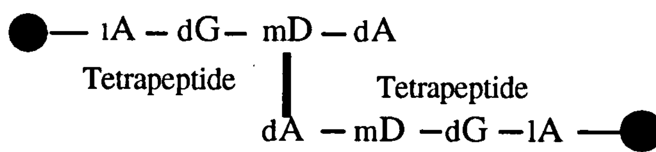
Trimeric cross-bridges are produced by cross-linkage of a third peptide side chain to an acceptor molecule already participating in a dimeric cross-bridge. This can involve LD-transpeptidation or DD-transpeptidation. By the same reactions cross-linking of a fourth chain can occur to form tetramers. The monomeric (uncrosslinked side chains) and dimeric forms of side chains are the most common, accounting for approximately 90% of the total side-chains in a sacculus. The proportion of trimers varies between 3% and 10%, while only 0.2% of side chains participate in tetrameric cross-bridges (Glauner *et al.*, 1988). While the strain variation of most peptidoglycan components identified by HPLC is estimated to be 10%, the total cross-linkage of the sacculus deviates by only 2%. Thus total

Figure: 1.5. Some Peptidoglycan Components Constructed by DD-transpeptidation (DD-cross-bridges).

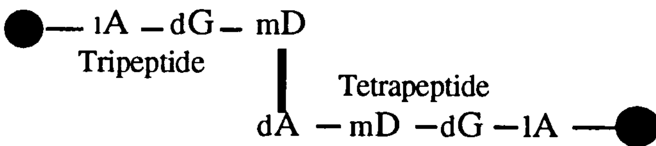
Dimer: penta-tetra Cross-bridge



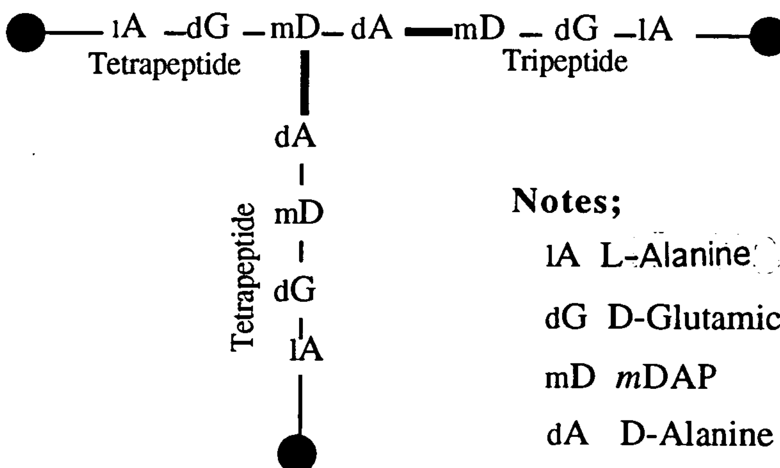
Dimer: tetra-tetra Cross-bridge



Dimer: tetra-tri Cross-bridge



Trimer: tetra-tera-tri



Notes;

lA L-Alanine

dG D-Glutamic acid

mD *m*DAP

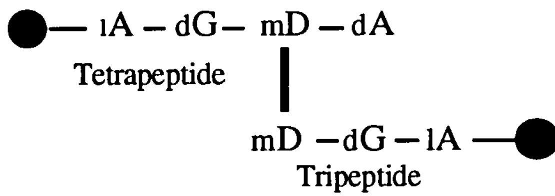
dA D-Alanine

● Glycan Chain (cross-section)

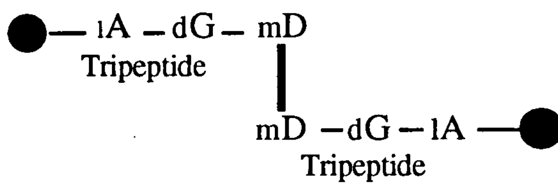
▬ DD-peptide bond

Figure: 1.6. Some Peptidoglycan Components Constructed by LD-transpeptidation (LD-cross-bridges).

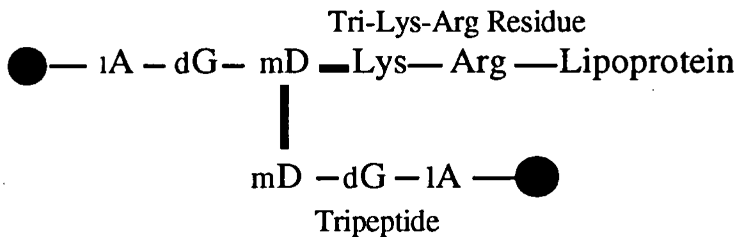
Dimer: LD-tetra-tri Cross-bridge



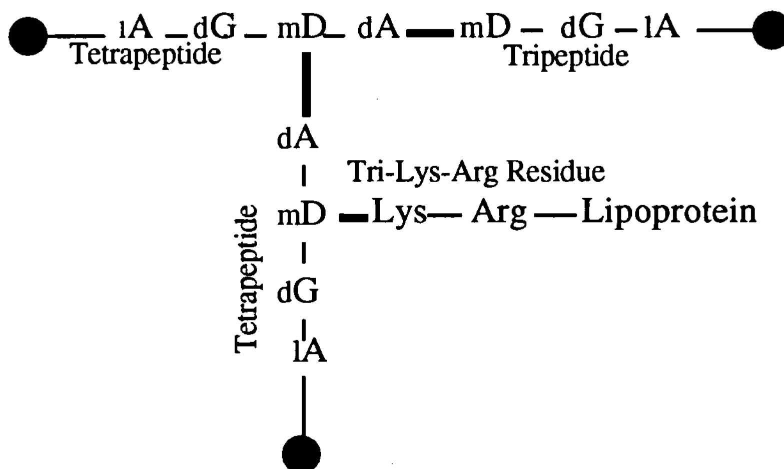
Dimer: LD-tri-tri (Dap-Dap) Cross-bridge



Trimer: Lipoptrotein attachment to LD-tri-tri Cross-bridge



Tetramer: tetra-tera-tri-tri,Lys,Arg



cross-linkage appears the most constant, and by inference the most regulated, parameter of the sacculus (Glauner, 1986; Holtje & Glauner, 1990).

Approximately 5.5% of peptides are involved in lipoprotein attachment (Figure:1.5). The connection of the sacculus to lipoprotein involves the lysyl-arginyl residues of its carboxy-terminus cross-linking to a tripeptide by either an 'LD' or 'DD' transpeptidation. Lipoprotein can participate in the formation of dimeric, trimeric or tetrameric cross-bridges. Lipoprotein attachment appears to favour the formation of LD-dimeric cross-bridges, and does not usually occur the end of sugar chains (Glauner *et al.*, 1988). It is also of particular interest that when a lipoprotein forms a trimer at the end of a sugar chain it produces a structure that inhibits one of the major murein hydrolases (Romeis *et al.*, 1993).

Glycine is found as a minor component of the sacculus. It substitutes for D-alanine at position four or five on the peptide side chain and is sensitive to hydrolysis by LD-Cpase and DD-Cpases. DD-Cpases catalyse hydrolysis of the terminal D-alanine in a pentapeptide side chain to generate a tetrapeptide (Izaki & Strominger, 1968). This is the most common form of side chain in the sacculus, but can be hydrolysed to a tripeptide and in turn to a dipeptide.

1.2.3 The 3-Dimensional Structure of Peptidoglycan.

Computer modeling of peptidoglycan structure predicts that individual saccharide residues within a glycan chain can adopt a variety of confirmations through different degrees of twisting (Barnickle *et al.*, 1983). The degree of twisting is determined by the stress subjected to the glycan strand. Ultimately, longitudinal extension of an individual glycan chain is restricted and consequently confers rigidity to the cell wall. Computer modeling has also predicted that individual peptide side chains will fold back on themselves to form a stable ring shaped conformation. This structure can be unwound by mechanical force. Therefore by unwinding under high stress and contracting under low stress a peptide cross-bridge can provide a high degree of flexibility.

From these models the complexity of saccular structure can begin to be envisaged, helical glycan chains twisted under different amounts of stress with peptide side chains protruding at different angles. The arrangement of glycan chains perpendicular to the long axis means they will be under twice the stress of the peptide side chains (Koch, 1990), while the formation of cross-bridges will alter the mechanical force on individual sugar residues. Models also predict that the formation of trimeric and tetrameric cross-bridges away from the ends of sugar

chains will force the chains into different planes (Holtje & Glauner 1990). Whether this actually occurs in vivo when individual sugar chains are also constrained by peptide cross-bridges is not known.

It is clear that the structure of peptidoglycan enables a highly flexible sacculus to be constructed from a single repeated sub-unit. The structure of this sub-unit means that a polymer can be produced that can expand and contract dynamically, yet still maintain rigidity.

1.2.4 Saccular Maturation.

Pulse-chase experiments have given insight into the normal maturation of the sacculus. As described earlier new peptidoglycan is extended rapidly at the rate of 9 disaccharides per minute, to a length of approximately fifty disaccharides. These new chains are cut back to produce an average saccular length of 30 disaccharides within 5 minutes (Glauner & Holtje, 1990). This process is performed by lytic transglycosylases and results in the sugar chains terminating in a 1-6 anhydromuramic acid residue. The side chains of nascent peptidoglycan are involved in rapid transpeptidation, 19.5% of them becoming involved in cross-bridges within 40 seconds. Pentapeptides are also subjected to rapid hydrolysis by DD-Cpases, which results in 89% being degraded to tetrapeptides within 60 seconds. The predominant link in new murein is a DD-tetra-tetra cross-bridge, but DD-tetra-tri cross-bridges are also formed. These tetra-tri cross-bridges are considered to represent new material being attached to tripeptides in the stress-bearing layer.

After the initial burst of transpeptidation, cross-linkage gradually increases to 24% in three hours. During this time trimeric, tetrameric and lipoprotein cross-bridges are formed. There is also evidence from pulse-chase experiments that attached lipoprotein is released by hydrolases and is then recycled to act as an acceptor in new cross-linkage reactions.

1.2.5 Recycling.

The recycling of murein has been demonstrated with H^3DAP (Goodell, 1985). Approximately 50% of *m*DAP is solubilised each generation, and 80-90% of this becomes incorporated into the sacculus. This process involves a periplasmic amidase releasing the muropeptide side chain (van Heijenoort *et al.*, 1975) and tetrapeptides being degraded to tripeptides by an LD-Cpase. The peptides and not the sugars (Goodell, 1985), are then transported across the cytoplasmic

membrane by an oligopermease (Goodell & Higgins, 1987) and a low affinity permease (Park, 1993). No free tripeptides have been detected in the cytoplasm so it is assumed tripeptides are recycled via the L-Alanine ligase and added to UDP-Muramic acid (Holtje & Glauner, 1990).

1.2.6 Model for Saccular Maturation.

The HPLC analysis of pulse-labelled peptidoglycan led to the first model for saccular maturation, based upon 'inside to outside' growth (Glauner & Holtje, 1990). This envisages three layers of peptidoglycan, the first anchored to the cytoplasmic membrane by the attachment of nascent murein to the lipid carrier molecule. The second layer represents a complete monolayer; the ‘stress bearing layer’, that envelopes the cell. In the first layer, strands of new murein are cross-linked to each other and also cross-linked to acceptors in the Stress-bearing layer (Glauner & Holtje, 1990).

The 'stress bearing layer' is the most cross-linked, forming connections to the layers above and below, as well as to lipoprotein in the outer membrane. The third-layer is the least cross-linked, possibly participating in lipoprotein attachment, and subject to degradation and recycling (Glauner & Holtje, 1990). This model has been adapted to fit the evidence from NMR and Fast Atom Bombardment (Labishinski *et al.*, 1991) that 70% of the sacculus is monolayered. It is now envisaged that only the Stress-bearing layer is a continuous monolayer, and that three layers are formed at growth points. At these growth points nascent murein is attached to the Stress-bearing layer above and as bonds in the Stress-bearing layer become hydrolysed the insertion of new material concomitantly pushes old material out of the Stress-bearing layer (Holtje, 1993).

1.2.7 Models for Saccular Growth

The existence of a single monolayer of peptidoglycan covering 80% of the cell's surface (Labischinski *et al.*, 1991) has had major implications on models for saccular growth. To maintain the cell's integrity during saccular expansion the synthesis of new material has to be co-ordinated with hydrolysis of existing cross-links. Such a 'make before break' (Koch, 1990) strategy for growth appears the only way a stress-bearing monolayer can expand.

Some general features of saccular growth have been established. These include the diffuse incorporation of new material during elongation and

localised incorporation at the leading edge of constriction during division (Wientjes *et al.*, 1989). It has still not been established conclusively whether single or double stranded peptidoglycan is inserted into the sacculus at growth points (Burman & Park, 1984; de Jonge 1989). However the most recent work suggests that single stranded peptidoglycan is inserted during elongation and that either double stranded peptidoglycan, or very rapid insertion of single stranded peptidoglycan occurs during division (de Jonge, 1989; Nanninga, 1991).

The 'hernia' model of saccular growth proposes that the curvature of the cytoplasmic membrane determines the activation of peptidoglycan synthesis and murein hydrolysases (Norris & Manners, 1993; Norris & Sweeney, 1993). The 'hernia' begins with a localised weakening of the sacculus by hydrolases which leads to the cytoplasmic membrane bulging (longitudinally and circumferentially) into the periplasm as a result of turgor pressure. Lateral diffusion of specific phospholipid species then establish a bulge which consists of a convex outer phospholipid monolayer, and concave inner phospholipid monolayer. This curved structure is proposed to positively affect the translocase and transferase responsible for the construction of the lipid linked bactoprenol dissacharide pentapeptide. Besides stimulating transport of peptidoglycan pre-cursors the hernia stimulates murein synthesis and hydrolysis. To fit Labischinski's (1991) observations the hernia is envisaged to result in the production of a triple layer of peptidoglycan. Once synthesis of new peptidoglycan is completed and the sacculus locally strengthened the hernia disappears. The authors propose this to result from the action of contractile proteins in the cytoplasm interacting with membrane proteins bound to the peptidoglycan (Norris & Manners, 1993).

Much of the detail for the hernia model remains vague. One of its premises is that the localised structure of the cytoplasmic membrane promotes transport of nascent peptidoglycan. This is supported by the lipid dependence of translocase activity (Geiss & Plopp, 1978) and the sensitivity of bactoprenol phosphate (lipid carrier) synthesis to lipid composition (Rogers *et al.*,1980). The influence of phospholipids on murein synthesising and hydrolysing enzymes has been reported. For one of the major murein synthetases (PBP1B) disruption of the cytoplasmic membrane results in detection of twice the level of enzyme that is detected in an intact membrane (Rojo *et al.*, 1984). Similarly alteration of the phospholipid composition of the cytoplasmic membrane can cause an increase in the detectible level of a murein hydrolase (PBP6) (Kuriki, 1981). It has also been reported that major murein synthesising and hydrolysing enzymes (PBPs) associate

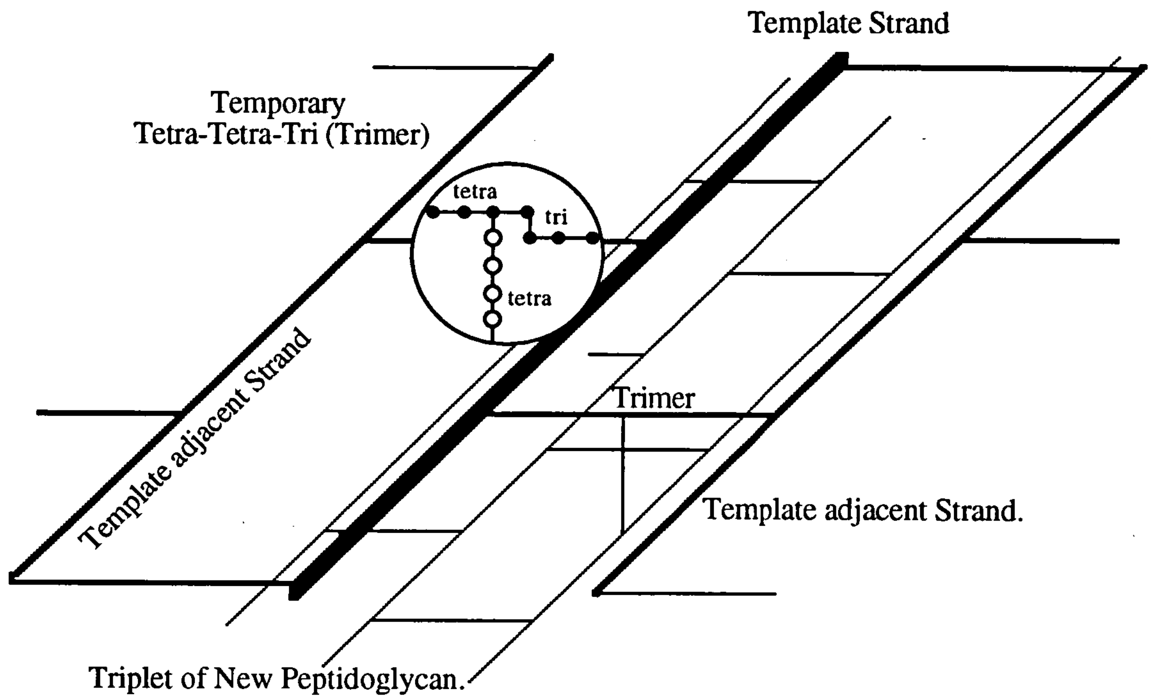
with different subsets of inner membrane vesicles (Leidenix *et al.*, 1989). This is an observation that Norris and Manners (1993) take to indicate a localisation of PBPs to distinct membrane domains.

The allosteric and multi-enzyme models proposed by Koch (1990) deal with the insertion of new material into the stress-bearing layer. The allosteric model requires tightly controlled allosteric murein hydrolases to recognise stressed bonds in the sacculus. This specific hydrolysis of cross-bridges under stress is partially determined by the lower energy of activation required for their cleavage but may also involve structural or conformational elements in the cross-bridges (Koch, 1990). Saccular growth begins with the synthesis of a single new strand beneath the Stress-bearing layer. This strand is cross-linked to the strands above. It is then proposed that only stressed bonds associated with unstressed bonds will be recognised and cleaved. These unstressed bonds indicate the attachment of new murein below, and upon hydrolysis they in turn become stressed due to saccular expansion. This transfer of stress then lifts the new material into the space provided by hydrolysis of the bond in the Stress bearing layer.

In the allosteric model, one new strand of peptidoglycan is attached to the strands above. Upon hydrolysis of cross-bridges the new strand becomes incorporated without the concomitant loss of old material. This method of growth fails to account for the extensive re-cycling of peptidoglycan which has been observed (Goodell, 1985).

The multi-enzyme model envisages a more co-ordinated process of incorporation and cleavage (Koch, 1990). Two strands will be inserted for every one replaced. The two new strands are first cross-linked together and to the sacculus above to produce a 'loose fabric' beneath the stress bearing layer. Murein hydrolases then cleave cross-bridges perpendicular to a single strand in the stress bearing layer and so release it for recycling. These cuts enable physical force (turgor pressure) to produce saccular expansion and pull two new strands into the stress bearing layer. The main premise of this model is that the synthetic and hydrolytic enzymes form a complex. The multi-enzyme complex synthesises new peptidoglycan as it hydrolyses old strands. The result is cylindrical expansion by inside to outside growth with two new peptidoglycan strands replacing every one hydrolysed. The single strand released is then free to be recycled.

Figure:1.7. The 3 for 1 Model of Saccular Growth.



Hydrolysis of Trimeric cross-bridges and incorporation of new material.

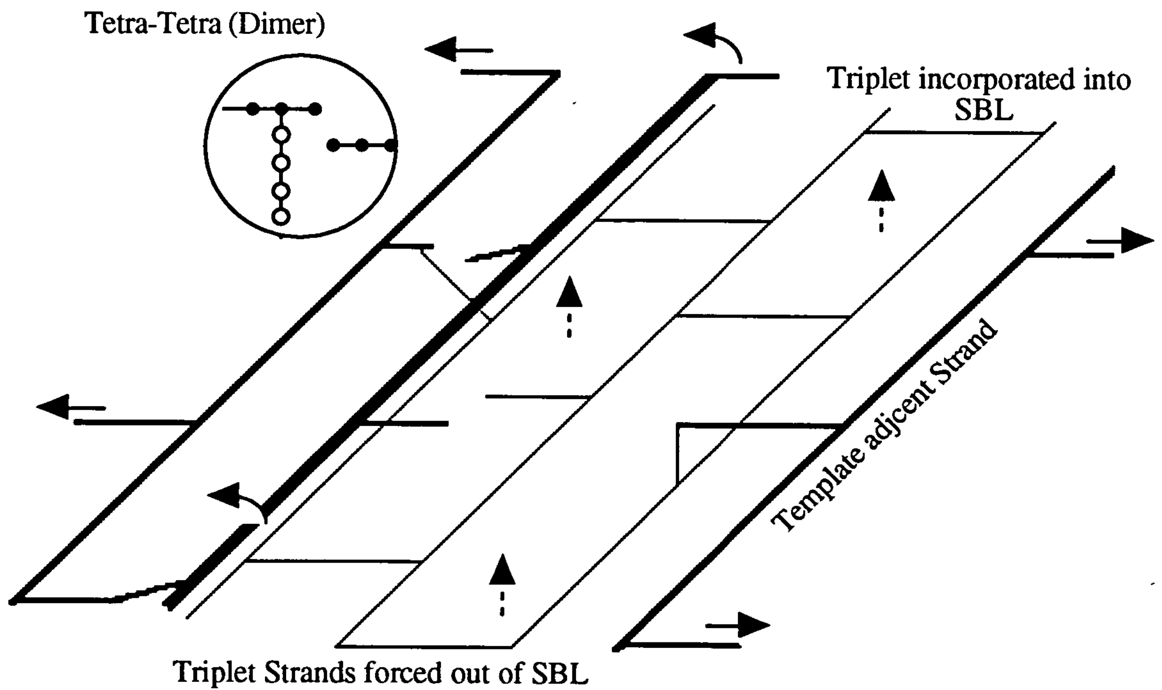
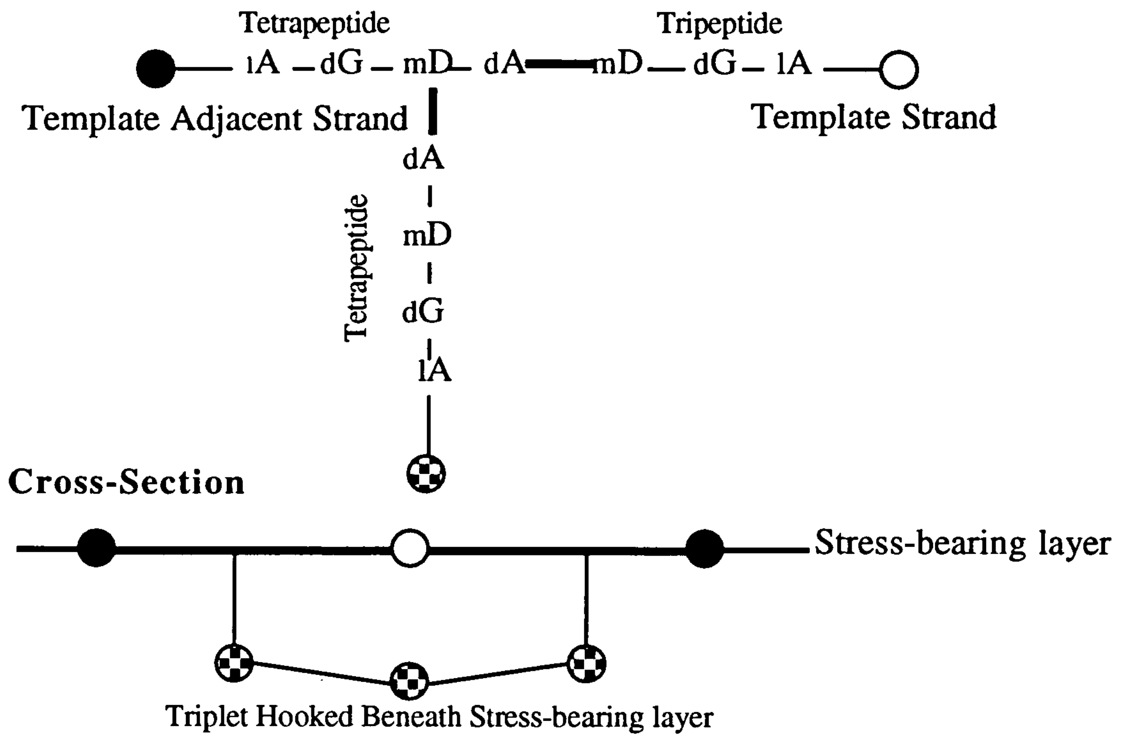
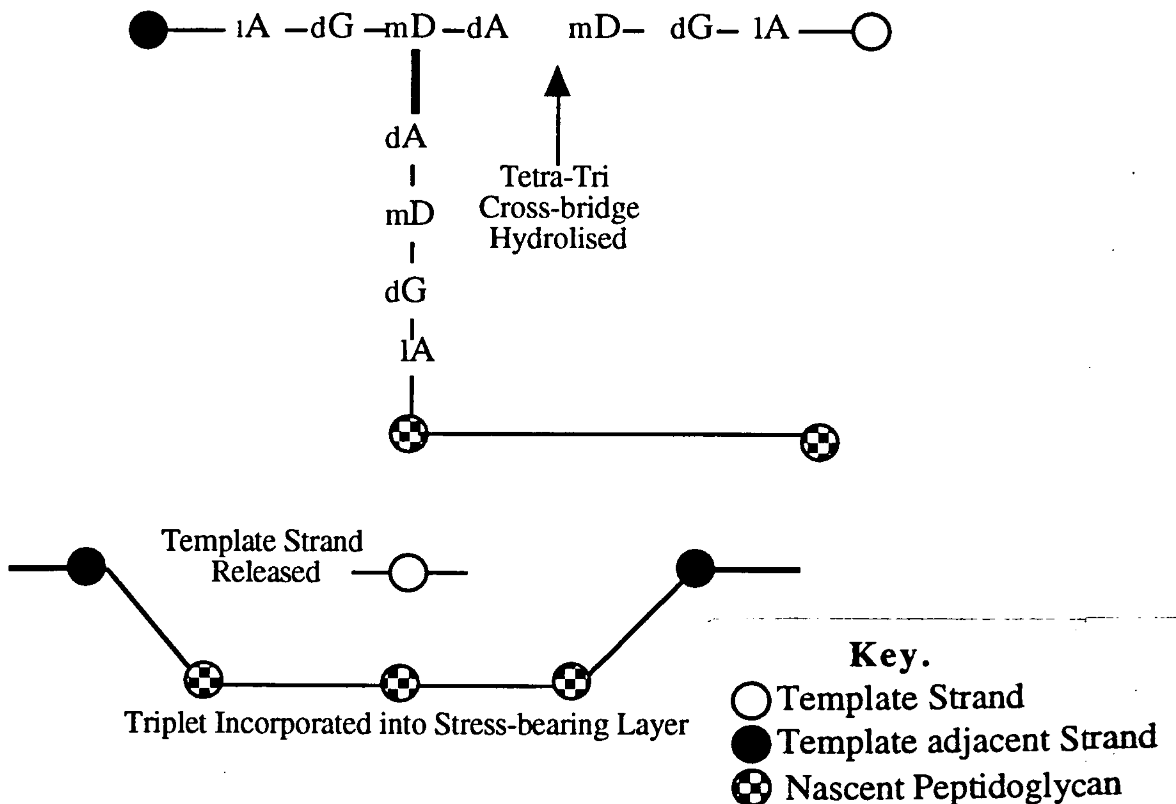


Figure: 1.8. Cross-Section of Template Defining Trimeric Cross-bridges



Hydrolysis of tetra-tri cross-bridge and incorporation of Triplet into the Stress-bearing layer



The 3 for 1 model is a development of the multi-enzyme model proposed by Koch (1990) (Figures:1.7, 1.8, 4.4). It accommodates safe expansion of the sacculus and accounts for recycling and the construction of a septum. It proposes that three glycan strands are incorporated for every one hydrolysed. Like the multi-enzyme model, new peptidoglycan is envisaged to be synthesised as a loose-fabric hooked underneath the stress bearing layer. Unlike Koch, Holtje proposes that this loose fabric consists of three parallel glycan strands (triplet) cross-linked together. The triplet is synthesised beneath a single 'template' strand in the Stress-bearing layer. The strands lying parallel to the template strand in the Stress-bearing layer (template adjacent strands) are linked to the template strand by dimeric cross-bridges. The template strands' side-chains participate in the dimeric cross-bridges as tripeptide acceptors. Consequently the template adjacent strands' side chains act as tetrapeptide donors. The precise structure of these cross-bridges is important as it defines which specific bonds are hydrolysed, and which side chains will participate in new cross-bridges.

The triplet of new murein is cross-linked together by DD-tetra-tetra cross-bridges. As the triplet is not under any stress it sits relatively compactly beneath the single template strand. The outermost triplet strands attach themselves to the stress bearing layer by cross-linking to the dimeric cross-bridges between the template and template adjacent strands (Figure:1.8). This results in the formation of temporary trimeric cross-bridges. Specific hydrolases are then proposed to recognise these trimers. Once recognised the tetra-tri bonds between the strands in the stress bearing layer are hydrolysed. This results in the triplet being lifted up into the stress bearing layer and the template strand being pushed outward for degradation and recycling (Figure:1.7).

Like the multi-enzyme model the essential features are 'make before break' (*ie.* synthesise new bonds before hydrolysing old) and inside to outside growth. This model was designed to accommodate the fact tht all tetra-tri cross-bridges and only 50% of tetra-tetra cross-bridges are degraded in one generation. By considering trimers as temporary structures it also accommodates the high turn over of trimeric cross-bridges.

The 3 for 1 model also encompasses construction of a septum. (Figure:4.4). During septation trimers and tetramers are enriched temporarily (Kraus & Holtje, 1985; Kraus *et al.*, 1987; De Jonge, 1989). The model suggests that this is the result of successive triplets being attached beneath each other as the cytoplasmic membrane constricts. This successive addition of material results from the increased rate of murein synthesis (Nanninga, 1991) not being accompanied by an identical

increase in hydrolysis. For each new triplet the central strand becomes the template strand for the triplet below. Hydrolysis of these template strands will lag behind the rate of ingrowth and will eventually liberate two poles.

Triplets added to the leading edge of the ingrowing septum are proposed to consist of shorter and shorter glycan chains due to the ever decreasing circumference of the invagination. This process will continue until only a small polar aperture remains. Sterical hindrance then prevents new peptidoglycan from being added and cross-links between adjacent strands are favoured, resulting in completion of the cell pole.

The 3 for 1 model represents the most recent and complete model for saccular growth. It attempts to explain the rapid turnover of tetra-tetra bonds, the transient increase in trimers during septation, and the shorter glycan chains observed in a septum (Romeis *et al.*, 1991). However its central premise, that three strands are inserted for every one hydrolysed, remains to be demonstrated.

1.3 The Murein Hydrolases.

Growth, maturation and recycling of murein require sets of synthesising and hydrolysing enzymes. Chain extension and peptide cross-linkage require transglycosylases and transpeptidases respectively. Hydrolases are required for insertion of new material (Holtje, 1993; Koch, 1990), recycling of old (Goodell, 1985) and the splitting of the septum (Figure:1.9).

The murein hydrolases include a number of enzymes that can act as autolysins and destroy the integrity of the sacculus (Holtje & Tuomanen, 1991). Uncontrolled autolysin activity is considered to be responsible for penicillin induced lysis (Kitano & Tomasz, 1979).

1.3.1 Glucosidases.

The murein hydrolases can be considered as three distinct groups, glucosidases, peptidases and carboxypeptidases. Glucosidases degrade sugar side chains and include the lytic transglycosylases which cleave the β-1-4 glycosidic bond between *N*-acetyl-muramic acid and *N*-acetyl-glucosamine (Holtje *et al.*, 1975). This cleavage is followed by an intramolecular transglycosylation of a muramic acid residue. The resulting transfer of the O-muramyl onto its own C6-hydroxyl group produces a 1-6 anhydromuramic acid. Two soluble lytic transglycosylases have been identified (Kusser & Schwarz, 1980; Romeis *et al.*,

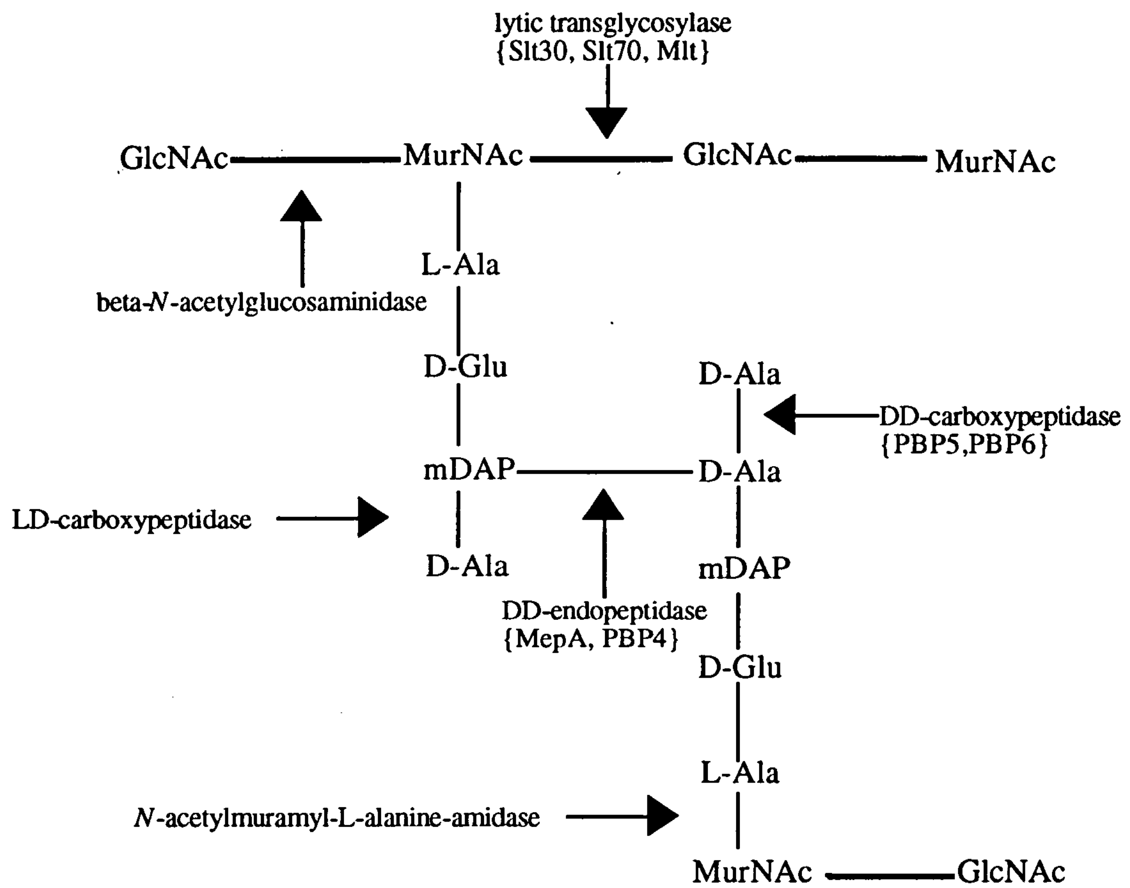
1993) and one membrane bound enzyme (Mett *et al.*, 1980). All these lytic transglycosylases have unique features. The Slt70 is active against isolated glycan chains and is found evenly distrubuted over the outer surface of the sacculus (Walderich & Holtje, 1991). It is postulated that the enzyme has a specific recognition site for a murein structure in addition to its active site. This second site is the target for its specific inhibitor bulgecin (Templin *et al.*, 1992). Inactivation of the Slt70 is not lethal but in conjunction with PBP3 inhibition produces bulges at the septum. A strain deleted for the *slt*70 gene also exhibits supersensitivity the PBP2 specific inhibitor; mecillinam. The Slt35 is the second soluble lytic transglycosylase and specifically recognises cross-linked glycan chains as its substrate (Engel *et al.*, 1993). The Mlt38 is only active when incorporated into the cell membrane and like the Slt35 is active on cross-linked glycan chains. The Mlt38 is uniquely sensitive to one particular murein structure. This structure, an anhydro form of lipoprotein containing cross-bridge (Romeis *et al.*, 1993) actually inhibits the Slt35. In Koch's (1990) and Holtje's (1993) models of saccular growth, carefully regulated murein hydrolases or 'smart enzymes' are required. The allosteric nature of the Slt70 and the sensitivity of the Mlt38 to certain murein structures, are both charcteristics that would be expected for such 'smart enzymes'.

The other glucosidase, β-*N*-acetylglucosaminidase (Maas *et al.*, 1964), is active on soluble murein fragments but not on isolated sacculi. Consequently this hydrolase is considered to be involved in recycling material and is not a potential autolysin (Holtje & Tuomanen, 1991).

1.3.2 Peptidases.

The peptidases include *N*-acetylmuramyl-L-alanine amidase, DD-endopeptidases, DD-Cpases and LD-Cpases. *N*-acetylmuramyl-L-amidase is only active on muropeptides and not intact sacculi (van Heijenoort *et al.*,1975). It cleaves the amide bond between muramic acid and the first amino acid of the peptide side chain L-alanine. The enzyme's activity has been reported to be dependent on the phospholipid composition of the cytoplasmic membrane (Vanderwinkle & Vlieghere, 1985). The DD-endopeptidase cleaves DD-cross-bridges of intact sacculi and consequently can be considered to be an autolysin (Hartman *et al.*, 1972). Two enzymes have been isolated with this activity. One of these is the penicillin-sensitive, PBP4 (Iwaya & Strominger, 1977), the other penicillin-insensitive, MepA (Keck & Schwarz, 1979; Keck *et al.*, 1990). The DD-endopeptidase activity is envisaged as being crucial in enabling new material to be incorporated into the

Figure: 1.9. The Murein Hydrolases of *E.coli*.



Stress-bearing layer (Koch, 1990; Holtje, 1993), and old material to be removed. The identification of LD-peptide bonds (Glauner, 1986) has also suggested an LD-endopeptidase might exist (Holtje & Toumonen, 1991).

The DD-Cpases catalyse the removal of the terminal alanine from a pentapeptide side chain, to produce a tetrapeptide (Strominger & Izaki, 1968). Three periplasmic penicillin-sensitive proteins have been ascribed DD-Cpase activity in *E.coli* . These are PBP4 (Matsuhashi *et al.*, 1977; Iwaya & Strominger, 1977; Koret *et al.*, 1990) PBP5 and PBP6 (Tamura *et al.*, 1976; Anamura &Strominger, 1980). The LD-Cpase cleaves the LD-bond between *m*DAP and D-alanine of a tetrapeptide side chain. Two of these enzymes have been identified, one periplasmic (Beck & Park, 1976; Ursinus *et al.*, 1992) the other cytoplasmic (Metz *et al.*, 1986).

1.4 The Penicillin-binding Proteins.

A penicillin-binding component of the bacterial cell was identified by 1949 (Maas & Johnson, 1949) and by 1957 it was established that the bacterial cell wall was the target for penicillin (Park & Strominger, 1957). The presence of distinct Penicillin-binding Proteins (PBPs) in *E.coli* was demonstrated by Spratt & Pardee (1975). The subsequent study of these penicillin-sensitive enzymes has driven research into the structure and metabolism of the murein sacculus.

PBPs by definition recognise and interact with penicillin, a substrate analogue of the D-alanyl-D-alanine dipeptide (Tipper & Strominger, 1965). Therefore the study of PBPs is a study of enzymes associated with one particular component of peptidoglycan. As a consequence of this PBPs include murein synthesisng and hydrolysing enzymes.

Exposure of the isolated cytoplasmic membrane to radiolabelled penicillin and the subsuquent separation of proteins by SDS-PAGE has led to the identification of 8 discrete proteins (Spratt, 1975; Spratt, 1977). These PBPs are numbered 1A, 1B, 2, 3, 4, 5, 6, and 7, according to decreasing molecular weight. They are all associated with the cytoplasmic membrane and are commonly divided into two groups. The High Molecular Weight (HMW) PBPs include 1A, 1B, 2, and 3. These are all murein synthesising enzymes, with an active site situated in the middle of the protein, and a cytoplasmic membrane anchor at their amino-terminus (Broome-Smith *et al.*, 1985; Adachi *et al.*, 1987;. Edelman *et al.*, 1987; Spratt & Bowler, 1987). The Low Molecular Weight (LMW) PBPs include 4, 5, and 6. PBP7 is excluded from this group as its enzyme activity remains to be fully elucidated. PBP4, PBP5, and PBP6 are all hydrolytic enzymes, that have their active sites

located close to their amino terminus (Crombie & Spratt, 1988). Sequence similarity suggests they have distinct but distant relationship to serine β-lactamases (Crombie & Spratt, 1988).

1.4.1 PBP1A & PBP1B.

PBP1A and PBP1B are the largest PBPs with molecular weights of 94kD and 89kD (Broome-Smith *et al.*, 1985) respectively. They have been demonstrated to be bifunctional transglycosylases and DD-transpeptidases (Suzuki *et al.*, 1985; Ishino *et al.*, 1980;. Nakayawa *et al.*, 1984). The two proteins are homologous (Broome-Smith *et al.*, 1985) and deletion of either of these proteins can be tolerated (Kato *et al.*, 1985; Youssif *et al.*, 1985). However inactivation of both proteins leads to rapid lysis (Tameki *et al.*, 1977). This has been suggested to indicate that they share a common role, and that the presence of either can compensate for the absence of the other (Suzuki *et al.*, 1978.).

PBP1B is anchored to the cytoplasmic membrane by an amino-terminal transmembrane sequence (Edelman *et al.*, 1987), and is reported to be preferentialy located to membrane adhesion sites (Bayer *et al.*, 1990). By contrast PBP1A appears to interact with the cell membrane via an uncleaved signal peptide (Broome-Smith *et al.*, 1985). PBP1B has been suggested be the major transpeptidase responsible for incorporation of new murein into the sacculus (Spratt, 1975; Spratt *et al.*, 1977). Deletion of PBP1B has been reported to produce supersensitivity to β-lactam antibiotics (Youssif *et al.*, 1985) and to slow the rate of growth in salt free media (Kato *et al.*, 1985). Inhibition of both PBP1A and PBP1B by β-lactams is the most effective trigger of autolytic wall degradation (Kitano & Tomasz, 1979). Since nearly all β-lactams show higher affinity for PBP1A rather than PBP1B it has been speculated that their ability to lyse cells is limited by the rate at which they bind PBP1B (Youssif *et al.*, 1985). The relatively low affinity of β-lactams for PBP1B may be explained in part by the existence of a fraction of the protein in a cryptic form. It has been estimated that up to 50% of all PBP1B is cryptic, and will only bind radioactive penicillin if the cytoplasmic membrane is disrupted with *n*-butanol or SDS before labelling (Rojo *et al.*, 1984).

Levels of PBP1B and the septum specific murein sytnthetase PBP3 are negatively regulated by the *mreB* operon (Wachi *et al.*, 1987). An unidentified chromosomal mutation that requires a functional PBP1B to suppress a temperature sensitive PBP3 allele has also been reported (del Portillo *et al.*, 1991). Along with

other experimental data (del Portillo *et al.*, 1989; del Portillo *et al.*, 1990) these observations have been proposed to indicate a role for PBP1B in division.

Another mutation mapping close to the *mreB* operon at 71 minutes has been reported to increase levels of PBP1A and PBP2 (Tameki *et al.*, 1977; Doi *et al.*, 1988). The identification of this *mraA* allele is proposed to indicate PBP1A and PBP2 interact during cell elongation (Matsuhashi *et al.*, 1990).

1.4.2 PBP2.

PBP2 has been demonstrated to catalyse a penicillin-sensitive transpeptidation in vitro, and penicillin-insensitive transglycosylation that requires the presence of a second protein RodA (Ishino *et al.*, 1986). The two genes for these proteins are located adjacent to each other and appear to be co-transcribed (Matsuzawa *et al.*, 1989). Inactivation of either of these proteins causes cells to lose their rod shape and grow as spheres (Spratt & Pardee, 1975). The two proteins are envisaged to form a functional complex which determines the cell's rod shape (Matsuhashi *et al.*, 1990). PBP2 has a molecular weight of 66 kD and is estimated to be present in 20 copies per cell (Spratt, 1975). PBP2 has been estimated to be responsible for between 50% and 70% of the total murein synthesised (Park & Burman, 1973; Rodriguez & de Pedro, 1990). This may be by synthesising peptidoglycan primers which are then extended by the action of PBP1A and PBP1B (Wientjes & Nanninga, 1991).

Inactivation of PBP2 with the specific β-lactam mecillinam (Lund & Tynberg, 1972) causes cells to become spherical, division to stop and cell viability to fall (Park & Burman, 1973; James *et al.*, 1975; Matsuhashi *et al.*, 1979.). However inhibition of PBP2 causes a poor induction of autolysins (Kitano & Tomasz, 1979) and consequently mecillinam is not highly bacteriolytic. Mutations in *cya*, *crp*, or *argS* have been shown to confer mecillinam resistance (Aono *et al.*, 1978;. Jaffe *et al.*, 1983; D'Ari *et al.*, 1988) and suppress deletions of either *rodA* (Ogura *et al.*, 1989) or *pbpA* (Vinella *et al.*, 1992). Study of a *pbpA* deletion strain showed that the viability conferred by these mutations was the result of elevated levels of the nucleoside ppGpp (Vinella *et al.*, 1992. Bouloc *et al.*, 1993). This was proposed to indicate a mechanism that normally co-ordinates ribosomal activity and growth rate with cell wall elongation (Vinella *et al.*, 1992).

The block to division produced by mecillinam (James *et al.*, 1975) and the demonstration that complete deletion of PBP2 is lethal (Vinella *et al.*, 1992), has been argued to implicate PBP2 in division (Bouloc *et al.*, 1993). This

explanation remains speculative; however it is clear that PBP2 is essential for lateral cell wall synthesis and the recovery of cells from stationary phase (de la Rosa *et al.*, 1985. Pisabarro *et al.*, 1987).

1.4.3 PBP3.

Like PBP2, PBP3 is involved in morphogenesis. Inactivation of PBP3 produces filamentous cells blocked in division but capable of lateral cell wall synthesis (Spratt, 1975; Botta & Park, 1981; Schmidt *et al.*, 1981.). The protein has been demonstrated to catalyse a penicillin sensitive transpeptidation and weak penicillin-insensitive transglycosylase reaction in vitro (Ishino & Matsuhashi, 1981). It has been suggested that, like PBP2 and RodA, PBP3 normally forms a functional complex with a second protein that is responsible for transglycosylase activity. FtsW has been proposed to be this second protein (Matsuhashi *et al.*, 1990). The *ftsW* DNA sequence shows significant similarity with *rodA*, is located close to *ftsI* in the two minute region, and mutants have the filamentous phenotype that would be expected.

PBP3 is synthesised as a precursor and processed in the periplasm by cleavage of its first eleven COOH-terminal amino acids (Hara *et al.*, 1989). Its amino-terminus behaves as a non-processed, non-modified signal peptide which drives it to the membrane (Gomez *et al.*, 1993) and anchors it there (Bowler & Spratt, 1989). The similarity of its transmembrane structure to other division proteins including FtsQ, FtsL, and FtsN has led to the speculation that it may form part of a functional complex (Dai *et al.*, 1993; Nanninga *et al.*, 1991). The existence of a PBP3 complex has also been suggested by the division block produced when a mutant PBP3 is expressed in a wild type cell (Broome-Smith *et al.*, 1985). PBP3 remains the only penicillin-sensitive peptidoglycan synthetase that is essential under all circumstances (Hara & Park, 1993). Despite this, it is one of the least abundant, present in 50 copies per cell, and is the only PBP to undergo *relA* dependent degradation at the end of active growth (de la Rosa *et al.*, 1983).

1.4.4 PBP4.

PBP4 is a 49.6 kD protein present in 110 copies per cell (Spratt, 1975). It has no membrane anchoring sequence and appears to exist as both a loosely membrane-associated and as a soluble form (Mott, 1992). It has been attributed membrane bound '1B' and soluble '1C' DD-Cpase activity (Iwaya & Strominger,

1977). PBP4 has been suggested to have three distinct enzyme activities; DD-endopeptidase, DD-Cpase (Matsuhashi *et al.*, 1977; Iwaya & Strominger, 1977) and DD-transpeptidase (de Pedro & Schwarz, 1981). The loss of secondary transpeptidase activity in a PBP4 deletion strain (de Pedro & Schwarz 1981) was later shown to have been wrongly attributed to PBP4 and to result from a second mutation (Glauner, 1986). The cloning of *dacB* and overproduction of PBP4 confirmed its activity as a DD-endopeptidase and DD-Cpase *in vivo* (Koret *et al.*, 1990).

The *dacB* coding sequence includes the characteristic SXXK, SXN and KTG motifs common to all PBPs (Mottl, 1992). The spacing of the SXXK and SXN motifs is unusually large, with an extra domain of 188 amino acids seperating them. Deletion of this domain brings the motifs into alignment with those of PBP5 and PBP6, and does not interfere with penicillin-binding (Mottl, 1992). It remains unclear whether this domain has any role in determining an aspect of PBP4 activity. PBP4 is also distinct from other PBPs in the ability of boronic acid to compete with its binding of penicillin (Then *et al.*, 1990).

Along with the penicillin-insensitive MepA, PBP4 is thought to be involved in hydrolysis of cross-bridges to enable insertion of new peptidoglycan and expansion of the sacculus (Holtje, 1993). DD-endopeptidase activity increases prior to division (Hackanbeck & Messer, 1977) and is enriched in minicell membranes (Goodell & Schwarz, 1977), thus implicating it in division. A strain deleted for PBP4 (Iwaya & Strominger, 1977), and strains with reduced DD-endopeptidase activity (Kitano *et al.*, 1980) have been demonstrated to be penicillin tolerant. It has also been noted that phenotypic tolerance produced by slow growth is accompanied by decreased DD-endopeptidase activity (Cozens *et al.*, 1989). Consequently it would appear that PBP4 has a role in penicillin induced autolysis.

1.4.5 PBP5 & PBP6.

PBP5 and PBP6 share 62% sequence similarity (Broome-Smith *et al.*, 1988) and have been demonstrated to be responsible for membrane bound DD-Cpase '1A' activity (Tamura *et al.*, 1976; Amanuma & Strominger, 1980; Annamura & Strominger, 1984). Both are synthesised as pre-proteins and undergo processing before becoming located in the cytoplasmic membrane (Pratt *et al.*, 1986). They are anchored in the cell membrane by unusual non-hydrophobic amphipathic helices at their carboxy-termini (Jackson & Pratt, 1987).

In vitro PBP5 exhibits approximately 10 times the DD-Cpase activity of PBP6 (Amanuma & Strominger, 1984), and is considered to be the major DD-Cpase in vivo (Glauner, 1986). Gross overproduction of PBP5 causes cells to swell and become spherical before lysing (Markiewicz *et al.*, 1985). PBP5 is inessential (Spratt, 1980) and a deletion strain shows the expected accummulation of pentapeptide side chains (Glauner, 1986). Cells lacking a functional PBP6 are also viable (Broome-Smith & Spratt, 1982) but do not accummulate pentapeptides (Glauner, 1986). Overproduction of PBP6 has been reported to cause 'disordered cell shape' (Matsuhashi *et al.*, 1982), but recent work has established 100 fold overproduction to have no morphological affect (van der Linden *et al.*, 1992).

PBP5 and PBP6 are the most abundant PBPs, estimated to be present in 1,800 and 600 copies respectively (Spratt, 1975), and together account for 86% of all PBPs (Spratt, 1977). As early as 1968 Izaki & Strominger (1968) predicted DD-Cpase activity to be dispensible. This was because DD-Cpase activity could be inhibited by low concentrations of penicillin that did not affect growth. The construction of a mutant lacking both PBP5 and PBP6 confirmed that at least 90% of DD-Cpase activity was dispensible (Broome-Smith, 1983).

PBP6 has been reported to increase in stationary phase (Buchanan & Sowell, 1982) and to be regulated by the small DNA binding protein BolA (Aldea *et al.*, 1989). BolA is itself transcriptionally regulated by the stationary-phase sigma RpoS (Lange & Hengge-Aronis, 1991). It has been speculated that both RpoS and BolA may be involved in the stationary-phase regulation of PBP6 (Lange & Hengge-Aronis, 1991). PBP6 is the only PBP beside PBP1B to bind penicillin in the presence of *n* -Butanol or SDS (Rojo *et al.*, 1984). Unlike PBP1B, PBP6 binding to penicillin in the presence of chaotropic agents has not demonstrated the existence of an additional 'cryptic' fraction of the protein. However it has been reported that disturbed membrane structure of fatty acid auxotrophs can produce increased levels of PBP6 (Kuriki *et al.*, 1981).

PBP4, PBP5 and PBP6 share common features with class A serine β-lactamases, suggesting a common evolutionary origin (Crombie & Spratt, 1988). PBP5 and PBP6 also have short half lives for bound penicillin and have been described as exhibiting weak β-lactamase activity (Nicholas & Strominger, 1988). The role of PBP5 and PBP6 in the cell cycle is not clear. It has been suggested that PBP5 stabilises murein by hydrolysing the terminal D-alanine and limiting the availability of donors for transpeptidation (de Pedro *et al.*, 1980). While PBP6 has been proposed to be responsible for changes in stationary phase peptidoglycan (van der Linden *et al.*, 1992). General DD-Cpase activity has been reported to increase

Table: B. The Penicillin-binding Proteins of *E.coli*.

PBP	Gene	Location (mins)	Size in kD	Copies per cell	Activity
PBP1A	*mrcA* (*ponA*)	74.2	93.5	230 (PBP1A & PBP1B)	transglycosylase DD-transpeptidase
PBP1B	*mrcB* (*ponB*)	3.2	88.8-94.1	230 (PBP1A & PBP1B)	transglycosylase DD-transpeptidase
PBP2	pbpA (mrdA)	14.4	70.9	20	transglycosylase DD-transpeptidase
PBP3	*ftsI* (*pbpB*)	2.5	63.5	50	transglycosylase DD-transpeptidase
PBP4	*dacB*	68.6	49.6	110	DD-endopeptidase DD-carboxypeptidase
PBP5	*dacA*	14	41.3	1800	DD-carboxypeptidase
PBP6	*dacC*	19	40.4	670	DD-carboxypeptidase
PBP7 PBP8	? ?	? ?	32 29	? ?	? ?

during division and to fall in FtsZ blocked filaments (Mirelman *et al.*, 1977). However its has also been reported that DD-Cpase activity remains constant throughout the cell cycle and that it is LD-Cpase activity that increases during division (Beck & Park, 1977).

1.4.6 PBP7 & PBP8.

PBP7 and PBP8 were first reported as occasionally found proteins' (Spratt, 1977), appearing only in exponential phase cultures. Peptide mapping established PBP7 to be a distinct protein (Tuomonen & Schwartz, 1987), and recent work has suggested PBP8 to be a breakdown product of PBP7 (T.Henderson *pers. comm.*). Its function remains to be fully elucidated, but preliminary work suggests that it may be a specialised peptidase (T.Romeis *pers. comm.*). The ability of Imipenem and other carbapenem antibiotics to lyse non-growing bacteria has been correlated with their high specificity for PBP7 (Tuomanen & Schwartz, 1987), but little else is known about PBP7 and PBP8.

1.5 Penicillin-insensitive Enzymes.

Several penicillin-insensitive enzymes involved in synthesis and hydrolysis of the murein sacculus have been identified. These include a number of the autolysins already described, such as MepA (Keck *et al.*, 1990), and the LD-transpeptidase (Ursinus *et al.*, 1992). It has also been inferred from the existence of LD-peptide cross-bridges that an LD-transpeptidase and LD-endopeptidase exist (Holtje & Tuomonen, 1991). This LD-transpeptidase may be the penicillin-insensitive transpeptidase reported in a PBP4 deletion strain by Glauner (1986). A penicillin-insensitive glycan polymerase incapable of cross-linking adjacent peptide strands has also been described (Hara & Suzuki, 1984).

It can only be speculated whether these penicillin-insensitive enzymes have discrete roles in the growth of the sacculus or if they offer a safety mechanism for penicillin-sensitive enzymes. However a discrete period of Penicillin Insensitive Peptidoglycan Synthesis (PIPs) during the cell cycle has been observed (Nanninga, 1991). This occurs after the formation of an FtsZ ring and prior to the action of FtsA or PBP3.

1.6 Regulation of Division Proteins.

The regulation of cell division proteins is just begining to be elucidated. Examples of transcriptional, translational and functional regulation exist. Most studies have concerned the cell division genes of the *mra* operon (Figure:1.1), and particularly *ftsZ*.

The *mra* operon of genes coding for peptidoglycan synthesising and cell division proteins consists of 16 open reading frames (ORFs), from which 14 protein products have been identified (Donachie, 1993). The ORFs share the same transcriptional orientation and are separated by either a few bases or overlap. Several promoters have been identified but only one transcriptional terminator (Beall & Lutkenhaus, 1987). This terminator defines the end of the operon (Figure: 1.1).

Seven distinct promoters capable of transcribing *ftsZ* have been located between *ddlB* and *envA* . The strength of these promoters varies and they have been proposed to modulate *ftsZ* expression in response to growth rate (Dewar *et al.*, 1989). In particular, expression of *ftsZ* from the promoters located in *ftsA* is inversely proportional to growth rate. Similarly transcription from the promoters in *ddlB* increases as growth rate falls (Smith *et al.*, 1992). One of the promoters in *ddlB* has a gearbox consensus which is characteristic of several other genes that respond inversely to growth rate (Aldea *et al.*, 1990)

As growth rate falls, cell volume decreases because of an increased rate of cell division per unit mass. It has been suggested growth rate dependent promoters in the *mra* operon are required to increase the rate of transcription of cell division division proteins during this process (Donachie *et al.*,1984). Consequently the amount of division proteins per unit mass will increase to enable increased division. However the actual amounts of FtsA and FtsZ have been reported to remain at a constant concentration which is independent of growth rate (*ie.* does not alter with respect to unit mass) (Wang & Gayda, 1992; Bi & Lutkenhaus, 1993). Therefore it is not clear whether protein levels are controlled by another mechanism (Donachie, 1993) or if the growth rate dependency of promoters is not significant. Expression of *ftsZ* from one of the promoters internal to *ddlB* has been demonstrated to be positively regulated by another factor; the *sdiA* gene product (Wang *et al.*, 1990). Like growth rate regulation it is not clear what the significance of *sdi* regulation is because *sdi* can be deleted without any obvious effect.

Normal expression of *ftsZ* appears to require a far upstream region of the *mra* operon (Bi & Lutkenhaus, 1991), possibly resulting in the production of a single 18 kD transcript of the entire *mra* operon (Hara & Park, 1993). The regulation

of the levels of FtsQ (25 molecules/cell), FtsA (50 molecules/cell), and FtsZ (20,000 molecules/cell) from a single transcript is determined by differential translation (Mukherjee & Donachie, 1990). Translational control also appears to be responsible for the low levels of PBP3 and FtsW produced in vivo (Khattar & Roberts, *pers. comm.*).

Like *ftsZ*, *ftsI* (PBP3) also requires the production of a long transcript for full expression (Hara & Park, 1993). Transcription of *ftsI* is repressed by the 37 kD protein MreB (Doi *et al.*, 1988). *mreB* forms part of an operon (located at 71 minutes on the *E.coli.* chromosome) which includes three genes *mreB*, *mreC* and *mreD* (Doi *et al.*, 1988). While MreB is responsible for negative regulation of PBP3 (Wachi & Matsuhashi, 1989), a deletion covering all three *mre* genes produces increased levels of PBP1B and PBP3. It is not known how these proteins regulate PBP levels. It is known that MreB is a cytoplasmic protein that shares homology with FtsA (Matsuhashi *et al.*, 1990) and shows structural similarity to actin (Bork *et al.*, 1992). FtsA has been suggested to have a regulatory role in division (Tormo *et al* 1986; Donachie *et al.*, 1984). However it is not clear whether this involves transcriptional control or physical interactions with FtsZ (Dewar *et al.*, 1992; Lutkenhaus *et al.*, 1992). A physical interraction has been favoured by some authors, due to the identification of an ATPase binding domain and a domain that could interact with other proteins (Pla *et al.*, 1993).

On the functional level, FtsZ assembly can be inhibited by interaction with the SOS inhibitor SulA or the MinCD proteins (Bi & Lutkenhaus, 1990). FtsZ also requires activation before exhibiting the GTPase activity that is assumed to be required for self-assembly. This activation can be achieved by K^+ or high concentrations of FtsZ (de Boer *et al.*, 1992; Mukherjee *et al.*, 1992). It remains unclear whether ionic changes or FtsZ concentration are responsible for assembly of the FtsZ ring in vivo.

How the septum specific activity of PBP3 is regulated is also not known. It has been reported that levels of PBP3 remain constant throughout the cell cycle (Wientjes *et al.*, 1983) and PBP3 does not become enriched in minicell membranes (Buchanan *et al.*, 1981). It has also been demonstrated that the normal C-terminal processing of PBP3 by the *prc* product is not essential for its function (Hara *et al.*, 1991). Some experimenters have suggested that the supply of specific murein pre-cursors may regulate PBP3 activity (Markaewitz *et al.*, 1982; Pisabarro *et al.*, 1986). PBP3 has also been suggested to interact with FtsA (Tormo *et al.*, 1986) and FtsZ (Ayala *et al.*, 1988) possibly forming a functional complex

(Nanninga, 1991; Guzmann *et al.*, 1992; Pla *et al.*, 1993). PBP3 may therefore be regulated by the assembly and activity of other members of such a complex.

The regulation and functioning of the other known cell division proteins is not well understood. However it is apparent from the phenotypes of division mutants that completion of one stage in septum formation is dependent on completion of the last (Begg & Donachie, 1985; Taschner *et al.*, 1988). In this sense the first event in septum formation, the assembly of the FtsZ ring, can be said to regulate the subsequent steps. Consistent with this FtsZ remains the only division protein that when overproduced can increase the number of division events per cell (Ward & Lutkenhaus, 1985).

1.7 Autolysis.

Bacterial autolysis is the result of murein degradation by endogenous autolysins. It can be induced by a number of mechanisms (Holtje & Tuomonen, 1991), including inhibitors of murein synthesis and chaotropic agents (Hartman *et al.*, 1974). The bacteriolytic effect of penicillin occurs in two stages (Tomasz, 1979). The first is the binding of penicillin and inhibition of the murein synthetases (Blumberg & Strominger, 1974). The second is the induction of autolysins and subsequent lysis of the cell wall (Tomasz, 1979; Pisabarro *et al.*, 1990). The rate of autolysis varies in direct proportion to growth rate (Tuomonen *et al.*, 1986). Non-growing bacteria are resistant to the majority of β-lactams, a phenomena called phenotypic tolerance (Stockman *et al.*, 1984). Studies with phenotypically tolerant amino-acid starved cells have established that penicillin-binding and induction of autolysis to be inhibited by the stringent response (Pisabarrow *et al.*, 1990). The stringent response is mediated by the *relA* gene product, and its induction shuts down murein synthesis by stopping phospholipid synthesis (Ishiguro, 1983). Consequently *rel*$^-$ amino-acid starved strains are sensitive to β-lactam antibiotics (Ishiguro, 1993).

Induction of autolysis also varies according to the specific PBPs that are inhibited (Spratt, 1983; Kitano & Tomasz, 1979). Inhibition of PBP1A and PBP1B produces a strong autolytic response (Kitano & Tomasz, 1979) and rapid cell lysis (Spratt, 1975). Inhibition of PBP2 produces spherical cells (Park & Burman, 1973) that are blocked in division (James *et al.*, 1975), but its inhibition causes poor induction of autolysis (Kitano & Tomasz, 1979). Inhibition of PBP3 blocks division and produces an intermediate level of autolysis (Kitano & Tomasz, 1979). Most β-

lactams produce autolysis by inhibition of a combination of HMW synthetic PBPs (Spratt, 1983). The resulting autolysis involves the action of lytic transglycosylases and endopeptidases (Kitano *et al.*, 1986; Romeis *et al.*, 1991). Experimental evidence has suggested PBP4 to be at least partially responsible for the endopeptidase activity during autolysis (Iwaya & Strominger, 1977; Kitano *et al.*, 1980. Kitano *et al.* 1979; Cozens *et al.*, 1989). DD-endopeptidase and lytic transglycosylase activity have also been implicated in division (Hakenbeck & Messer, 1977; Templin *et al.*, 1992). The normal role of autolytic activity during division is presumed to be reshaping of the sacculus and septum splitting (Kohlrausch & Holtje, 1991; Holtje, 1993).

The involvement of the same autolysins in cell division and β-lactam-induced autolysis has been suggested because of the appearence of isolated sacculi from penicillin treated cells (Schwarz *et al.*, 1969). These sacculi had cuts which were localised to the cell centre and ran perpendicular to the long axis of the cell. It has also been observed that inhibition of PBP2 and PBP3 produces swelling at potential division sites (Botta & Buffa, 1981), and that PBP1A/1B induced autolysis is co-incident with division (Nanninga *et al* ., 1990; de Portillo *et al.*, 1989). It would therefore appear that at least some β-lactams induce autolysis by disturbing the synthetic and hydrolytic enzymes involved in construction of the septum.

1.8 An Outline of Division.

The study of Fts mutants, PBPs, murein hydrolases and the structure of the murein sacculus has provided an insight into the synthesis of the lateral cell wall and construction of a septum. A brief outline of how the cell wall grows and forms a septum can be constructed from our present knowledge.

Growth begins with recovery of cells from stationary phase. The initiation of elongation and continued extension of the cell wall requires PBP2 (de la Rosa, 1985;. Pisabarro, 1985). PBP2 has been estimated to be responsible for 50% of total murein synthesis (Park & Burman 1973; Botta & Buffa, 1981), and is proposed to form a functional complex with RodA (Matsuhashi *et al.*, 1990). Synthesis of a lateral cell wall involves the insertion of single stranded peptidoglycan (de Jonge, 1989) at multiple sites (Wientjes & Nanninga, 1989). This process has been proposed to involve the formation of peptidoglycan primers by PBP2 that are then extended by the action of PBP1A and PBP1B (Nanninga *et al.*, 1990).

After doubling in cell length, the earliest stage in division is defined by the formation of an FtsZ ring (Bi & Lutkenhaus, 1991). FtsZ resembles eukaryotic cytokinetic proteins and is proposed to be contractile (Lutkenhaus, 1993) pulling in the cytoplasmic membrane as it contracts (Donachie, 1993). This mechanism would be responsible for the ability of L-forms that lack a rigid cell wall to divide.

The formation of the FtsZ ring is followed by a brief period of PIPs (Nanninga *et al.*, 1990; Nanninga, 1991). During this stage and throughout constriction, double stranded peptidoglycan is incorporated preferentially at the leading edge of constriction (Wientjes & Nanninga, 1989; de Jonge, 1989). The next stage in division is defined by FtsQ, PBP3 and FtsL. Inactivation of any of these proteins produces smooth filaments with occasional constrictions. FtsQ, PBP3 and FtsL share similar structures, are present in approximately 50 copies per cell, and are all located within the same operon. They have been suggested to form a functional complex (Guzman *et al.*, 1992) responsible for septal peptidoglycan synthesis.

FtsA has been suggested to act after FtsQ and PBP3 (Begg & Donachie, 1985). FtsA has also been implicated in the activity of PBP3 by the isolation of a temperature sensitive *ftsA* allele that alters the affinity of PBP3 for penicillin (Tormo *et al.*, 1986). More recently a new Fts protein, FtsN, has been demonstrated to extragenically suppress mutations in both *ftsA* and *ftsI* (Dai *et al.*, 1993). In the this outline it would be proposed that FtsA acts after the initiation of PBP3/FtsQ/FtsL peptidoglycan synthesis, but is required for continued PBP3 activity.

The two final stages in division are defined by FtsK and EnvA. FtsK inactivation arrests septation at a late stage characterised by filaments with deep invaginations (K.Begg, *pers.comm.*). EnvA inactivation produces chains of cells incapable of completing the final stage of septation, the splitting of two completed septa.

Prior to division, murein hydrolases (Goodell & Schwarz, 1977; Hackenbaeck & Messer, 1977; Beck & Park, 1977; Karibean *et al.*, 1981.) and phospholipid synthesis have been reported to increase (Carly & Ingram, 1981; Pierucci, 1979). Ultimately the result of all these co-ordinated proteins is the formation of a triple layered (Labischinski *et al.*, 1991) hemispherical cell wall at the pole. This septal murein has been reported to be more cross-linked (Olijhoek *et al.*, 1982), at least temporarily (de Jonge, 1989), and to consist of shorter glycan chains (Romeis *et al.* 1991).

CHAPTER 2

MATERIALS & METHODS

2.0 Bacterial Strains.

Table:2.1 Bacterial Strains.

Strain	Genotype	Source/Reference
AB2497	F-, *thr*-1 *leu*-6 *lac*Y1 *gal*K2 *ara*-14 *xyl*-5 *mtl*-1 *pro*A2 *his*-4 *arg*E3 *rps*L-31 *tsx*-33 *sup*E37 *thy*A12 *thy*R14.	Laboratory Stock.
Toe23	Like AB2497, but *ftsI*23 (Ts)	K. Begg.
Toe23b	Like Toe23, but *leu*::Tn*10*	K. Begg
Toe23c	Like Toe23, but *leu*::Tn*9*	K. Begg
KJB1	Like Toe23, but *rodA*sui.	
AT1325	*lip*9*thi*-1 *his*-4 *pur*B15 *pro*A2 *mtl*-1 *xyl*-5 *gal*K2 *lac*Y1 *lip*-9 *rps*l35 *sup*E44.	Laboratory Stock
Ken222	Like AT1325, but *rodA*sui *lip*$^{+}$.	Begg *et al.*, 1986.
W3110	F-, inversion *rrnD-rrnE*, *sup*^{o} *Lmb-*	Laboratory Stock
W4	Like W3110, but *ilv*::Tn*5*	This Work.
W5	Like W3110, but *cya*$^{-}$	This Work.
W6	Like W3110, but *cya*$^{-}$ *lip*::Tn*5*	This Work.
DE1	Like W3110, but *cya*$^{-}$ *rodA*sui	This Work.
A4	Like AB2497, but Δ*dacB*::Spec/Strep.	This Work
A5	Like AB2497, but Δ*dacA* ::Km^{R}.	This Work.
A45	Like AB2497, but Δ*dacB*::Spec/Strep Δ*dacA* ::Km^{R}.	This Work.
SP1070	*his supF* Δ*dacA*::Km^{R} *srl*::Tn*10* (Tc^{S}).	B.Spratt & Broome-Smith 1985.

Strain	Genotype	Source/Reference
D456	Like Sp1070, but Δ*dacB*::Spec/Strep	This Work.
ED3184	*his supF*	T.Henderson (Broome-Smith 1985.)
E6c	Like ED3184, but *leu*⁻::Tn9 Δ*dacC*	This Work.
D46	Like E6c, but Δ*dacB*::Spec/Strep.	This Work.
T4	Like A4, but *leu*::Tn*10 ftsI*23	This Work.
T5	Like A5, but *leu*::Tn*10 ftsI*23	This Work.
T45	Like A45, but *leu*::Tn*10 ftsI*23	This Work.
T456	Like D456, but *leu*::Tn*10 ftsI*23	This Work.
K33	Like E6c, but *leu*::Tn*10 ftsI*23	Ken Begg.
T56	Like SP1070, but *leu*::Tn*10 ftsI*23	This Work.
T46	Like D46,but *leu*::Tn*10 ftsI*23	This Work
RH90	F-, Δ(*arg-lac*) *U169* *araD*139 *rpsL*150 *pts,F*25 *flbB*5301 *rpsR deo*C *relA*1 *rpoS*359::Tn*10.*	Langge *et al.*, 1993.
ABrs	Like AB2497, but *rpoS*359::Tn*10.*	This Work.
Trs	Like Toe23, but *rpoS*359::Tn*10.*	This Work.
NM621	Like C600, but *leu pro* B1 *hsdR mcrA*⁻ *mcrB*⁺ *recD*(1009) *supE tsx tonA*	N.Murray. Whitaker *et al.*, 1988.
DL307	F-, *recD*	D.Leach.
CA8306	HfrH *thi*, Δcya854	K.Begg.
AT1325	*thi his purB pro lip*9 Tn5	K.Begg.

2.0.1 Plasmids and Phage.

Table:2.2 Plasmids

Plasmid	Description	Source/Reference
pBS47	Km^r, carrying the *pbpA* gene derivative of pSC105.	B.Spratt
pBS59	Km^r, carrying the *dacA* gene derivative of pSC105.	B.Spratt
pBS110	Ap^r, carrying the *dacC* gene derivative of pSC105.	B.Spratt
pBK18-1	Ap^r, 1.8 kb *SmaI-EcoRI* fragment carrying *dacB* inserted into pUC18.	W.Keck
pHP45Ω	Ap^r, 2.3 kb plasmid carrying Ω fragment (Spc^r/Sm^r).	Prentki & Krisch, 1984
pBK4Ω	pBK18-1 with 2 kb Ω fragment inserted in the *dacB* gene.	This Work
pACYC184	Tc^r, Cm^r, 4.244 kb low copy number cloning vestor	Laboratory Stock.
pAX607	pACYC184 with a 9 kb chromosomal insert from 67 minute region cloned into the *BamHI* site (Tc^S).	T.Ogura (Hiraga *et al.*, 1989)
pSUF	pACYC184 with a 2.1 kb *BamHI-ScaI* insert carrying *SufI* cloned between *BamHI* and *NruI* . Tc^S	This Work.
pJF118EH	Am^r, cloning/expression vector *lac*Iq, tac promoter.	Furste *et al.*, 1986.

2.0.2 Phage Stocks.

Table:2.3 Phage

Bacteriophage	Description	Source/Reference
P1	Wild type transducing phage	Laboratory Stock.
λ cIts857		N.Murray.
λ cIts857 *E*am1009	Like λ cIts857, but amber mutation 1009 in gene *E*.	Katsura 1986.
λ cIts857 *E*am1010	Like λ cIts857, but amber mutation . 1010 in gene *E*	Katsura 1986
λ cIts857 *E*am1012	Like λ cIts857, but amber.mutation 1012 in gene *E*.	Katsura 1986
λ2001	General cloning vector, used in the construction of the Kohara library.	Kohara phage Collection.
λ Kohara	As 2001, but *red*$^-$ *gam*$^-$ due to the presence of 20 kb insert.	Kohara phage Collection.

2.1 Chemicals and Enzymes.

All chemicals used were of laboratory reagent grade or better. Unless indicated otherwise they were purchased from the following suppliers.

BDH Ltd., Poole. Dorset.
Boehringer Corporation (London) Ltd. Lewes. Sussex.
Fisons PLC., Loughborough. Leicestershire.
Sigma Chemical Company Poole Dorset.

Enzymes for DNA manipulation were obtained from;

Amersham International PLC., Amersham. Buckinghamshire.
Boehringer Corporation (London) Ltd. Lewes. Sussex.
IBI Ltd., Cambridge.
Pharmacia LKB Biotechnology. Milton Keynes. Buckhamshire.

Cronex photographic film (Dupont) was routinely used for autoradiography. Oligonucleotides were synthesised on an Applied Biosystems DNA synthesiser, by the Oswel DNA service at Edinburgh University. Protein Sequencing was carried out on an Applied Biosystems 477A Protein Sequencer by WELMET at Edinburgh University, and the SERC Sequencing Centre at Aberdeen University.

Murein Analysis and DD-Cpase assays were performed in Joachim Holtje's Laboratory at the Max-Plank Institute for Entwickslungbiologie in Tubingen. The separation of muropeptides was performed by reversed phase chromatography of borohydride-reduced compounds on a ODS (C18) column (Glauner, 1988). This system uses sodium phosphate as a buffer and methanol as an organic modifier. Quantitative analysis of murein composition is achieved using a linear gradient from 50 mM sodium phosphate, pH 4.31, to 75 mM sodium phosphate, pH 4.95, containing 15% methanol. HPLC is performed for 135 minutes with a flow rate of 0.5 ml/minute and column temperature of 55°C. The column used was a 250 x 4.6mm 3.µm Hypersil ODS column (Bischoff, Leonberg, Germany). Separated muropeptides are identified and quantified using UV detection at 205 nm.

The HPLC Equipment includes a Waters gradient HPLC system including, Model 660 solvent programmer, two Model 6000 A pumps with rinseable pump heads and a Model 710B automatic injection unit. Muropeptides are detected by a Model 450 UV detector, and quantified using a Model 730 data module. The loading and running of samples were performed by S.Kormer,T.Romeis or A.Ursinus.

Radioactive isotopes including [phenyl-4(n)-^{3}H] BenzylPenicillin, [α^{32}P] dCTP, and [gamma-^{32}P] ATP (3000 Ci/mM), were purchased from Amersham. Kohara "Genome Mapping Membranes" were obtained from the Takarra (Biomedicals) Shuzo Co. Ltd. (Kyoto, Japan). Scanning densitometry of PBP gels was performed at SmithKline Beecham using a LKB Ultrascan (model 2202) Laser Densitometer by G.Hill.

2.1.1 Media and Stock Solutions.

Dried media was obtained from Oxoid Ltd. Basingstoke. Hampshire. and from Difco Laboratories. Michigan USA.

L-Broth (LB):	Difco Bacto Tryptone	10 g
	Difco Bacto Yeast Extract	10 g
	NaCl	5 g

	pH to 7.2 with NaOH Made up to 1 litre with distilled water.	
L-Broth Agar:	L-Broth Agar, 15 g Difco Agar Made up to 1 litre with distilled water.	
L-Broth Top-Agar:	L-Broth, 6.5 g Difco Agar Made up to 1 litre with distilled water.	
Nutrient Broth (NB):	Oxoid No.2 Nutrient Broth Distilled water added up to 1 litre	25 g
NB Agar:	Nutrient Broth, Davis NZ Agar . Made up to 1 litre with distilled water.	 12.5 g
VB Minimal Media:	20x VB Salts 20% Carbon Source Supplements as required Distilled water added up to 1 litre.	25 ml 5 ml
VB Minimal Agar:	VB Minimal Media, Difco Agar. Made up to 1 litre with distilled water.	 15 g
MacConkey Agar:	MacConkey Base 20% Carbon Source Made up to 1 litre with distilled water.	 50 ml

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
	Made up to 1 litre with distilled water.	
Phage Buffer:	Na_2HPO_4	7 g
	KH_2PO_4	3 g
	NaCl	4 g
	$MgSO_4$ (0.1M)	10 ml
	$CaCl_2$ (0.1M)	10 ml
	1% gelatin solution	1 ml
	Made up to 1 litre with distilled water.	
Bacterial Buffer:	KH_2PO_4	3 g
	Na_2HPO_4	7 g
	NaCl	4 g
	$MgSO_4.\&H_2O$	2 g
	Made up to 1 litre with distilled water.	

2.1.2 Other Stock Solutions.

10x TBE:	Tris base	108 g
	Boric acid	55 g
	Na_2EDTA	9.3 g
	Water added to 1 litre. (pH 8.2)	
TE:	Tris	10 mM
	EDTA	1 mM
Agarose Gel Loading Buffer:	10x TBE.	10 ml
	Ficoll	3 g
	Bromaphenol blue	10% w/v

Phenol.	Phenol (Analr)	250 g
	1M Tris-HCl pH7.8	110 ml
	m-Cresol	14 ml
	β-Mercaptoethanol	0.5 ml
	8-Hydroxyquinoline	0.28 g
	Water	14 ml.

Phenol was mixed for 30 minutes then stored at -20°C. Before use the upper aqueous layer was removed and the remaining solution was equilibrated with an equal volume of TE. This mixture could then be stored at 4°C for up to a month.

20x SSC:	3M NaCl
	0.3M Trisodium Citrate.
	adjusted to pH 7 with NaOH.

Cell Fixing Solution:	Phosphate peptone buffer	80ml
	Formaldehyde	20 ml

This solution is filtered through a Millipore 0.45 μm filter to remove any particles that may interfere with Coulter count analysis.

2.1.3 Stock Solutions of Antibiotics.

Table: 2.3 Antibiotic Solutions

	Solvent	Sterilisation	Stock Solution	Final Concentration
Ampicillin.	H_2O	F	100 mg/ml	50 μg/ml
Chloramphenicol	Ethanol.	F	20 mg/ml	20 μg/ml
Kanamycin Sulphate.	H_2O	F	25 mg/ml	50 μg/ml
Spectinomycin.	H_2O	F	50 mg/ml	25 μg/ml
Streptomycin.	H_2O	F	100 mg/ml	200 μg/ml

All stock antibiotic solutions were stored at -20°C, and the final concentrations were sometimes varied depending upon specific experimental requirements.

2.1.4 Minimal Media Supplements.

Table: 2.4 Amino Acids and Media Supplements.

	Solvent.	Stock Conc. mg/ml	Sterilisation	Final Conc. µg/ml
Amino Acids				
DL-Alanine HCl	H_2O	10	Autoclaved	100
L-Arginine	H_2O	2	Autoclaved	20
L-Asparagine	H_2O	10	Autoclaved	100
L-Aspartic acid	H_2O	10	Autoclaved	100
L-Cysteine HCl	H_2O	2	Autoclaved	20
L-Glutamic acid	H_2O	10	Autoclaved	100
L-Glutamine	H_2O	10	Autoclaved	100
Glycine	H_2O	10	Autoclaved	100
L-Histidine HCl	H_2O	2	Autoclaved	20
L-Isoleucine	H_2O	2	Autoclaved	20
L-Leucine	H_2O	2	Autoclaved	20
L-Lysine HCl	H_2O	10	Autoclaved	100
DL-Methionine	H_2O	2	Autoclaved	20
L-Phenylalanine	1mM NaOH	2	Autoclaved	20
L-Proline	H_2O	3	Autoclaved	30
DL-Serine	H_2O	10	Autoclaved	100
DL-Threonine	H_2O	10	Autoclaved	100
L-Tryptophan	H_2O	2	Autoclaved	20
L-Tyrosine	10mM NaOH	2	Autoclaved	100
DL-Valine	H_2O	4	Autoclaved	40
L-Isoleucine/D-Valine	H_2O	2/4	Autoclaved	20/40
Casamino acids	H_2O	100	Autoclaved	5000
Purine/Pyrimidines				
Adenine	30mM HCl	2	Autoclaved	20
Thymine	H_2O	2	Autoclaved	40
Uracil	H_2O	2	Autoclaved	20
Vitamins				
Biocin	H_2O	0.1	Filtered	0.5
Nicotinic Acid	H_2O	0.5	Filtered	1
Thiamine HCl (B1)	H_2O	1	Filtered	2

2.2 General Bacterial Techniques.

Bacteria were cultured in the media defined in section 2.1.1. Routinely, a fresh overnight culture was inoculated into sterile glassware containing the appropriate media, and the strain incubated on a shaker in the 37°C room or a water bath shaker set to the required temperature. Growth was monitered using a Perkin-Elmer spectrophotometer set to read the OD at 540 or 560 nm. For short periods of time cells were stored on plates or as liquid cultures in screw cap bottles. For longer storage cells were stored frozen as TSS prepared competent cells.

Glassware and media for bacteriological work and DNA manipulation was sterilised by autoclaving in the departmental media room.

2.2.1 Minimum Inhibitory Concentrations.

The MICs determined in this thesis were carried out by Smith Kline and Beecham using their standard procedures. Two replicates were performed and the results expressed represent the lowest concentration of antibiotic (µg/ml) to inhibit growth in liquid media. Fresh overnight cultures were diluted 1:250 in 50 µl aliquots of L-broth and pipetted into a microtitre plate. For each strain 12 wells were inoculated. A double dilution series of the specific antibiotic was prepared, and 50 µl of each dilution administered seperately to wells 1 to 11; well 12 acted as a control. The microtitre plate was then incubated for 18 hours at 37°C and the wells examined for growth.

2.2.2 Sizing and Counting of Bacterial Cells.

A Coulter counter 2B (Coulter electronics) and Coulter channelyser model C-1000 were used for determining the size and number of bacterial cells. Cutures were grown in L-broth filtered through a millipore 0.45 µm filter. Routinely a sample of 100 µl was taken and fixed by mixing with an equal volume of fixing solution (Bacterial buffer 80%, Formaldehyde 20%). The fixing solution had also been filtered to remove any particles that would interfere with sizing. The sample could then be stored indefinitely in a sealed tube. For sizing the sample was first diluted with 1.8 ml of a filtered buffer (0.85% NaCl, 0.08% Sodium Azide) vortexed and then dispensed into a glass vile. The electrode was lowered into the solution and the sample read. The channelizer enables the cells to be placed into separate channels according to their size.

From this distrubution of different sizes the mode and median cell size of a culture could be determined.

2.2.3 Transformation.

Competent cells of *E.coli* were prepared by the method of Chung *et al.* (1989). A fresh overnight culture grown under appropriate selection was diluted into 25 ml of L-both and grown to an OD_{540} of between 0.3 and 0.4. The culture was then harvested in a sterile universal bottle and centrifuged at 4000 rpm for 10 minutes. The supernatant was discarded and the cells resuspended in one tenth their original volume of ice cold TSS buffer. Cells could either be stored in frozen aliquots at -70°C or used immediatly for transfomation.

Transfomation was carried out by the addition of between 100 and 500 ng of DNA to 100 μl of competent cells. The mixture was then gently mixed and incubated on ice for 30 minutes, after which 0.9 ml of LBG (L-Broth + 20mM glucose) was added and the cells left to express antibiotic resistance at an appropriate temperature for one hour. At the same time an aliquot of competent cells with no DNA was prepared in parallel to provide a control. After expression both the transformation and control cells were plated out onto the appropriate selective media and incubated overnight.

TSS Buffer:	Difco Bacto Tryptone	10 g
	Difco Yeast Extract	5 g
	NaCl	10 g
	PEG3350	100 g
	$MgSO_4$	20 mM
	DMSO	50 ml
	PIPES buffer pH 6.5	10 mM
	Distilled water to 1 litre	

2.2.4 Transformation by Electroporation.

For plasmids that were difficult to transform into a strain by the method of Chung *et al.* (1989), electroporation was used. A 1 ml cuture of the desired strain was grown to an OD_{540} of approximately 0.5. This was then harvested at 4500 rpm (10 minutes) in a bench centrifuge. The pellet was resuspended in 1 ml of sterile distilled H_2O, and then transferred to a 20 ml universal bottle. A further 18 mls of cold sterile distilled H_2O was added, and the suspension was then centrifugated at 4500 rpm for

10 minutes. The pellet was then resuspended in chilled sterile distilled H_2O, and the process of washing begun again. After 3 to 4 washes the pellet was resuspended in 1 ml of a chilled 10% glycerol solution. Cells could either be aliquoted and stored frozen at -70°C, or used directly for electroporation.

For electroporation 150 µl aliquots of cells were chilled on ice. DNA was then added (1-10 µg in approximately 5 µl of TE) and the cells incubated on ice for a further minute. The cells and DNA were then transferred to the bottom of a pre-chilled electroporation cuvette. Electroporation was performed using a Gene Pulser II fitted with a Pulse Controller (Bio Rad Laboratories ltd., Hemel Hempstead Hartfordshire). The 25 µF capacitor was charged to a potential of 2.5 kV and the Pulse Controller set at 100-400 Ω. The cuvette was then placed in the safety chamber, and the cuvette pushed into position between the contacts from the Gene Pulser. The cuvette was pulsed once for a time constant of 4.5 to 5 msecs (field strength 12.5 kV/cm). The cuvette was then removed from the chamber and the cells immediately resuspended in 1 ml of L-broth (supplemented with 5% glucose) using a pasteur pipette. This suspension could then be plated onto the appropriate media.

2.2.5. Chromosomal Transformation with Linear DNA.

Wild type *E.coli* are normally resistant to transformation with linearised DNA due to exonuclease activity. However, *recD* mutants have been demonstrated to be easily transformed with linearised plasmid DNA (Russell *et al.*, 1989). In this work DL304 (*recD*) was transformed with gel purified DNA by the TSS method. The only adaptation was the use of large quantities of DNA. In the experiments carried out in this thesis these quantities were as large as 5-10 µg per aliquot (100 µl) of competent cells.

2.3 Phage Techniques.

2.3.1 P1 Transduction.

The recipient strain was grown in 5 ml of L-broth under appropriate selection. The culture was harvested in late log-phase by centrifugation, and the bacterial pellet resuspended in one tenth of the original volume of L-broth supplemented with 2.5 mM $CaCl_2$. For transduction, 100 µl of the appopriate P1 lysate was added to an Eppendorf tube containing a 100 µl aliquot of resuspended bacteria. Controls containing 100 µl of either resuspended bacteria or P1 lysate were prepared at the

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same time. The transduction and the two controls were incubated at 30°C for 20 minutes. If the selection for transductants was a change in auxotrophy, then 0.4 ml of phage buffer was added to each tube and 0.2 ml aliquots from each tube plated onto appropriate minimal agar plates. When transductants were selected for their antibiotic resistance, 0.8 ml of L-broth was added to each tube, and the tube incubated at 30°C with shaking for 60 minutes. As with auxotrophic markers, 0.2 ml aliquots were plated onto the appropriate media. Plates were then incubated at the appropriate temperature overnight, and transductants routinely streaked to purity on the same media used for selection.

2.3.2 Preparation of P1 Lysates.

The appropriate strain was grown to late log-phase in L-Broth supplemented with 5 mM $CaCl_2$. A 200 µl aliquot of cells was then mixed with approximately 10^6 P1 phage, and the tube incubated at 30°C for 30 minutes. Then 3 ml of pre-warmed LB top agar was added to the mixture, the solution vortexed, and poured onto a LB plate. The plate was then incubated overnight. The P1 was harvested by adding 5 ml phage buffer to the plate and then scraping up the top agar and pouring it into a sterile universal. Chloroform was added to this solution and the universal vortexed. The chloroform and agar were separated from the lysate by centrifugation in a bench top centrifuge (4500 rpm for 10 minutes). The supernatent (lysate) was then collected in a sterile half ounce bottle and two drops of chloroform added. The lysate could then be titred and stored indefinately at 4°C.

2.3.2 Preparation of λ Lysates.

The appropriate bacterial strain was grown to late log-phase in L-broth supplemented with 20 mM $MgSO_4$ and 0.2% maltose. λ phage (approximately 10^6 particles) were then added to a 200 µl aliquot of cells, and the Eppendorf tube incubated at 37°C for 5 minutes. The rest of the procedure followed was identical to the preparation of a P1 lysate with one exception. For λ lysates Phage buffer was used instead of L-broth to harvest the top agar.

2.3.4 Amplification of λ Phage.

Amplification of Kohara phage began by spotting a drop of lysate onto a freshly prepared lawn of NM621. The plate was incubated overnight and the resulting

plaque separated from the rest of the lawn using a sterile scalpel. This agar was transferred to a sterile test tube and vortexed with 1 ml of phage buffer and a single drop of chloroform. The supernatant was then harvested and used to produce a plate lysate.

2.4 Isolation of DNA.

2.4.1 Purification of genomic DNA.

Small scale preparation of genomic DNA was prepared by the method of Redfield and Campbell, (1987). A 1.5 ml overnight culture of the appropriate strain was harvested using the bench centrifuge (4500 rpm for 10 minutes). This was then resuspended in 0.5 ml of TEL buffer. TEL consists of 40 mM Tris (pH 8), 20 mM EDTA, and lysozyme added to a final concentration of 10 mg/ml. The resuspension was then incubated for 30 minutes at 37°C. This mixture was then extracted twice with an equal volume of phenol, and once with an equal volume of chloroform. The aqueous phase was then separated and 15 µl of 5 M NaCl and 1 ml of 100% ethanol added. The DNA was ethanol precipitated overnight at 4°C, then dried and resuspended. This method harvested approximately 50 µg of DNA.

2.4.2 Large Scale Preparation of Plasmid DNA

A fresh overnight culture of the required plasmid carrying strain grown under appropriate selection, was used to inoculate 500 ml of growth media. This was then incubated with vigourous shaking overnight and harvested in polypropylene bottles by centrifugation at 5,000 rpm for 10 minutes in a GSA rotor at 4°C. The supernatant was then discarded and the pellet resuspended in 200 ml of chilled TE, before being pelleted once more at 5,000 rpm.

The supernatant was again discarded and the pellet resuspended in 4 ml of a Tris-sucrose solution before being transferred to a 50 ml polypropylene centrifuge tube. 1 ml of a 20 mg/ml lysozyme solution was than added and the tube left on ice for 5 minutes and mixed frequently by gentle swirling. For DH1 the protocol was adapted and lysozyme digestion carried out at room temperature. Once completed, 1 ml of 0.5 M EDTA (pH 8) was added followed by 0.8 ml of RNaseA solution. The mixture was incubated on ice, or in the case of DH1 at room temperature for 5 minutes. Then 5 ml of two times Triton mix was added, the tube covered with parafilm and the whole mixture inverted several times, and incubated on ice for 10 minutes. Triton lysis mix

consists of 100 mM Tris-HCl (pH8), 125 mM EDTA, and 0.2% (W/V) Triton X-100. The lysate was then centrifuged at 15,000 rpm in a Sorvall SS-34 rotor for 20 minutes at 4°C. This procedure pelleted the cell debris and most of the chromosomal DNA. Then the cleared lysate was carefully collected in a sterile 25 ml measuring cylinder before CsCl and Ethidium Bromide was added for isopycnic centrifugation.

For Sorvall 50Ti crimp seal tubes 17.1 g of CsCl and 0.342 ml of stock Ethidium Bromide solution was added to 23 ml of lysate. This gives a CsCl density of 1.55 g/ml and ethidium bromide concentration of 200 g/ml. Isopycnic centrifugation was performed in a Sorval 50-B ultracentrifuge at 38,000 rpm for 60 minutes with a run temperature of 20°C. After centrifugation the plasmid and chromosomal DNA could be visualised as discrete bands by UV illumination. The denser plasmid DNA formed the lower band. This was carefully removed using a syringe and wide bore needle. The ethidium bromide in the sample was then extracted five to eight times using an equal volume of isobutanol (isobutanol was stored over CsCl staturated TE). The sample could then be dialysed extensively against TE at 4°C.

Plasmid DNA was precipitated by adding 3 M Sodium Acetate to 1/10 of the final volume, and then adding 2 volumes of abolute ethanol. After incubating at 4°C the solution was centrifugated at 15,000 rpm for 15 to 30 minutes. The DNA was then resuspended to the desired final volume in TE. The purity and quantity of DNA could then be checked by determining the optical density of the solution at 260 nm and 280 nm.

2.4.3 Small scale preparation of Plasmid DNA.

Minipreparations of plasmid DNA was performed using a modification of the Brinboim and Dolly alakaline lysis method. Routinely plasmid DNA was extracted from a 5 ml fresh overnight culture grown under the appropriate selection. This culture was centrifugated at 4500 rpm for 10 minutes and the pellet resuspended in 100 μl of TEG (1% glucose, 10 mM EDTA, and 25 mM Tris-HCl (pH8)). Then 200 μl of a solution containing 0.2 M NaOH and 1% SDS was added; the tube inverted several times, and then incubated on ice for 5 minutes. Next 150 μl of 3 M sodium acetate (pH 5) was added, and the solution mixed by vortexing. The Eppendorf tube was incubated on ice for a further 5 minutes, and then centrifuged for 10 minutes in a microcentrifuge. The supernatant was transferred to a fresh Eppendorf tube and extracted with an equal volume of phenol/chloroform (phenol saturated with TE pH 8, added to an equal volume of chloroform). After mixing the phenol/chloroform with the sample and separating the phases by microcentrifugation, the upper aqueous phase

was transferred to a fresh Eppendorf tube and the lower phase discarded. The whole procedure was then repeated using phenol instead of phenol/chloroform. The upper aqueous phase was again transferred to a fresh Eppendorf tube and the DNA could then be precipated by the addition of 2 volumes of absolute ethanol, and centrifugation. The pellet was washed with 70% ethanol and then resuspended in TE containing RNaseA (20 µg/ml).

2.4.4 Preparation of Kohara phage lysates and phage DNA

NM621 was used as the host strain for amplification of all Kohara phages as it carries a *recD* deletion.

A fresh overnight of NM621 was used to inoculate 200 ml of L-broth supplemented with 10 mM of $MgSO_4$, and then incubated at 37°C. The OD_{540} was followed until the cultures density reached approximately 0.5. Then the appropriate phage lysate was added to a multiplicity of infection (m.o) of between 0.1 and 1. The cultures OD was again followed until it began to drop, usually between 90 minutes and 4 hours. At its lowest OD $CHCl_3$ was added, approximately 0.5mls for a 200 ml culture and the flask left to shake for a further 10 minutes. Then 8 g of NaCl was added and left to dissolve. Once any initial precipitate had disappeared DNAse and RNAse was added to a final concentration of 1 ug/ml and the culture left at room temperature for one hour. The solution was then transferred to a 250 ml centrifuge bottle and spun in a Sorvall RC5B:GSA rotor at 10,000 rpm for 10 minutes. The supernatant was decanted into a 500 ml flask which contained 20 g of PEG6000, and the flask was then left in the cold room overnight.

The precipitate formed contained macromolecules and phage debris which was precipitated by centrifugation at 10,000 rpm for 10 minutes. The supernatant was carefully decanted and discarded, any final supernatant was removed with a pasteur pipette. 5 ml of Phage buffer was then added and the pellet then left to shake gently in the cold room until it had totally disaggregated. To ensure complete resuspension the pellet was taken up and expelled from a pasteur pipette several times. It was then centrifuged at 5000 rpm for 10 minutes. The supernatant was transferred to a sterile bottle and now the concentrated lysate could be purified by CsCl gradient centrifugation.

Three solutions of 1.3 g/cc, 1.5 g/cc, and 1.7 g/cc CsCl density were prepared. A step gadient was set up in a 14 ml polypropylene centrifuge tube, by layering 2mls of first the 1.3 g/cc solution and then in turn the 1.5 g/cc, and 1.7 g/cc density onto the bottom of the tube. The phage lysate was then added to the top and the tubes centrifuged at 30,000 rpm for 35 minutes in a MSE 6x14 swinging bucket rotor.

The Phage concentrates as an opaque band near the 1.5 g/cc layer and was collected using a syringe and needle to pierce the side of the tube. The concentrated phage was dialysed against two changes of TE. DNA was then isolated by extracting with an equal volume of phenol three times and precipitating with sodium acetate and ethanol.

2.5 DNA Manipulation.

Except where stated otherwise the methods used for DNA manipulation are modifications of Sambrook *et al.*, 1989.

2.5.1 DNA Quantification.

DNA concentrations were determined from the 260 nm absorption of a suitable dilution of the sample. The 260 nm absorption of a solution containing double stranded DNA at a concentration of 50 µg/ml is known to be 1. Therefore the concentration of DNA in a sample can be calculated. The purity of a DNA solution can be determined from the ratio of its OD at 260 nm and 280 nm. For pure double stranded DNA the $OD_{260/280}$ is close to 1.8.

2.5.2 Restriction of DNA by Endonuclease digestion.

DNA was usually digested with restriction endonucleases for 1 to 2 hours, using a 2 to 10 fold excess of the enzyme and under the conditions recommended by the manufacturer. The final volume of reactions was routinely 10 times the volume of enzyme added (*ie.* 20 µl). Digestion products were either separated by gel elctrophoresis or phenol extracted. They were then ethanol precipated and resuspended for further manipulation.

2.5.3 Agarose Gel Electrophoresis.

For the analysis and purification of restriction products DNA molecules were size separated by electrophoresis in submerged agarose gels. All the DNA electrophoresis was performed using Pharmacia gel elcetrophoresis tanks and 1x TAE buffer. The appropriate amount of agarose (routinely between 0.8% and 1.5%) was added to TAE buffer and the solution heated to boiling point in a microwave oven. The solution was then allowed to cool to approximately 60°C and poured into a casting tray. A plastic comb was inserted at one end of the tray, and the gel left to cool. Samples were added to 5x TAE loading buffer and incubated at 70°C for 5 minutes. Once the gel had set and the comb had been removed the wells could be loaded with sample when dry, or when the tray was submerged beneath TAE in the elcetrophoresis tank. Depending on the size of gel, the percentage of agarose used and the fragments to be separated, the gel would be run for 4-8 hours at a constant current of 15-70 mA.

Once electrophoresis was completed the gel was removed and stained in a solution of 2 µg/ml ethidium bromide for 15 minutes. The gel was then washed in two changes of distilled water for between 30 and 60 minutes. DNA fragments could then be visualised on using a UV transilluminater and if necessary photographed using a Polaroid positive/negative film.

2.5.4 Purification of DNA from Agarose Gels.

DNA was isolated from agarose gels using the Geneclean kit, a product of Bio101 Inc. (La Jolla. California.USA). The agarose containing the band was cut out and dissolved at 55°C in 2.5 times its own volume of a 6 M sodium iodide solution. A suspension of the silica matrix glass milk was added. In the presence of 6 M sodium iodide the DNA attaches to the matrix. The matrix is then precipitated using a microfuge and the pellet washed in New-wash solution (a Tris/NaCl/EDTA/ethanol solution). The solution is then centrifuged again and the washing procedure repeated. A final wash is performed and all the New-wash removed with a pasteur pipette. The pellet was finally resuspended in 5 µl of TE at 50°C, liberating the DNA from the matrix. The matrix was then precipitated by centrifugation, and the supernatant containing the DNA transferred to a fresh Eppendorf tube.

2.5.5 Ligation of DNA.

Ligation reactions usually contained 200–300 ng of dephosphorylated cut vector DNA, and a five fold gene excess of the DNA fragment to be inserted. For cohesive DNA termini 1x Boerhinger Mannheim ligation buffer and 0.2 units of T4 DNA ligase were added to the DNA, in a final volume of 10 μl. For the ligation of blunt ended termini the same procedure was followed using 2 to 5 units of T4 ligase. All ligations were performed overnight at 4°C, and between 2 and 5 μl used to transform the appropriate strain of *E.coli.*

2.5.6 "Filling in" recessed 3'termini.

The filling of recessed termini produced by restriction was perfomed with the Klenow fragment of polymerase 1. Reactions routinely consisted of a final volume of 20 μl containing 1 μg of DNA, 1x Klenow buffer, the appropriate dNTPs at a concentration of approximately 20 μM, and 2 units of Klenow enzyme. Reactions were performed at 16°C for 60 minutes and the reaction stopped by incubating at 70°C for 5 minutes and diluting out the enzyme with 200 μl TE. The reaction mixture was then phenol extracted and the DNA ethanol precipitated. The resulting blunt ended DNA could then be resuspended and used for cloning.

2.5.7 Southern Blotting Procedure.

DNA separated by gel electrophoresis was stained and photographed before being transferred to Hybond-N (Nylon membrane). Bidirectional transfer was then performed by the method of Smith & Summers, (1980).

The first step was to depurinate the gel by soaking it in 2 volumes of 0.25 M HCl for 15 minutes. This was then repeated with fresh HCl for a further 15 minutes. The gel was washed in dH_2O and then the DNA denatured by soaking the gel in 2 volumes of 0.5 M NaOH, 1.5 M NaCl for 15 minutes. Denaturating was completed by repeating this step with fresh solution for a further 15 minutes. The gel was then soaked for 30 minutes in two changes of neutralising solution (1 M ammonium acetate, 10 mM NaOH, pH 8).

A Hybond-N membrane, cut to the appropriate size, was positioned on top of 4 pieces of Whatman 3MM paper saturated with 20x SSC. The gel was then removed and inverted onto this Hybond-N membrane. Any air bubbles between the membrane and gel were then carefully excluded. Then 3-4 sheets of pre-wetted (20x SSC) 3MM

paper, 6 cm of dry paper towels and finally a large glass plate, were all placed on top of the gel. This arrangement was then inverted so another wad of paper towels and glass plate could be added. To maintain an even contact between all the layers, a large weight was then placed on top of this symetrical 'sandwich'. Transfer was typically performed overnight. The membrane was then removed, briefly air dried and the DNA UV-crosslinked.

2.5.8 Hybridisation and Prehybridisation of Membranes.

Solutions;

50x Denhardt's Solution.
1% Ficoll.
1% Polyvinylpyrolidone.
1% Bovine Serum Albumin.

20x SSC (Stock)
3M NaCl
0.3M Trisodium Citrate.
Adjusted to pH 8

Hybridisations were routinely performed using a Tecne hybridisation oven, and hybridisation tubes. During the work contained in this thesis two types of hybridisation were performed. The first involved random-primed probes that matched the target sequence exactly. For these experiments high stringency was used with Formamide added to the hybridisation buffers to lower the melting temperature. The second type of hybridisation involved end labelled degenerate oligos as probes. In these experiments the formamide was excluded from the buffer, and stringency was controlled by the temperature at which the hybridisation was performed.

The general procedure in both types of experiment was the same. The filter was prehybridised in filtered prehybridiation buffer for 30-60 minutes at the desired temperature. The probe was then boiled and added to the prehybridisation buffer. Hybridisation was then performed overnight and then the buffer discarded. In a series of washing steps the filter was immersed in gradually more stringent solutions.

For random-primed probes all the steps were performed at 37°C, and the solutions used were as follows;

Prehybridisation buffer.

20x SSC	1 ml.
Formamide	5 ml
50x Denhardt's Solution	200 μl.
Sheared Salmon testes DNA (10 mg/ml)	250 μl.
10% SDS	500 μl.
dH_2O	3.05 ml.
	Final Volume:10 ml.

The washing procedure for random-primed probes involved two 15 minute washes with a solution consisting of 50% (v:v) formamide, 2x SSC, 0.5% SDS. This procedure was repeated for a solution of 2x SSC, 0.5% SDS. The two final washes were performed for 30 minutes using a 2x SSC solution.

For end-labelled oligos the hybridisation procedure is described in the text of the relevant results chapter. The initial melting temperature (Tm) for oligonucleotides was estimated from the equation;

$Tm = 81.5 - 16.6 (\log_{10} [Na^+]) + 0.41 (G + C \%) - 600/S$

where S = oligo length - number of inosines.

Once hybridisation and washing was completed the filter was sealed in clingfilm and exposed to photogographic film at -70°C.

2.6 Radio-labelling of DNA.

2.6.1 Random-Priming.

Approximately 5-10 μg of plasmid DNA was digested and the fragment to be random-primed separated by gel electrophoresis in low-melting point agarose. The fragment was then visualised by staining with ethidium bromide and the appropriate band cut out of the gel. The gel slice was placed in a plastic tube and dissolved by adding 3 ml of dH_2O and heating the tube in a boiling water bath for 10 minutes. At this stage the DNA could be stored at -20°C indefinately.

After boiling the fragment, the DNA was left to equilibrate at 37°C for 5-10 minutes. Then the reaction mixture was set up as follows;

Oligolabelling buffer (OLB)	10 μl
Bovine Serum Albumin (10mg/ml)	2 μl
$\alpha^{32}P$ at 10 mCi/ml.	5 μl

DNA fragment.up to 32.5 μl.
ddH$_2$O to a final volume of 50 μl.
2 units DNA polymerase I Klenow fragment.

This solution was incubated overnight at room temperature. The reaction was then stopped by adding 200 μl of STOP solution Random-primed probes were separated from unincorporated material using 'Elutip-d' columns (Schleicher & Schuell Dassel Germany) according to the manufacturer's instructions.

OLB was made from the following solutions;
O: 1.25 M Tris-HCl, 0.125 M $MgCl_2$ pH 8.
A: 1 ml solution O and 18 μl β-mercaptoethanol and 5 μl of each of dATP, dTTp, dGTP (nucleotides at 0.1 M in 3 M Tris-HCl, 0.2 mM EDTA, pH 7).
B: 2 M Hepes (pH to 6.6 using NaOH)
C: Hexadeoxyribonucleotides (Pharmacia P-L 2166) (at 90 OD units/ml in TE)

Solutions A:B:C are mixed in the ratio 1:2.5:1.5 to make OLB.

STOP contains 20 mM NaCl, 20 mM Tris-HCl (pH 7.5), 2 mM EDTA, 1 μM dCTP.

2.6.2 End-labelling Oligonucleotides.

The reaction mixture was set up as follows;
10–25 pM of oligonucleotide
2.5 μl of 10x Forward Exchange buffer.
2 μl of [$\gamma^{32}P$] ATP (10 mCi/ml. 3000 Ci/mM)
2 units of T4 Polynucleotide kinase.
dH$_2$O to a final volume of 25 μl.

Forward exchange buffer (10x) consists of 500 mM Tris-HCl (pH 7.5), 100 mM $MgCl_2$, 50 mM DTT. The labelling reaction was allowed to proceed overnight

and stopped by placing the reaction tube in a 70°C water bath for 15 minutes. The probe was then cleaned on a Nic column according to the manufacturers instructions (Pharmacia LKB Biotechnology Upsala Sweden).

2.7 Protein Techniques.

Proteins where usually separated by SDS-polyacrylamide gel electrophoresis using a discontinuous buffer system as originally described by Laemmli (1970). Except were described otherwise all SDS-PAGE was performed with a 4% Stacking gel and 10% Resolving gel. Routinely 11 x 14 cm gels were run on a Hoeffer Scientific Instruments (SE 600) gel apparatus.

Resolving and Stacking gels were prepared fresh at 4°C according to the following recipe;

10% Resolving Gel	
Acrylamide (Stock solution 40%)	6.25 ml.
4x Resolving gel buffer	6.25 ml.
10% SDS	0.25 ml.
7.5% Ammonium persulphate	0.25 ml.
Distilled water	12 ml.
TEMED	15 µl.

4% Stacking Gel.	
Acrylamide (Stock solution 40%)	1.0 ml.
4x Stacking gel buffer	2.5 ml.
10% SDS	0.1 ml.
7.5% Ammonium persulphate	0.1 ml.
Distilled water	6.3 ml.
TEMED	10 µl.

The Ammonium persulphate and TEMED were added last and if necessary the solutions degassed in a side arm flask. The gel apparatus was set up in a casting base, with two 11 x 14 cm glass plates separated by 0.75 cm spacers. The resolving gel was then carefully poured into the sealed space using a pipette. Isobutanol saturated with 1x resolving gel buffer was then layered onto the top of the resolving gel. The gel was then left to polymerise at room temperature. The isobutanol was poured off and the top of the gel briefly washed with dH_2O. The stacking gel was then poured on top of the

resolving gel and a comb carefully inserted. The stacking gel was left to polymerise and then the comb carefully removed.

Unpolymerised material was washed out of the wells using dH_2O expelled from a syringe. Then 1x running buffer was poured into the wells and the samples loaded. The apparatus was set up according to the manufactures instructions, and the buffer reservoirs filled with running buffer. Gels were normally run for between 6 and 16 hours at 20-60 mA constant current. The plates were then removed from the apparatus and then carefully separated. The gel was removed and placed in staining solution for 45 minutes with gentle agitation. Then the gel was transferred to destaining solution, and after several changes of destain individual protein bands became visable. The gel was removed and dried down on blotting paper using a vacuum gel drier at 80°C for one hour.

2.7.1 Preparation of Sample.

Protein samples were mixed with an equal volume of 2x SDS-PAGE loading buffer and briefly boiled. The sample was then briefly centrifuged and loaded using a 50 µl Hamilton syringe.

2.7.2 Standard SDS-PAGE Solutions.

Acrylamide: 37 g Acrylamide, 1 g NN'Methylene bis-acrylamide. Made up to 100 ml with dH_2O, filtered and stored.

4x Stacking Gel Buffer: 15.25 g Tris base, dissolved in 200 ml dH_2O, adjusted to pH 6.8 with HCl and made up to a final volume of 250 ml, then filtered and autoclaved.

4x Resolving Gel Buffer: 45.5 g Tris base, dissolved in 200 ml dH_2O, adjusted to pH 8.8 with HCl and made up to a final volume of 250 ml, then filtered and autoclaved.

10x Running Buffer: 30.2 g of Tris base, 144 g of glycine dissolved in 600 ml of dH_2O, made up to a final volume of 1 litre and filtered. In the final 1x buffer SDS was added to 0.1%.

Staining Solution: 9% (v:v) Acetic acid, 45% (v:v) Methanol and 0.1% (w:v) Coomassie blue.

Destaining Solution: 7% (v:v) Acetic Acid, and 5% (v:v) Methanol.

2x PAGE Loading Buffer: This was made up from 4x Stacking gel buffer (125 μl), 10% SDS (300 μl) 50% glycerol (200 μl), 2-mercaptoethanol (50 μl), 0.1% bromophenol blue (200 μl), up to a final volume of 10 ml with dH_2O (125 μl).

2.8. Protein Digestion and Peptide Isolation.

Prof. J.V. Holtje supplied me with protein samples purified by Norcardicin-A affinity chromatography, and demonstrated to exhibit LD-carboxypepitdase activity. Three samples, of varying purity, were supplied over a period of 12 months.

2.8.1 Protein and Peptide Separation for *N*-terminal sequencing.

Separation of proteins for blotting was carried out by modified SDS-PAGE. The modifications were primarily designed to minimise the chances of N-terminal blockage and included substituting Pierazine di-Acrylamide (PDA) (Bio-Rad. Richmond. California) for Bis-Acrylamide as a cross-linking agent. The substitution was done on a gram for gram basis.

50 nM of glutathione solution was added to the upper reservoir of running buffer, and the gel pre-run for between 1 hour to 2 hours (Mighty Small vertical gel apparatus Hoeffer) at 12 mA constant current. The buffers used during pre-running were then discarded. Samples were loaded and fresh buffer containing 0.1 nM sodium thioglycolate used to fill both buffer reservoirs. The samples were then slowly run into the stacking gel at 7 mA constant power for one hour before operating as normal.

Once run, the gel was removed and prepared for electroblotting.

2.8.2 Electroblotting.

Purified peptides separated by modified SDS-PAGE were electroblotted onto Polyvinylidene diflouride (PVDF) membranes (Millipore). Transfer was performed using a Bio Rad Transfer cell, and a 10mM CAPS, 10% methanol transfer buffer (pH 11). Immediatly after protein separation was complete the SDS-PAGE gel was removed and soaked in transfer buffer for 5 minutes. The PVDF membrane was briefly rinsed in methanol and then stored in transfer buffer. Six pieces of whatman 3MM blotting paper were cut to size (approximately 3 cm bigger than the gel on all sides) and soaked in transfer buffer. Three piece were then layed on one side of the

transfer cassette. The SDS-PAGE gel was placed on top of these three sheets of 3MM paper and then the PVDF membrane placed directly on top of the gel. The final three pieces of blotting paper were placed on the PVDF membrane, and air excluded from this 'sandwich' by carefully rolling a 25 ml pipette over the blotting paper. The transfer cassette was then closed and placed in the transfer cell. The transfer buffer was added and the power pack and cooling system connected. Operating times and voltages varied, and are discussed in detail in the appropriate results chapter.

Once transfer was completed membrane was washed briefly in ddH_2O and then stained with amido-black, air dried and stored at -20°C. Bands required for sequencing were then cut out and could be sequenced directly in an Applied Biosystems Sequenator.

2.8.3 Protein Digests

All proteinases were obtained from Boehringer Mannheim and were of sequencing grade. For digestion with Endoproteinase Lys-C a Tris-HCl (25 mM/l), EDTA (1 mM/l) buffer pH 8.5 was used, and the reaction allowed to proceed overnight at 37°C. For digestion with Endoproteinase Glu-C a ammonium carbonate (25 mM/l) buffer pH 7.8 was used, and the reaction allowed to proceed overnight at 25°C. For Trypsin and Elastase digests a standard protease buffer was used.

Protease Buffer:
20 mM Tris-HCl.
10 mM $MgCl_2$
1 mM EDTA
200 mM NaCl
1 mM $CaCl_2$ (Final buffer pH 8).

Elastase and Trypsin were made fresh for each experiment. A small amount of solid was dissolved in protease buffer and the absorption of the solution at 280 nm determined. A 1% solution of Trypsin has an absorption at 280 nm of 14.3, a 1% solution of Elastase has an absorption at 280 nm of 22 (Carey, 1989). Using these standards the concentration of the stock solution could be determined. For Lys-C and Glu-C aliquots of enzyme at a known concentration were supplied by the manufacturer.

2.9 Preparation of Bacterial Membranes.

A fresh overnight culture was grown under appropriate conditions and used to inoculate between 200 ml and 500 ml of culture, depending on the experiment. The cultures were then transferred to a shaking incubator set to a suitable temperature and the OD_{560} of the culture monitored. For temperature shift experiments a cell culture of 500 ml was grown at 30°C up to an OD_{540} of between 0.1 and 0.2 and then split into two equal aliquots. Half was transferred to a pre-chilled polypropylene centrifuge bottle and stored on ice while the other half was transferred to a sterile flask already shaking at 42°C. To this second flask an equal volume of broth pre-heated to 54°C was slowly added using a funnel and gentle agitation. Once completed the culture was shifted to a 42°C water bath and grown for a further 80 to 100 minutes depending on the strains doubling time. Then the culture was collected in a pre-chilled centrifuge bottle and stored on ice.

For experiments requiring no temperature shift, smaller cultures were grown at 37°C to an OD_{540} of approximately 0.5. For the examination of stationary-phase cells, cultures were collected 9 to 10 hours after the OD had stopped rising.

Once collected the cultures were harvested by centrifugation at 10,000 rpm in a GSA rotor for 20 minutes at 4°C. Cells were then resuspended in 20 ml of phosphate buffer, transferred to a sterile universal, vortexed and precipitated by centrifugation for 10 minutes at 7000 rpm. The pellet was then resuspended in 12 ml of ice cold phosphate buffer, stored on ice and sonicated immediatly.

Sonication was carried out using an MSE sonicater and 10 mm diameter probe. The machine was adjusted to give an output of 6-7 microns and the probe pre-chilled in an ice-bucket. The probe was cleaned prior to use and between samples by brief sonication in sterile distilled water. For each membrane sample the probe was positioned in the middle of the universal and lowered to lie just below surface. The samples were sonicated for 30 seconds, care being taken to avoid bubbles forming, and the sample returned to ice for a minute before the sonication was repeated again. Usually the sample could be seen to clear sufficiently after two sonications, but for concentrated solutions a third sonication was required.

To remove unwanted cell debris and any unbroken cells the samples were transferred to 20 ml corex tubes and centrifuged in an SS-34 rotor at 5000 rpm for 10 minutes. The supernatant was carefully collected and transferred to Beckman 50Ti Sorvall crimp seal tubes. The tubes were then centrifuged at 40000 rpm (15°C) in an ultracentrifuge for 1 hour. The supernatant was discarded and the membrane pellet

carefully resuspended in 50–100 µl of phosphate buffer by extensive vortexing. The membrane preparation was then transferred to an Eppendorf tube, and frozen at -70°C.

2.9.1 Estimation of Protein Concentration.

The concentration of membranes was estimated by the method of Lowry *et al.* (1951). A series of dilutions of the sample were prepared and a set of standards of known protein concentration. The standards routinely used contained between 10–50 µg of Bovine Serum Albumin.

Each sample (approximately 10–20 µl) was added to 2 ml of freshly prepared Solution A, and then incubated at 37°C for 10 minutes. Then 200 µl of a Solution B was added, and the samples incubated at room temperature for a further 30 minutes. The samples OD at 680 nm was then determined, and a curve plotted for the BSA standards. The OD_{680} of the suitable membrane dilution enables the protein concentration of the sample to be estimated from the standard curve.

Solution A:	2% $CuSO_4$	500 µl
	4% Potassium Sodium Tartrate.	500 µl
	2% Na_2CO_3 (disolved in 0.1N NaOH)	49.9 ml.

Solution B: 1:1 Folin Reagent : dH_2O

2.10 Radio-Labelling of Penicillin-binding Proteins.

In these studies PBPs were radio-labelled using Benzyl [^{3}H] penicillin supplied by Amersham. This was supplied as 250 µl aliquots with specific activity of 10-30 Ci/mmol. The penicillin solution was supplied as 3 parts acetonitrile to 7 parts 0.02 M phosphate buffer. The volatile acetonitrile was removed by passing a gentle stream of nitrogen over the solution for 10 minutes. Once this was completed the volume of the solution had usually dropped to aproximatly 175 µl and the concentration of penicillin increased proportionately. Assuming the initial activity had been 20 Ci/mmol and the molecular weight of the free acid to be 334, the 250 µl supplied could be estimated to contain approximately 5 µg of Benzyl [^{3}H] penicillin once the acetonitrile was removed. The following protocol therefore assumes that the stock concentration of Benzyl [^{3}H] penicillin to be 28.6 µg/ml.

Labelling routinely involved 38 µg of membranes at a concentration of 5 mg/ml aliquoted into eppendorfs. To each sample 8 µls of Benzyl [^{3}H] penicillin was

added to give a final concentration of aproximatly 5 µg/ml. The tube was then vortexed and incubated at 30°C for 10 minutes. Then 7.5 µl of Penicillin G solution (20 mg/ml), 6 µls of 10%SDS and 0.5 µl of Phosphate buffer were added. The tube was vortexed again and incubated for a further 20 minutes at room temperature. To precipitate the outer membrane and peptidoglycan the tube was then centrifuged in a microcentrifuge (approximately 12,000 rpm) for 15 minutes. The supernatant was collected and added to SDS-PAGE loading buffer before being boiled for 4 minutes and left to cool. The membrane proteins were then separated by standard SDS-PAGE.

Once SDS-PAGE was completed the gel was stained and destained as normal. After destaining the gel was soaked in the flourographic agent Amplify (Amersham). After treatment for 30 minutes at 37°C the gel was then dried and exposed to Hyperfilm MP (Amersham) at -70°C. The use of hyperfilm and the pre-treatment of the gel with amplify reduced the length of exposure, however on average gels still had to be exposed for 6 to 21 days.

2.11 Preparation of Murein Sacculi

The isolation of bacterial sacculi was carried out at the Max-Planc in Tubingen by a method modified form the one published by Glauner (1988).

Fresh overnight cultures were grown under appropriate selection and diluted 1 to 500 into fresh media. Temperature shift experiments were then performed by the same method described for the isolation of bacterial membranes. However because of the large amounts of material lost during the isolation of sacculi, the initial volume of culture harvested had to be increased. Typically the volumes used were 1000 ml for initial cultures, with 700ml harvested and 300 ml shifted to 42°C. For strains not requiring a temperature shift 500 ml cultures were used, and the cells harvested in mid-log phase.

Harvested cultures were rapidly cooled on ice, transferred to a polyproylene tubes, and centrifuged at 9000 rpm in a GSA rotor. The pelleted cells were resuspended in 10 ml distilled water and then boiled in 8% SDS for 20 minutes. The solution was left to cool before centrifuging at 100,000 rpm using a Beckman TL-100 ultracentrifuge and TLA100.3 rotor. The pellet was carefully and thoroughly resuspended in distilled water using a glass rod and vortexer before being centrifuged in the TL-100 rotor again. The process of washing by thorough resuspenion and centrifugation was repeated between 5 and 8 times until the assay for SDS was negative.

SDS Assay

0.7% Methylene Blue

0.7 M Sodium Phosphate (pH7.2)

Chloroform

335 µl of sample supernatant, or water for the blank was aliquoted into a test tube. To this 170 µl of sodium phosphate solution; 8 µl Methylene Blue and 1 ml of chloroform were added. The tube was then vortexed and the solution examined for a colour change. A clear or pink discolouration indicated that samples were free from SDS, a blue discolouration indicated SDS still contaminated the sample.

Once completely washed the sacculi were resuspended in 1 ml of distilled water and could be digested with α-amylase to remove glycogen. Since all commercially available α–amylase is contaminated with lysozyme a specific buffer has to be used.

α–amylase buffer;

10 mM Tris-HCl

10 mM NaCl

0.32 M Imidasol (adusted to pH 7 with HCl)

To each sample 300 µl of buffer and 5 µl of α-amylase was added and the tube incubted at 37°C for 2 hours. The murein was then incubated with 6 µl of Pronase at 60°C for a further hour and the reaction stopped by boiling in 2% SDS (final concentration) for ten minutes. The sample was left to cool and the process of washing to remove the SDS begun again. At this stage the washing procedure is performed in 1 ml of distilled water and centrifuged using the TL-100.2 rotor and tubes.

Once free of SDS the pellet was resuspended in 0.5 ml of distilled water. Next the murein was digested with Cellosyl (final concentration 20 µg/ml) overnight at 37°C. The solution was then boiled for 10 minutes and left to cool in the cold room before being centrifuged in a microcentrifuge for 15 minutes. The supernatant was decanted and the pellet discarded. Sodium borate was added to the supernatant in small quantities and the sample allowed to reduce for 30 minutes. Excess borohydride was then destroyed by the addition of 20% phosphoric acid (approximately 10 µl).

When the evolution of borohydride was complete, the sample was adjusted to pH 3.5 using phosphoric acid. A sample of 10-100 µl (approximately 10-50 µg) could then be injected into the HPLC column for separation.

2.11.1 Separation of Muropeptides by HPLC.

The preparation and running of the HPLC columns used in this work was performed by Stefan Kormer or Astrid Ursinus. Muropeptides are separated on a 3 μm Hypersil ODS column (250 x 4.6 mm) kept at 55°C in a Pharmacia Column Jacket. The column is equilibrated with buffer A (50 mM sodium phosphate pH 4.310, 0.8 mg/ml sodium azide) at a flow rate of 0.5 ml/min for 40 minutes. The sample is then injected and eluted with a linear gradient starting with buffer A and reaching buffer B (75 mM sodium phosphate pH 4.950, 15% methanol). Separated muropeptides were identified and quantified using UV detection at 205 nm.

2.12 DD-carboxypeptidase assays.

For these assays UDP-muramyl pentapetide purified from *Bacillus cereus*, and the synthetic muropeptide bisacetyl-L-Lys-D-Ala-D-Ala were used as substrate. Between 0.2 and 1 nM of substrate was incubated with isolated membranes for 60 minutes at 37°C in 0.05 of Tris-HCl buffer (5 mM, pH 7.4) containing 0.1 mM dithiothreitol, 0.1% Triton X-100 and 0.02% sodium azide. The reaction is stopped by boiling and then applied to the HPLC column used for muropeptide separation. The products are then eluted with a 50 mM sodium phosphate buffer (pH 6) and UDP-muramyl tetrapeptide (elution time 2.5 minutes) and UDP-muramyl pentapeptide (elution time 4 minutes) quantified by their UV absorbance.

CHAPTER 3

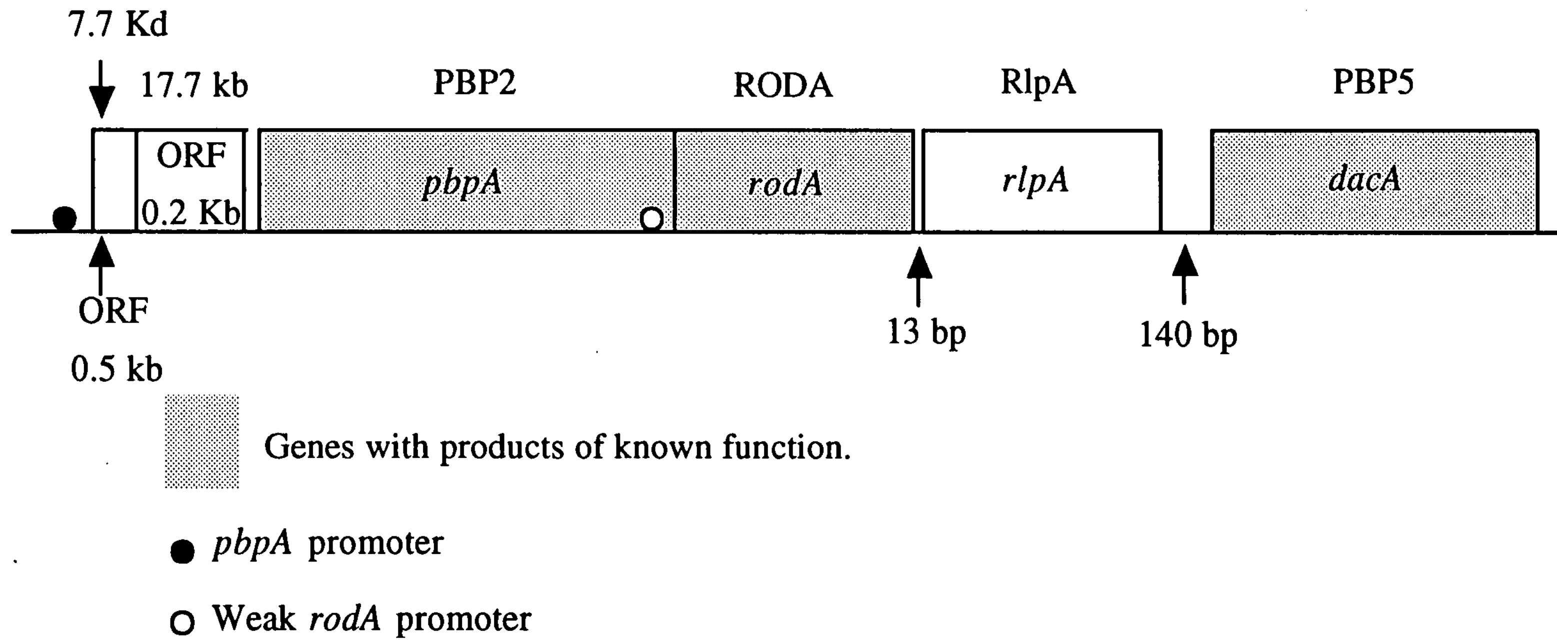
THE STUDY OF KJB1

The study of the prokaryotic cell cycle has been based upon the isolation of temperature-sensitive mutants blocked at various stages of division. Through these studies some of the basic steps have been placed in sequence. These include the initiation of chromosome replication, partitioning of nucleoids, formation of an FtsZ ring, construction of a septum and the final step of splitting two covalently linked cell poles to produce individual daughter cells.

The work contained in this thesis began with the isolation of a temperature-sensitive mutation in septum formation (Begg *et al.*, 1986). This allele, *ftsI*23, encodes a temperature-sensitive form of PBP3; a septum specific peptidoglycan synthetase (Ishino & Matsuhashi, 1981). Selection for suppressors of *ftsI*23 resulted in the isolation of a mutation in the *rodA* gene (*rodA*sui). The *rodA* gene forms part of the 15 minute cluster of peptidoglycan synthesising genes (Figure: 3.1) (Tameki *et al.*, 1980) and the RodA protein is proposed to form a functional complex with PBP2; thus enabling the synthesis of a rod shaped cell wall and allowing cell elongation (Matsuhashi *et al.*, 1990). The suppressed strain KJB1 carried two mutations, *ftsI*23 and *rodA*sui, which affected peptidoglycan synthesis. The *ftsI*23 allele forms long filaments at the restrictive temperature. The *rodA*sui mutation interferes with the formation of a normal lateral cell wall, and forms spherical cells. The initial hypothesis for the suppression of *ftsI*23 by *rodA*sui was that the altered RodA protein was increasing PBP3 activity via a direct interaction (Begg *et al.*, 1986).

Further work determined *rodA*sui to be an amber mutation at Gln111 (Begg *et al.*, 1990). The original parent of KJB1, AB2497, carried a suitable suppresser (*supE*37). Therefore, rather than producing a functionally altered RodA, the *rodA*sui allele was responsible for reduced levels of wild type protein. It had originally been observed that the phenotype of the *rodA*sui mutation was strain dependent. These strains carried different suppressor tRNAs of varrying efficiency. Therefore it was assumed the pressure of different suppressors altered cell shape by changing the level of RodA. KJB1 carried mutations in two separate peptidoglycan synthesising systems, one for elongation of the sacculus the other for division. The levels of RodA could be altered by the presence of different amber suppressors with the result that cell shape would alter from rod to sphere. Instead of a functional interaction, the suppression observed for KJB1 was explained as a balance between lateral (RodA) and septal (PBP3) cell wall synthesis. This balance would be disturbed by reducing the level of RodA and would favour PBP3 cell wall synthesis. The synthetic activity of PBP3, normally responsible for the hemispherical murein of the septum, would then produce ovoid or spherical cells. Therefore in KJB1 suppression of *ftsI*23 would result from reduced lateral cell wall synthesis (RodA) balancing impaired septum

Figure: 3.1. 15 minute (*mrdB*) operon.



synthesis at 42°C. This explanation implied that the two systems competed for a common substrate. When RodA was impaired the increased availability of substrate increased the efficiency of an impaired PBP3.

However the presence of *rodA*sui in AB2497 produced increased levels of PBP2 and PBP5 (B.Spratt *pers.comm.*). Both these proteins are encoded by genes in the proposed *rodA* operon at 15 minutes.

3.1 Experiments with Competing Antibiotics.

In the 'balance hypothesis' PBP2 and PBP3 activity had central roles. Suppression of reduced PBP3 activity by reduced PBP2 activity should therefore be demonstrable with specific inhibitors.

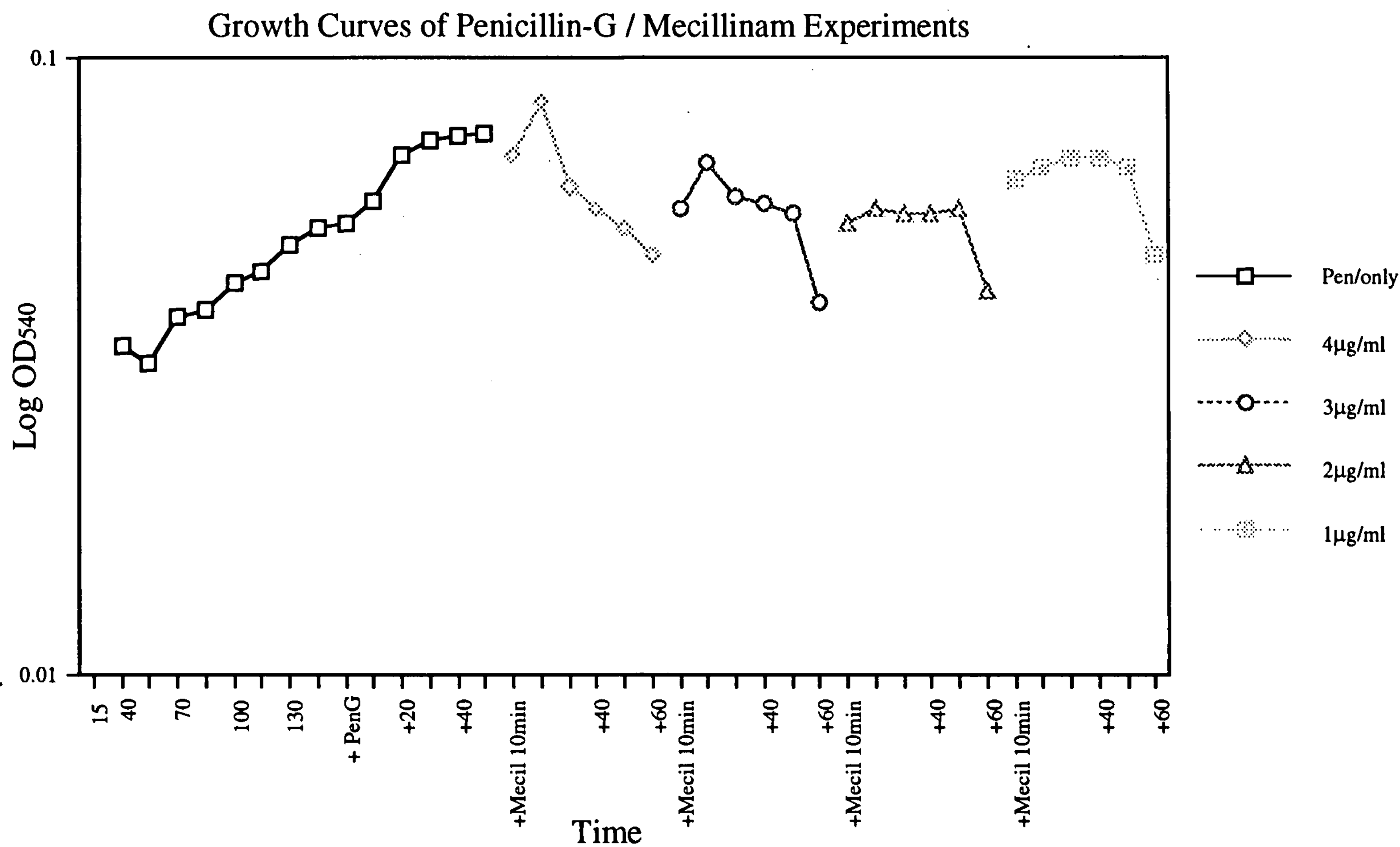
Mecillinam is the most specific β-lactam antibiotic known, binding exclusively to PBP2 at low concentrations (Spratt, 1975). Penicillin-G is not as specific as mecillinam but at low concentrations binds preferentially to PBP3.

Cells were grown from a fresh overnight of AB2497 and inoculated into 50 mls of LB. They were incubated in a shaking water bath at 37°C until they reached early log-phase, approximately an OD_{540} of 0.2. Division was then inhibited by the addition of 30 unit/ml Penicillin-G and growth monitored for another 40 minutes. The culture was then split and mecillinam added at a final concentration of 5 µg/ml. Growth was then monitored for a further 40 minutes and samples taken for observation under the microscope.

In these experiments mecillinam failed to induce division in Penicillin-G induced filaments. The cells continued to filament and eventually became swollen at their centre before lysing. This was identical to the phenotype described for PBP2 temperature-sensitive mutants treated incubated at their restrictive temperature with the PBP3 specific antibiotic furazlocillin (Schmidt *et al.*, 1981). The mecillinam alone was not sufficient to induce lysis in the control culture, or even to significantly slow the growth rate.

In an attempt to overcome the synergistic affect of penicillin and mecillinam, lower concentrations of mecillinam were used. In these experiments AB2497 was grown to early log-phase in minimal media. Penicillin-G was then added at a concentration of 30 unit/ml and the cells monitored for a further 30 minutes. At this point the culture was split and mecillinam added to final concentrations between 1 µg/ml and 4 µg/ml. Minimal media was used to slow the growth rate and reduce the

Figure: 3.2. Attempts to mimic KJB1 Suppression with specific antibiotics.



observation of any transient increase in division, that may be obscured by autolysis.

The growth rate of the Penicillin-G only control slowed and the OD_{540} of the culture stopped increasing (Figure:3.2). The culture given 4 μg/ml of mecillinam began to lyse after 10 to 20 minutes. The lower concentrations of mecillinam also caused the OD_{540} to fall and the cells to lyse. No transient increase in division was produced by even the lowest concentrations of mecillinam. It was observed that even in minimal media and at low concentrations mecillinam acted synergistically with Penicillin-G

Inhibition of PBP2 and PBP3 can be achieved using temperature-sensitive PBP2 alleles and furazlocillin (Schmidt *et al.*, 1981), or a temperature-sensitive PBP3 allele and mecillinam (Gutman *et al.*, 1986). In both these situations inhibition of PBP2 and PBP3 has been reported to act synergistically to promote autolysis. Further attempts to mimic suppression with specific antibiotics were abandoned.

3.2 Construction of a strain lacking functional RodA.

The initial work with KJB1 centred on reduced levels of RodA and consequently PBP2-RodA lateral wall synthesis. PBP2 assays had suggested the *rodA*sui mutation also caused increased levels of PBP2 and PBP5. The genes for both these proteins lie alongside *rodA* in the 15 minute cluster of peptidoglycan synthesising genes (Figure:3.1) (Stoker *et al.*, 1983). To determine if RodA was essential for suppression, it was decided to construct a strain with no RodA. This was achieved by transducing *rodA*sui into the suppressor free strain W3110. The intention was then to transduce *ftsI*23 into the deletion and determine if suppression could be achieved in the absence of RodA.

Initially the 15 minute cluster of morphogenes had been identified through work on mecillinam resistant mutants that showed a spherical phenotype (Iwaya *et al.*, 1978). Subsequent work on temperature-sensitive alleles of *pbpA*, and *rodA* described their characteristic spherical, mecillinam resistant phenotype. Attempts to produce a *pbpA* (PBP2) deletion strain had proved unsuccessful except in the presence of secondary mutations (Ogura *et al.*, 1989; Vinella *et al.*, 1992). Similarly construction of a RodA deletion was expected to be lethal. The secondary mutations that confer viability to PBP2 deletion strains include *cya*$^-$ and *crp*$^-$, which have also been reported to be responsible for mecillinam resistance (Aono *et al.*, 1979). It was therefore decided to construct a strain lacking any functional RodA in the presence of a *cya*$^-$

allele. This mutation was chosen as its wild type phenotype could be restored by the addition of cAMP (Ogura *et al.*, 1989).

3.2.1 Transductions.

W3110 (*sup* ^{O}) was transduced to kanamycin resistance using a P1 lysate of GC2880 (*ilv* :: Tn5). Kanamycin resistant transductants were purified and screened for the *ilv*$^{-}$ phenotype. W4 (*sup* ^{O} *ilv* :: Tn5 Kanamycin) was then transduced with the P1 lysate of CA8306 (*ilv*$^{+}$ *cya*$^{-}$), and *ilv*$^{+}$ transductants selected on minimal media. The *ilv*$^{+}$ transductants were purified and checked for kanamycin sensitivity. The presence of a *cya*$^{-}$ phenotype was then demonstrated using MacConkey agar.

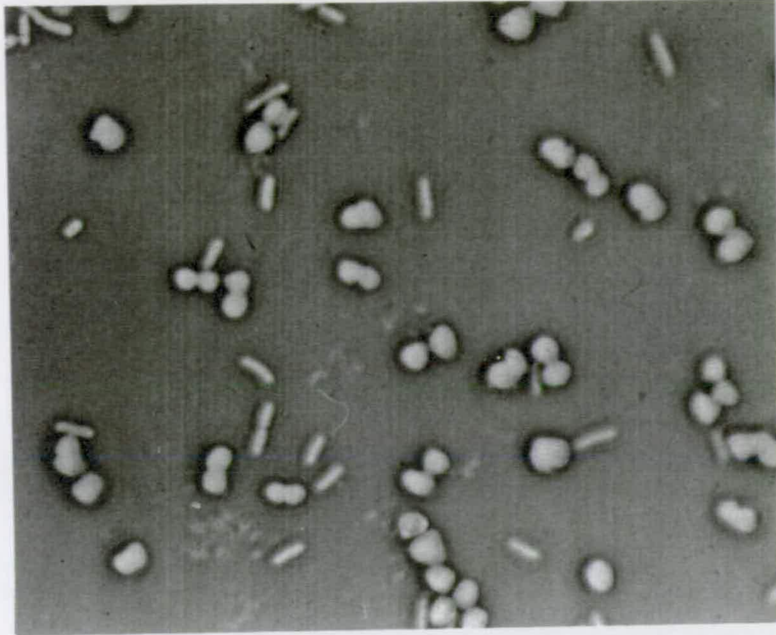
W5 (*sup* ^{O} *ilv*$^{+}$ *cya*$^{-}$) with a P1 lysate of AT1325 (*lip*$^{-}$::Tn5) and kanamycin resistant transductants selected. Again transductants were purified and screened for the *lip*$^{-}$ phenotype. *lip*$^{-}$ transductants require α-lipoic acid and will only grow on minimal media supplemented with DL-6,8 Thioctic acid (final concentration of 1µg/ml). The strain constructed (W6) by this series of transductions was *sup*^{O} and *cya*$^{-}$, and carried the *lip*$^{-}$:: Tn5 at 15 minutes. W6 was then transduced with a P1 lysate from Ken222 (*rodA*sui) and transductants selected for growth on minimal media without thiotic acid.

Transductants came up slowly after 48 hours and colonies consisting of spherical cells were patched onto the same media to check their auxotophy. Colonies were then screened for kanamycin sensitivity by replica plating. The *sup*^{O} phenotype was confirmed with two λ strains that required the presence of amber suppressors to complete their lytic cycles, λnam and λpam. These both failed to plate on the spherical transductants and W3110, while the control wild type λ did. The resulting strain was named DE1 and had the appropriate markers and the expected spherical phenotype.

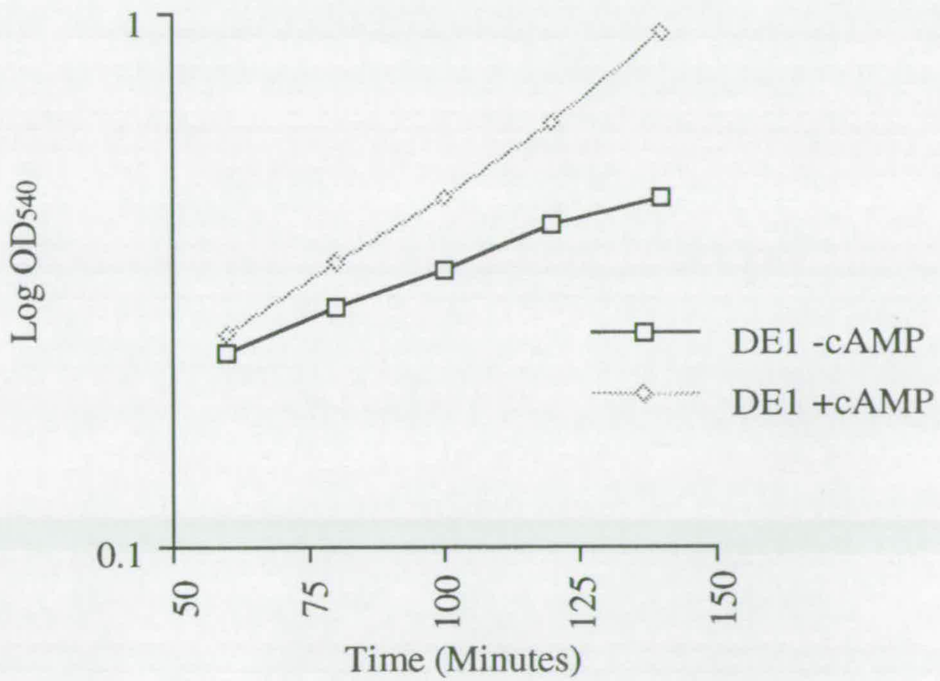
3.2.2 RodA is not essential.

DE1 cells appeared as large, osmotically stable, spheres (Figure:3.3). W3110 was sensitive to mecillinam at concentrations of 1 µg/ml. DE1, and its immediate parent, W6 (*lip*$^{-}$, *cya*$^{-}$, *rodA*$^{+}$), were viable in the presence of mecillinam at a concentration of 50 µg/ml. This is twice the level of resistance reported for previous *cya*- strains (D'Ari *et al.*, 1988). DE1 grew in the presence and absence of cAMP (1 µg/ml). This is in direct contrast to *pbpA* deletions (Ogura *et al.*, 1989) and *rodA* deletions constructed in *Salmonella typhimurium* (Costa & Anton, 1993). Though viable in *cya*$^{-}$ backgrounds these deletions are lethal when cAMP is added.

Figure: 3.3 Phenotype of DE1 (*rodA*sui)



Note: DE1 (spherical) and AB2497 (rod-sape) cells from exponential-phase liquid cultures



In the presence of cAMP median cell size, as determined by the coulter counter, doubled. cAMP also increased the growth rate from a doubling time of 100 minutes to 45 minutes (Figure:3.3). This did not match the 30 minute doubling time of the wild type, W3110. It was also noticed that addition of cAMP did not completely restore the growth rate of W6. This is probably due to exogenous cAMP not fully restoring the *cya*$^{+}$ phenotype.

DE1 continued to display mecillinam resistance in the presence or absence of cAMP. The concentration of cAMP added was identical to that reported to restore wild type mecillinam sensitivity to *cya* mutants (Aono *et al.*, 1979). The morphological changes produced by *cya* mutations are known to be strain specific. This strain dependency for causing cell shape changes, suggests strain dependency for affecting RodA-PBP2 activity and possibly mecillinam sensitivity. Therefore strain variation could explain the discrepancy between DE1 and the *cya*$^{-}$ strains of Iwaya *et al*, (1978). However mutations in *rodA* are known to confer mecillinam resistance in *E.coli* (Tameki *et al.*, 1980), and *Salmonella typhimurium* (Costa & Anton, 1993). Consequently the continued resistance of DE1 in the presence of cAMP is probably due to the *rodA*sui allele.

3.2.3 Attempts to construct a *ftsI*23, *rodA*sui, sup^{o}, strain.

The spherical cells produced by deletion of RodA were presumed to result from synthesis of hemispherical septal peptidoglycan. This is the same explanation previously proposed for spherical cells produced by deletion of PBP2, mecillinam treatment or overproduction of PBP5 (Markiewicz *et al.*, 1982). The 'balance hypothesis' implied competition between PBP2-RodA and PBP3 for substrate. Consequently it would be predicted that deletion of RodA would provide increased substrate for PBP3 and suppress temperature-sensitive mutations of PBP3. The possibility that RodA would disturb the balance between elongation and division and be lethal was discounted because DE1 was viable in the presence of cAMP.

A TOE23 (*ftsI*23 *leu*-::Tn*10*) lysate was used to transduce DE1 and W3110. The transductants were selected for tetracycline resistance at the permissive temperature. The transduction of tetracycline resistance into DE1 was three times less efficient than its transduction into the wild type W3110.

After being purified on tetracycline plates, 50 colonies were patched at the permissive and restrictive temperature in the presence and absence of tetracycline. The PBP3 allele (*ftsI*23) co-transduces with *leu* at a frequency of 60%. Approximately

50% of the W3110 transductants failed to grow at 42°C, all the DE1 transductants formed colonies at both temperatures. The absence of a class of temperature-sensitive transductants suggested that *ftsI*23 in a *rodA* deletion strain was either suppressed or inviable. Microscopic examination of tetracycline resistant DE1 transductants revealed no evidence of a suppressed phenotype. Along with the reduced transduction frequency this suggested an *ftsI*23 Δ*rodA* strain was not viable. To check this it was attempted to construct the strain in reverse *ie.* to transduce *rodA*sui into a W3110 *ftsI*23 strain.

W23 (W3110 *ftsI*23) was transduced to *lip*::Tn*5*. This strain was then transduced to *lip*$^+$ using the Ken222 (*rodA*sui) lysate. Again a low efficiency of transduction was observed, and the 27 transductants screened maintained a temperature-sensitive phenotype but with no indication of spherical morphology.

These transductions were repeated twice more, using minimal media to see if a slower growth rate would allow the maintenance of the *rodA*sui allele in the presence of *ftsI*23, but no temperature-sensitive spherical cells were obtained. The phenotypes obtained and frequency of transduction observed were consistent with the transduction of only the linked marker without the *rodA*sui allele.

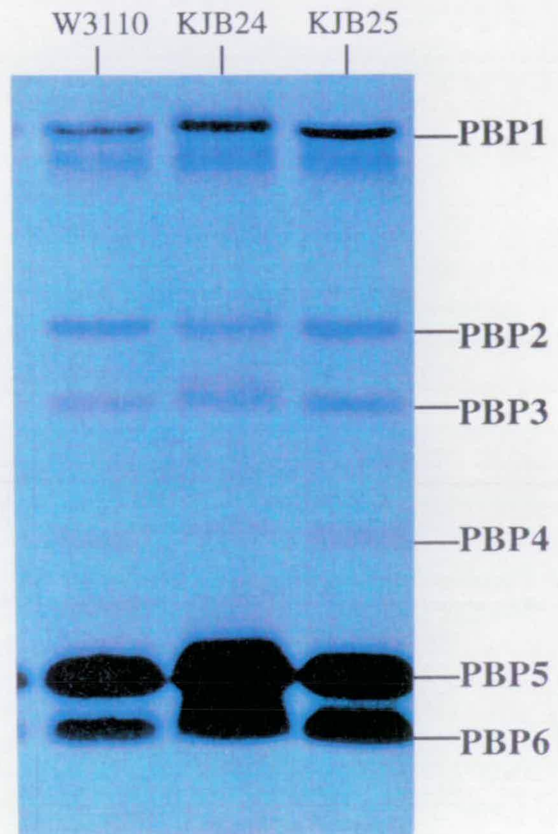
It was concluded that a strain deleted for RodA was viable but could not tolerate the presence of the *ftsI*23 allele. The inviability of a strain with impaired PBP2-RodA and PBP3 activity, is consistent with the synergistic affect of inhibition of PBP2 and PBP3 on autolysis (Schmidt *et al.*, 1981; Gutman *et al.*, 1986).

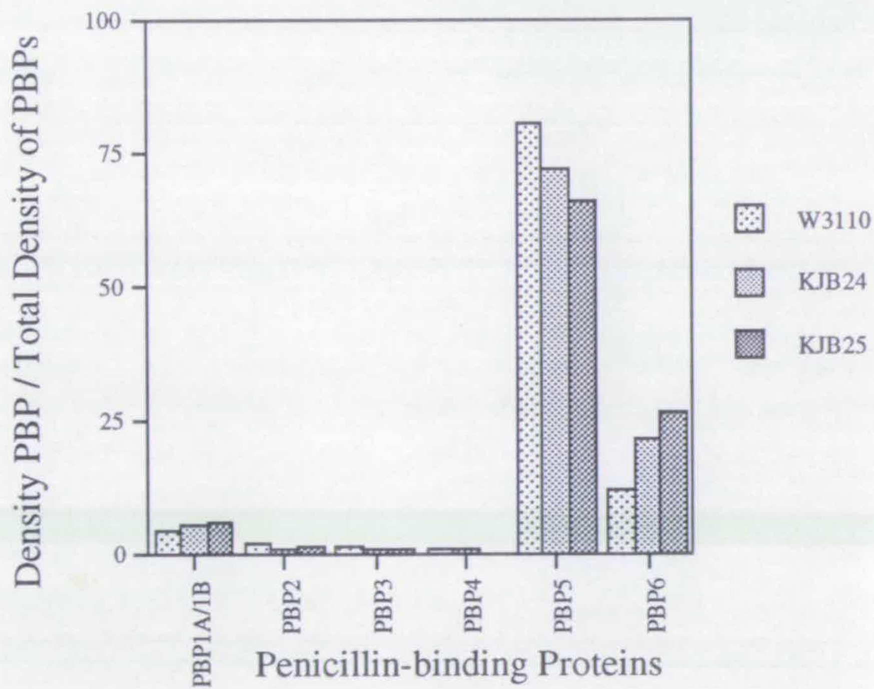
3.3 PBP analysis of strains producing different levels of RodA.

Attempts to mimic the proposed balance between crosswall and septum synthesis with specific antibiotics had failed. The construction of a strain completely lacking RodA and carrying *ftsI*23 also proved unsuccessful. Consequently no evidence had been found to support the balance hypothesis.

The PBP gels prepared by B.Spratt had suggested the presence of the *rodA*sui allele affected the levels of two PBPs. No previous *rodA* mutations had been reported to affect PBP levels (Tameki *et al.*, 1980). To clarify this it was decided to determine the affects of varying levels of RodA on the level of PBPs. Three strains were used; W3110, a wild type control; KJB24, a *cya*$^+$ version of DE1 constructed by K.Begg; and K25, a derivative of K24 carrying the amber suppresser *supE*37. These three

Figure: 3.4PBP assay of strains expressing different levels of RodA.





strains should vary in the amounts of RodA they produce. This assumption was supported by the cell shape which varied between rod shape (W3110); ovoid (KJB24), and spherical (KJB25). Two membrane preparations were made from each strain and their protein concentrations determined. The membranes were labelled using saturating concentrations of H^3-PenG, and then the inner membrane was solubilised. The membrane proteins were separated by SDS-PAGE and the gels dried and exposed to photographic film.

For each replicate experiment the gel was exposed for 10 days and 30 days. The images were then scanned using a densitometer and the average density for the two replicates determined. The protein concentration of each sample had been determined by a Lowry assay (Lowry *et al.*, 1956) so that the labelling and running of membranes was standardised. However, to avoid the effect of any variations in protein concentrations, the density of an individual PBP bands was expressed as a proportion of the total density of all the PBP bands in an individual track.

Table: 3.1. Proportion of PBPs in W3110, KJB24 and KJB25.

	W3110 30 Day	W3110 10 Day	KJB24 30 Day	KJB24 10 Day	KJB25 30 Day	KJB25 10 Day
PBP1A	11.79	4.28	12.68	5.2	17.5	6
PBP1B	3.21		4.69		6.57	
PBP2	5.75	1.74	3.35	0.61	6.61	1.19
PBP3	2.78	1.1	3.12	0.68	4.9	0.75
PBP4	0.34	0.94	0.54	0.53	0.97	-
PBP5	55.32	80.33	52.71	71.75	34.38	65.71
PBP6	20.84	11.94	22.89	21.42	29.29	26.46

Note: Figures represent the density of each PBP band expressed as a proportion of the total PBPs. Values were obtained from two replicate experiments with the gels exposed for either 10 or 30 days.

The relative proportions of PBPs fall within the range of values previously reported (Spratt, 1975; de la Rosa *et al.*, 1982; Tuomanen *et al.*, 1986; Dreihuis & Wouters,1987). The problem of obtaining a linear response between the radiation and the image scanned is partially overcome by the use of pre-flashed film and exposing at -70°C (Laskey & Mills, 1975). The proportion of the most abundant PBPs decreased between the 10 and 30 day exposure. This suggested saturation of the photographic

emulsion had occured by 30 days and therefore the figures for the 10 day exposure were chosen for analysis.

The original PBP profiles for KJB1 and TOE23 indicated an increase in PBP2 and PBP5 associated with the presence of *rodA*sui allele (Begg *et al.*, 1990). The profiles of W3110, KJB24 and KJB25 were not consistent with this. The levels of PBP5 and PBP2 were highest in the wild type and lowest in KJB25, a *rodA*sui strain carrying a suitable amber suppresser (Figure:3.4). KJB24 (*rodA*sui *sup°*) had an intermediate level of PBP2 and PBP5 (Figure:3.4).

The difference between the proportions of PBPs in my assays and the original assays may reflect strain variations. It has been reported that AB1157 does not exhibit the stationary phase increase in PBP6 normally associated with other strains (de la Rosa *et al.*, 1983). KJB1 is derived from AB1157 therefore it is possible that KJB1 may also respond differently to the *rodA*sui allele. Variations in growth-phase (Buchanan & Sowell, 1982) media and growth rate (Dreihuis & Wouters, 1987) are all known to affect the levels of PBPs. The original KJB1 assays by B.Spratt were performed on cells harvested in late exponential-phase growth, in these assays mid-exponential phase cultures were harvested. This would not be expected to cause any significant difference in the proportions of individual PBPs. The possibility that the increased levels of PBP2 and PBP5 may be the result of an interaction between *rodA*sui and a second mutation in KJB1 could not be discounted. In particular an interaction between *rodA*sui and *ftsI*23 could be responsible. Such an explanation would explain why no other *rodA* mutation has been reported to affect PBP levels (Tameki *et al.*, 1980; Spratt *et al.*, 1980.).

In these experiments the levels of PBP6 were higher in both the strains carrying *rodA*sui. This was most distinct in KJB25 where it represented over twice the level in W3110. Like PBP5, PBP6 is considered to be a DD-Cpase (Amanuma & Strominger, 1980). The total proportion of PBP5 and PBP6, remained constant in all three strains despite their individual values changing. This may represent regulation of the total DD-Cpase activity available. This is discussed further in chapter two.

3.4 DD-carboxypeptidase Theory

It has been proposed that the 14.2 minute cluster of genes involved in peptidoglycan synthesis form an operon (Tameki *et al.*, 1980; Matsuzawa *et al.*, 1989). This is based upon the close proximity of six open reading frames (Figure:3.1)

and is supported by the demonstration of efficient transcription of *rodA* from the *pbpA* promoter (Matsuzawa *et al.*, 1989). This proposed operon includes the genes for PBP2 (*pbpA*) and PBP5 (*dacA*). PBP assays of KJB1 revealed elevated levels of both PBP2 and PBP5. Further PBP assays suggested this increase may require the presence of the *ftsI*23 allele. Therefore it was possible that increased levels of PBP2 and PBP5 may be involved in the suppression of *ftsI*23. While PBP2 could be postulated to interact with reduced PBP3 activity and enable division, there was more support in the literature for PBP5 overproduction being involved in septation.

PBP5 has been identified as one of two proteins responsible for "Carboxypeptidase 1A" activity (Amanuma and Strominger, 1980). Also termed DD-carboxypeptidase or Carboxypeptidase 1, this activity catalyses the removal of the terminal D-Alanine from a pentapeptide side chain to produce a tetrapeptide. The first evidence for these enzymes having a role in division came from analysis of ether permeablised cells blocked in division. It was apparent that these filamentous cells showed an increased degree of peptidoglycan cross-linkage and reduced DD-Cpase activity (Mirelman *et al.*, 1977). Membranes derived from minicells and therefore enriched for septal material were demonstrated to exhibit twice the normal DD-Cpase activity (Goodell & Schwarz, 1977). Subsequent work on PBP5 (Markeiwitz *et al.*, 1982) revealed its overproduction to induce a morphological change from normal rod shaped bacteria to ovoid or spherical cells. The shape change was accompanied by increased peptidoglycan cross-linkage similar to that found with mecillinam treated cells or a *pbpA* (PBP2) temperature-sensitive allele (Spratt, 1977). The authors therefore proposed that the hemispherical murein determining the shape of these cells was synonymous with septal murein. The formation of septal murein by overproduction of PBP5 was considered to be the product of increased levels of substrate for the septum specific transpeptidase PBP3. This implied that PBP3 would use tetrapeptides as the preferred acceptors for its cross-linking reaction.

Suppression by *rodA*sui could therefore result from elevated levels of PBP5 increasing the availability of tetrapeptides. These tetrapeptides acting as preferred acceptors would enable residual PBP3 activity to complete septation. The existence of residual PBP3 in *ftsI*23 filaments had been inferred from the sensitivity of KJB1 to furazlocillin at 42°C (K.Begg *pers.comm.*).

3.4.1 Experiments to examine the affect of DD-carboxypeptidase overproduction.

DD-Cpase activity has been ascribed to three proteins in vitro, PBP4, PBP5 and PBP6 (Iwaya & Strominger, 1977; Amanuma & Strominger, 1980). Of these PBP5 is considered to have four times higher specific activity than PBP6 (Amanuma & Strominger, 1980), with which it shares 63% sequence identity (Broome-Smith *et al.*, 1988). PBP4 is also proposed to have DD-endopeptidase activity in vivo (Matsuhashi *et al.*, 1977; Iwaya & Strominger, 1977) but does not share the same degree of homology that PBP5 and PBP6 do (Mottl, 1992).

Gross overproduction of PBP5 is lethal and consequently Markeiwitz *et al.* (1982) used a low copy number vector producing 10x the normal level of PBP5. Some of the original *dacA* (PBP5) plasmids along with a plasmid carrying *dacC* (PBP6) were kindly supplied by Brian Spratt. The plasmid pBK18-1 expressing the PBP4 protein was supplied by W.Keck. Extragenic multi-copy suppression of *ftsI*23 with each of the three putative DD-Cpases was examined.

Table:3.2. Transformation of TOE23 with plasmids overproducing Peniillin-binding proteins

Plasmid	Insert	Protein	Growth at 42°C
pBS47	*pbpA*	PBP2	–
pBS59	*dacA*	PBP5	+
pBS110	*dacC*	PBP6	+
pBK18–1	*dacB*	PBP4	–

Note; Growth at 42°C indicates the suppression of TOE23 on solid media without selection. +; indicates growth and suppression.

3.4.2 Extragenic suppression of *ftsI*23.

TOE23 was transformed with each of the plasmids, and transformants isolated at 30°C using appropriate selection. Colonies were purified and then streaked out at the restrictive temperature in the presence and absence of selection. Any suppression was checked by repitition and then confirmed by curing the plasmid and rescreening for the temperature-sensitive phenotype.

As the table shows, both PBP5 and PBP6 overproduction caused multi-copy suppression of *ftsI*23 on solid media (Figure:3.5). In broth lysis was still observed

Figure:3.5 Suppression of *ftsI23*.

Plate 1

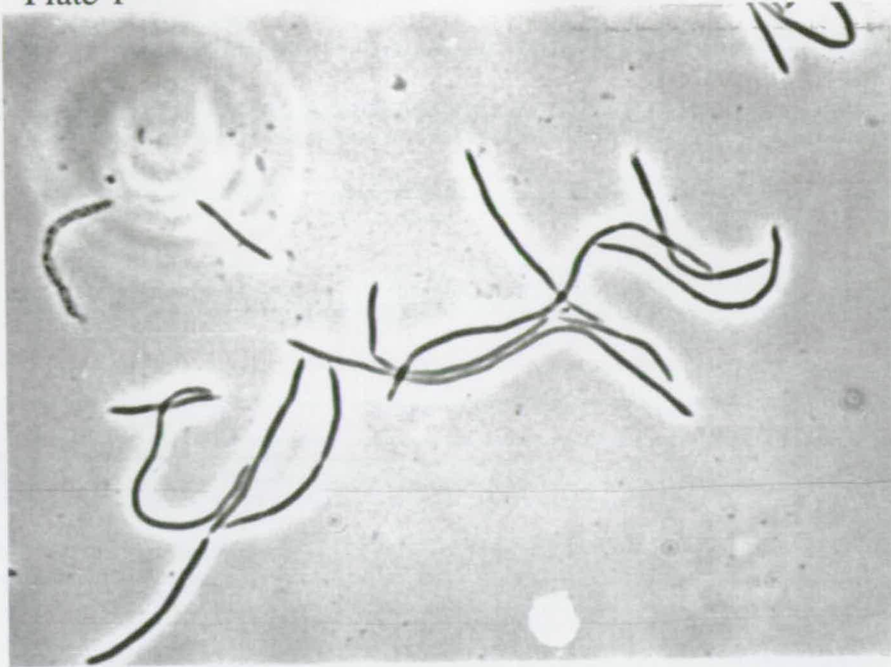
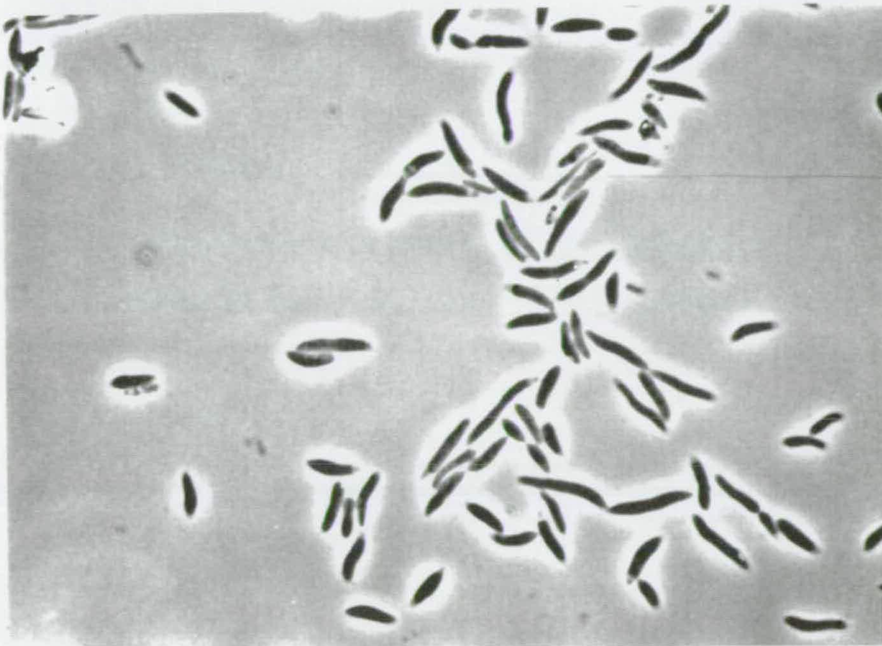


Plate 2.



Note: Photographs taken of bacteria growing on solid media without selection at 42°C. Plate 1; is of TOE23 cells, Plate 2; is of TOE23 cells carrying pBS59 (*dacA*).

upon shifting an exponentially growing culture. By contrast increased PBP4 or overproduction of PBP2 do not suppress.

3.4.3 Suppression is the result of increased DD-Cpase activity.

Of the three proteins which have been ascribed DD-Cpase activity in vitro (Amanuma & Strominger, 1980) both PBP5 and PBP6 suppressed *ftsI*23; a result consistent with the hypothesis that PBP3 preferred tetrapeptide side chains as acceptors (Markeiwitz *et al.*, 1982).

PBP5 has been consistently attributed DD-Cpase activity. Mutations in *dacA* (PBP5) reduce DD-Cpase activity (Matsuhashi *et al.*, 1976), purified PBP5 exhibits DD-Cpase acivity in vitro (Awaya and Strominger, 1980) and murein synthesised in vivo by a *dacA* deletion strain is enriched in pentapeptides (Glauner, 1986). This contrasts with the inconsistent experimental data on PBP6.

PBP6 was first demonstrated to have DD-Cpase activity at the same time as PBP5 (Tamura *et al.*, 1976). This activity was estimated to represent one third of PBP5's specific activity (Amanuma and Strominger, 1980). Matsuhashi *et al.* (1978) reported a PBP5 deletion strain that had no demonstratable 1A DD-Cpase activity despite the presence of PBP6. This was contradicted by the isolation of a PBP6 with demonstrable DD-Cpase from the same strain (Amanuma and Strominger, 1984). Isolation and sequencing of the PBP5 (*dacA*) and PBP6 (*dacC*) genes revealed a 63% sequence identity, supporting the concept of a common function (Broome-Smith *et al.*, 1988). However analysis of the peptidoglycan synthesised in vivo by a PBP6 deletion strain showed no increase in the amount of pentapeptides (Glauner, 1986). Recently gross overproduction of PBP6 was demonstrated to have no distinct morphological affect (van der Linden *et al.*, 1992). The PBP6 purified from this overproducing strain also failed to exhibit any DD-Cpase activity in vitro. Therefore the activity of PBP6 in vivo is not certain. However the extragenic suppression of *ftsI*23 in these experiments and the proteins' 63% sequence similarity support Annamura & Strominger's (1980 & 1984) assertion that PBP5 and PBP6 share a common function.

3.4.4 The function of PBP4 in vivo.

PBP4 has been attributed endopeptidase, transpeptidase and DD-Cpase 1B and 1C activity. The original isolation of mutants lacking a PBP4 was achieved independently by two groups using nitrosoguanidine mutagenesis (Matsuhashi *et al.*,

1977; Iwaya & Strominger, 1977). Both groups demonstrated the loss of 1B and 1C DD-Cpase activity, and reduced endopeptidase activity. Matsuhashi *et al.* (1977) also reported altered transpeptidase activity. Subsequent work by DePedro *et al.* (1980), DePedro *et al.* (1981) and Goodell and Schwarz (1983) all used DL64, the strain isolated by Matsuhashi *et al.* (1977). DL64 was later shown to contain a temperature-sensitive penicillin-insensitive transpeptidase (Glauner, 1986). This fact invalidated the previous suggestion (DePedro *et al.*, 1980, DePedro *et al.*, 1981) that PBP4 functioned as a transpeptidase in vivo. The best data on the role of PBP4 has come from the sub-cloning of *dacB*, and overexpression of PBP4. This confirmed PBP4 overproduction to increase DD-Cpase 1B/C and endopeptidase activities in vivo (Koret *et al.*, 1991).

Three putative DD-Cpases were tested for extragenic suppression of *ftsI*23. The two membrane-bound DD-Cpases were capable of suppression, but PBP4 was not. This failure to suppress *ftsI*23 with a plasmid overproducing PBP4 that has been demonstrated to increase tetrapeptides and tripeptides in vivo was not consistent with our hypothesis. In contrast with the highly homologous sequences of PBP5 and PBP6 the structure of PBP4 is quite novel. PBP4 has an additional domain of 188 amino acids in its primary structure (Mottl, 1992). This may indicate a specific role which prevents the enzyme from carrying out the correct DD-Cpase activity at the correct location or time to supply substrate for PBP3. Comparison of the murein synthesised by PBP5 and PBP4 overproducing strains does suggest a difference in their activities (Glauner, 1986; Koret *et al.*, 1990). Overproduction of either PBP4 or PBP5 leads to a reduction in pentapeptides and accumulation of tripeptides. However the form in which tripeptides accumulate differs. The increased tripeptides of a PBP4 overproducing strain are present as increased dap-dap cross-bridges. These cross-bridges result from penicillin-insensitive transpeptidation and are considered to be formed from tripeptides released from the stress bearing layer by the action of endopeptidases (Glauner & Holtje 1990). Tri-tri bonds also appear to increase as part of a rescue mechanism when the integrity of the sacculus is threatened (deJonge 1989). By contrast strains overproducing PBP5 accumulate tripeptides in tetra-tri cross-bridges which can be catalysed by penicillin-sensitive transpeptidases (Glauner, 1986). This suggested that the two enzymes have distinct roles in supplying tripeptides, and provided an explanation for the failure of PBP4 to suppress.

3.5 Tripeptides are the preferred substrate for PBP3.

Multi-copy suppression of *ftsI*23 by the two major DD-Cpases PBP5 and PBP6, supported the proposed model of Markeiwitz *et al.* (1982). However both these relatively abundant PBPs are dispensible (Broome-Smith, 1985). Therefore either the role of tetrapeptides is not essential or other sources of substrate are available for septum synthesis.

In direct contrast to Mirelman *et al.* (1977), Beck and Park (1977) showed that there was no increase in DD-Cpase activity during division. They did demonstrate cycling of an associated enzyme activity; the LD-carboxypeptidase. Similarly work with *envC* chain forming mutants revealed a six fold increase in LD-Cpase activity during completion of septation (Karibean *et al* 1981). This enzyme cleaves the L-D peptide bond between *m*DAP and D-Ala on a tetrapeptide side chain to produce a tripeptide. The LD-Cpase activity has been demonstrated in *E.coli* (Izaki & Strominger, 1968) *Streptococcus faecalis* (Coyette *et al.*, 1974) and *Gaffkya homari* (Hammes & Seidel, 1978).

Cells treated with D-cycloserine accumulate tripeptides and produce highly cross-linked hemispherical murein, the same phenotype that is produced by PBP5 overproduction (Pisabarro *et al.*, 1986). It has also been demonstrated that ether permeabilised cells incorporate exogenous tripeptides by a furazlocillin-sensitive (PBP3) transpeptidation (Pisabarro *et al.*, 1986). Both these observations suggest that PBP3 can utilise tripeptides as acceptors. Therefore it was proposed that suppression by overproduction of PBP5 and PBP6 could be the result of increased tetrapeptides being degraded to tripeptides by the LD-Cpase. The increased tripeptides would then act as the preferred acceptor for reduced levels of PBP3.

D-cycloserine is a structural and functional analogue of D-alanine and interupts the synthesis of the two terminal residues of the pentapeptide side chain, leading to accumulation of tripeptides (Neuhaus & Hammes, 1981). Its addition leads to a reduction in cytoplasmic pentapeptides and increase in tripeptides. Ken Begg used normally lethal levels of this antibiotic to enable growth of TOE23 (*ftsI*23) on plates at 42°C. Therefore it appeared that tripeptides were in fact the preferred acceptors.

3.5.1 Attempts to demonstrate D-cycloserine suppression for liquid cultures.

Tripeptides appeared to be the preferred substrate for PBP3 dependent transpeptidation and the use of D-cycloserine had provided a mechanism for inducing growth and division on solid media. It was attempted to mimic this and induce division in a liquid culture of *ftsI*23 filaments by adding D-cycloserine. A series of experiments were performed using varrying concentrations of the antibiotic and analysing results both microscopically and using the coulter counter.

3.5.2 D-cycloserine does not suppress liquid culutres.

Experiments to repeat for liquid cultures the suppression produced by D-cycloserine on solid media failed. There was no evidence of division from microscopic analysis of filaments treated with D-cycloserine. Similarly, Coulter-counter analysis provided no evidence for division being triggered by addition of D-cycloserine. The failure to reproduce suppression in liquid media is similar to the situation for PBP5 and PBP6 suppression.

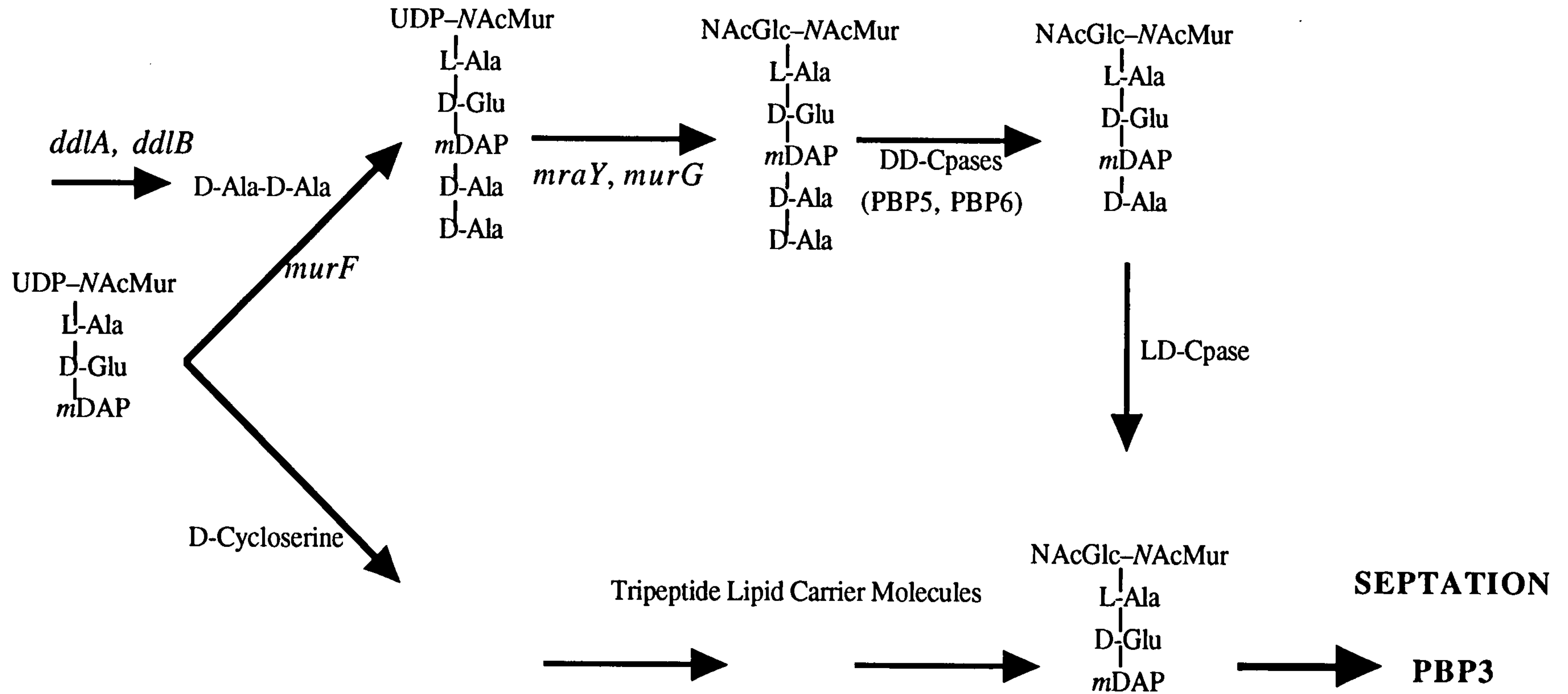
3.6 Summary

The original hypothesis for growth and division for KJB1 at the restrictive temperature proposed a balance between two competing peptidoglycan synthesising systems (Begg & Donachie, 1986). Attempts to mimic this balance with specific antibiotics failed, and confirmed the synergistic effect of PBP2 and PBP3 inhibition on induction of autolysis (Gutmann *et al.*, 1986). To examine whether RodA was required for suppression, a strain completely lacking RodA was constructed. This strain carried a *cya-* allele but grew as spherical cells in the presence of cAMP. It was concluded that RodA was not essential. It was also established that a sup^{o} *ftsI*23 strain could not accept the *rodA*sui allele by transduction. This supports previous observations that inhibition of PBP2-RodA activity and PBP3 has a synergistic affect on autolysis. PBP assays had suggested that either PBP2 or PBP5 could be responsible for KJB1 suppression (Begg *et al.*, 1990). Extragenic multi-copy suppression of *ftsI*23 by the two membrane-bound DD-Cpases, PBP5 and PBP6 was demonstrated on plates. The other proposed DD-Cpase, PBP4 (Koret *et al.*, 1990) could not suppress. K.Begg also demonstrated that D-cycloserine could suppress

*ftsI*23 on solid media. Attempts to repeat D-cycloserine suppression in liquid media failed.

It is proposed that tripeptides act as preferred acceptors for PBP3 mediated transpeptidation, required during septation (Figure: 3.6). Suppression of *ftsI*23 is achieved by the supply of increased substrate to residual enzyme activity at 42°C, enabling septation to take place. The key enzymes in this process would appear to be the DD-Cpases and the LD-Cpase. Subsequent chapters describe attempts to investigate their roles. Chapter 4 concerns a second known suppressor of temperature-sensitive *ftsI* (PBP3) mutations; SufI. It was considered possible that this suppressor could be the LD-Cpase or a regulator of DD-Cpase activity. Chapter 5 describes attempts to construct a strain deleted for all three proposed DD-Cpases. If the periplasmic control of tetrapeptides was essential this strain would be expected to be inviable. Chapter 6 describes more direct attempts to isolate the gene for the LD-Cpase.

Figure: 3.6. Preferred acceptor hypothesis.



CHAPTER 4

SUPPRESSION OF *ftsI*23 BY *sufI*

When it had been established that over production of either of the two DD-carboxypeptidases (PBP5 and PBP6) could suppress *ftsI*23 work was begun on a second known suppressor.

An *ftsI* mutation specific suppressor carried by the Clarke-Carbon plasmid pLC4-14 was first reported by Takeda *et al.* (1981). Further work on a new mutation causing abnormal chromosome segregation, *parC* (Kato *et al.*, 1988), led to characterisation of this plasmid and in the process to identify an *ftsI* suppressor named, *sufI*. The *sufI* gene was shown to suppress when present on a multi-copy plasmid and to encode a 55 kD protein which was processed before becoming localised in the periplasm. Along with two other genes, *parC* and a 25 kD open-reading frame, *sufI* appeared to be part of an operon which mapped at 65.3 minutes on the *E.coli* chromosome. A strain carrying a chromosomal disruption of the *sufI* gene proved viable and the protein was considered dispensible. Later work on the nucleotide sequence of *sufI* revealed an open-reading frame of 1,476 basepairs coding for a polypeptide of 492 amino acid residues with deduced molecular weight of 54,178 (Yamamoto *et al.*, 1989). Two 60 amino acid domains in the *C*-terminal region of *sufI* and in the *N*-terminal region of *ftsI* were found to be similar. It was suggested that the shared similarity spanned a region of *ftsI* corresponding to a peptidoglycan transglycosylase domain. This region of similarity indicating transglycosylase activity for the SufI protein, was then proposed to explain the suppression of *ftsI* mutants. The problem with this proposal is that it is based upon identification of a transglycosylase domain. The *ftsI* gene product; PBP3, has been demonstrated to exhibit a penicillin-insensitive transglycosylase activity but only in the presence of a low molecular weight contaminant (Ishino & Matsuhashi, 1981). Consistent with these observations is the suggestion that; as with PBP2 and RodA, a second protein capable of transglycosylase activity forms a complex with PBP3 (FtsW) (Ikeda *et al.*, 1989). Identification of a PBP3 transglycosylase domain has been attempted by selecting for plasmid borne PBP3 mutations which bind penicillin but cannot complement a chromosomal temperature sensitive *ftsI* allele (Spratt & Cromie, 1988). The mutants would be expected to include a class which can bind penicillin at a fully functional transpeptidase domain but have a mutated transglycosylase domain. However of ten mutations characterised nine mapped to the *C*-terminal transpeptidase domain and only one to the amino terminus. Even this single mutation maps to residue 190 and is outside the region that has sequence similarity to *sufI*. There is therefore no evidence for a transglycosylase domain shared by PBP3 and SufI.

Figure:4.1 Suppression of *ftsI23* by pAX607 (*sufI*)

Plate 1.

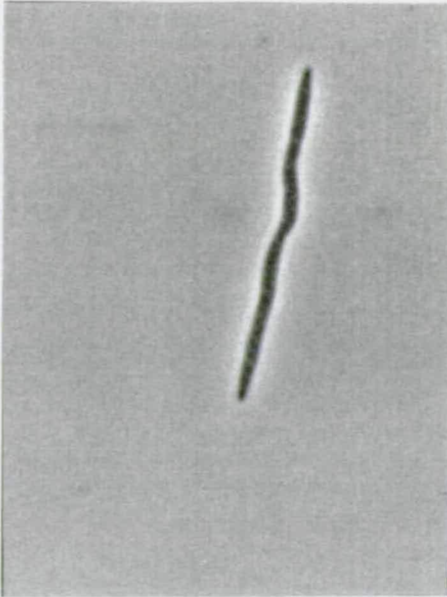
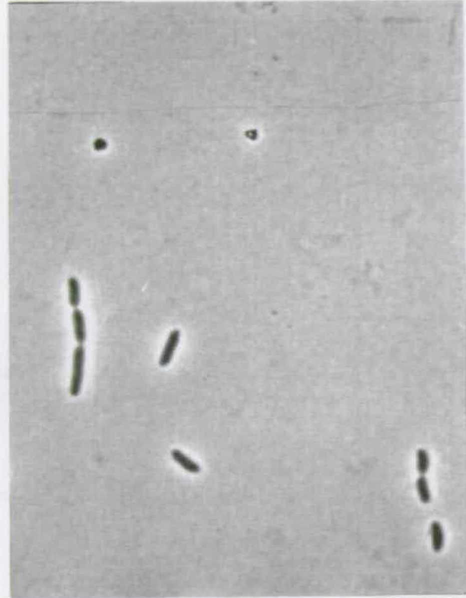


Plate 2.



Notes: Plate 1; TOE23 cells grown at 42°C in L-broth for 90 minutes. Plate 2; TS23 (ftsI23, pAX607) cells grown in L-broth at 42°C for 90 minutes. Photographs taken using phase-contrast microscopy.

4.0.1 Suppression by *sufI*.

A plasmid carrying a 9 kb *Bam*HI chromosomal fragment inserted into pACYC184, (pAX607) was kindly supplied by T.Ogura. This was used for most of the subsequent work on *sufI* because sub-cloning proved difficult. TOE23 was transformed at 30°C with pAX607 and chloramphenicol resistant colonies selected and patched onto LB plates at 30°C and 42°C. Suppression was checked by growth on plates and in liquid medium, and then confirmed by curing the plasmid and re-screening for the recovery of a temperature sensitive phenotype.

TOE23 was suppressed by pAX607, in the presence and absence of chloramphenicol. Liquid cultures shifted to the restrictive temperature clearly demonstrated that the filamentous phenotype of TOE23 was suppressed because the cells grew as chains of short rods (Figure: 4.1).

4.0.2 Deletion Studies.

Suppression of TOE23 by *sufI* was better than that caused by over-production of either PBP5 or PBP6. Yet, like PBP5 suppression, *sufI* suppression was reported to be allele specific (Kato *et al.*, 1988). It was decided to investigate if suppression was also caused by increasing DD-Cpase activity.

Strains were constructed that carried the *ftsI*23 allele together with deletions of the genes for each of the respective low molecular weight PBPs. This was done by transduction of the PBP4 and PBP5 deletions into the TOE23 background. KJB33 was constructed by Ken Begg and is described later.

Table: 4.1 Strains used in deletion studies.

Strain,	Background,	Source	Genotype
DT5	AB2497	This Work	*leu-* ::Tn10 , *ftsI* 23 , *dacA*:::Kan,
KJB33	AB2497	K.Begg	*leu-* ::Tn10 , *ftsI* 23 , *dacC* ,
DT4	ED3184	This Work	*leu-* ::Tn10 , *ftsI* 23, *dacB* ::Sp,

The strains were transformed with pAX607 and colonies selected and streaked to purity. Transformants were checked for suppression, by streaking on LB plates with and without selection at the permissive and restrictive temperatures. The plasmid was then cured and revertants screened for by plating at 30°C and 42°C.

The plasmid pAX607 failed to suppress *ftsI*23 in any of the DD-Cpase deletion backgrounds. This suggested that as with *rodA*sui, suppression was mediated by the activity of DD-Cpases. It was unexpected that PBP4 was required for suppression, as increased levels of PBP4 were unable to extragenically suppress *ftsI*23. It was then decided to assay PBPs in the presence of pAX607 (*sufI*) to ascertain the levels of the major DD-Cpases.

4.1 PBP Assays

TOE23 (*ftsI*23) and the isogenic strain AB2497 were transformed with pAX607. The four strains TOE23, TS23 (*ftsI*23, pAX607), AB2497, and AS2497 (pAX607) were inoculated and grown under non-selective conditions at 30°C. When the cultures reached early log phase they were shifted to 42°C and grown for a further 90 minutes. Fractions of the culture were harvested at both temperatures, membranes prepared, and the concentration of protein estimated by the method of Lowry *et al*, (1951). Penicillin-binding was carried out on 380 μg aliquots of membrane using ^{3}H-PenG at an initial saturating concentration of 28.6 μg/ml (Pierre *at al.*, 1990). Membranes were solubilised by sarkosyl in the presence of unlabelled ampicillin, and the insoluble outer membrane precipitated by centrifugation. The supernatent was added to SDS-PAGE loading buffer and boiled for 5 minutes before aliquots were loaded onto an SDS-PAGE system. Standard SDS-PAGE was performed and the gel was treated with the fluorographic agent PPO before being dried and exposed to pre-flashed film. Exposure was carried out over three time periods and each individual exposure was then subjected to scanning densitometry using a LKB Ultroscan Laser Densitometer.

4.1.1 PBP Densitometry

Quantitative estimation of Penicillin-binding Poteins by scanning densitometry of photographic images is problematical. Several workers have published data using this method, and their results demonstrate a wide degree of variation, produced by differences in media (Dreihaus & Weuters, 1987) or growth rate (Toumanen *et al.*, 1986). More generally problems lie in the assay itself, and particularly the analysis of the image. The fluorographic image is produced by the

formation of stable silver atoms through the absorption of beta-particles. Each silver halide grain has to absorb several particles before it reaches a 50% probability of being developed by processing. Each atom can also undergo thermal decomposition after absorbing the first particle and it is only when a second particle is absorbed that the crystal becomes stable and can accumulate energy as a linear response (Laskey & Mills, 1975). This problem is largely overcome by pre-flashing the film and exposing at -70°C. However if the exposure time is too short or too long the image produced will not have a linear relationship to the amount of radiation producing it.

For the range of levels of PBPs this is a problem and consequently the results of three exposures over 6 hours, 24 hours and 72 hours were used (Figure: 4.2). Possible variations in the amount of sample loaded into each track were corrected by expressing the densities as a proportion of either the total image for each track or as a percentage of the PBP5 band.

The six hour exposure produced inconsistant data. This is clearly demonstrated by comparison of the TOE23 and AB2497 strains carrying pAX607. At the permissive temperature both strains would be expected to have the same proportions of PBP5 and PBP6. This was observed for the 24 and 72 hour exposures, however at 6 hours a large discrepency was apparent. This was considered to result from the non-linearity of the film response to low levels of radiation. The greatest band density corresponded to PBP5 in TS23 grown at 30°C. The absolute values for this band in the respective exposures were 4,284,000 (6 hours), 27,687,000 (24 hours), and 56,797,000 (72 hours). These figures show saturation had not occured by 24 hours. By 72 hours the proportion of PBP6 to PBP5 in most of the tracks had increased by comparison with 24 hours, suggesting the emulsion had approached saturation for PBP5. Therefore the 1 day exposure was considered the most reliable indication of the levels of PBP5 and PBP6.

Key to Figure:

Plate 1: 72 Hour Exposure.

Lane 1; AB2497, 30°C
2; AB2497, 42°C
3; AS2497 (pAX607), 30°C
4; AS2497 (pAX607), 42°C
5; TOE23, 30°C
6; TOE23, 42°C
7; TS23 (pAX607), 30°C
8; TS23 (pAX607), 42°C

Plate 2: 24 Hour Exposure.

Lane 1; AB2497, 30°C
2; AB2497, 42°C
3; AS2497 (pAX607), 30°C
4; AS2497 (pAX607), 42°C
5; TOE23, 30°C
6; TOE23, 42°C
7; TS23 (pAX607), 30°C
8; TS23 (pAX607), 42°C

Figure:4.2 PBP analysis of strains carrying pAX607 (*sufI*).

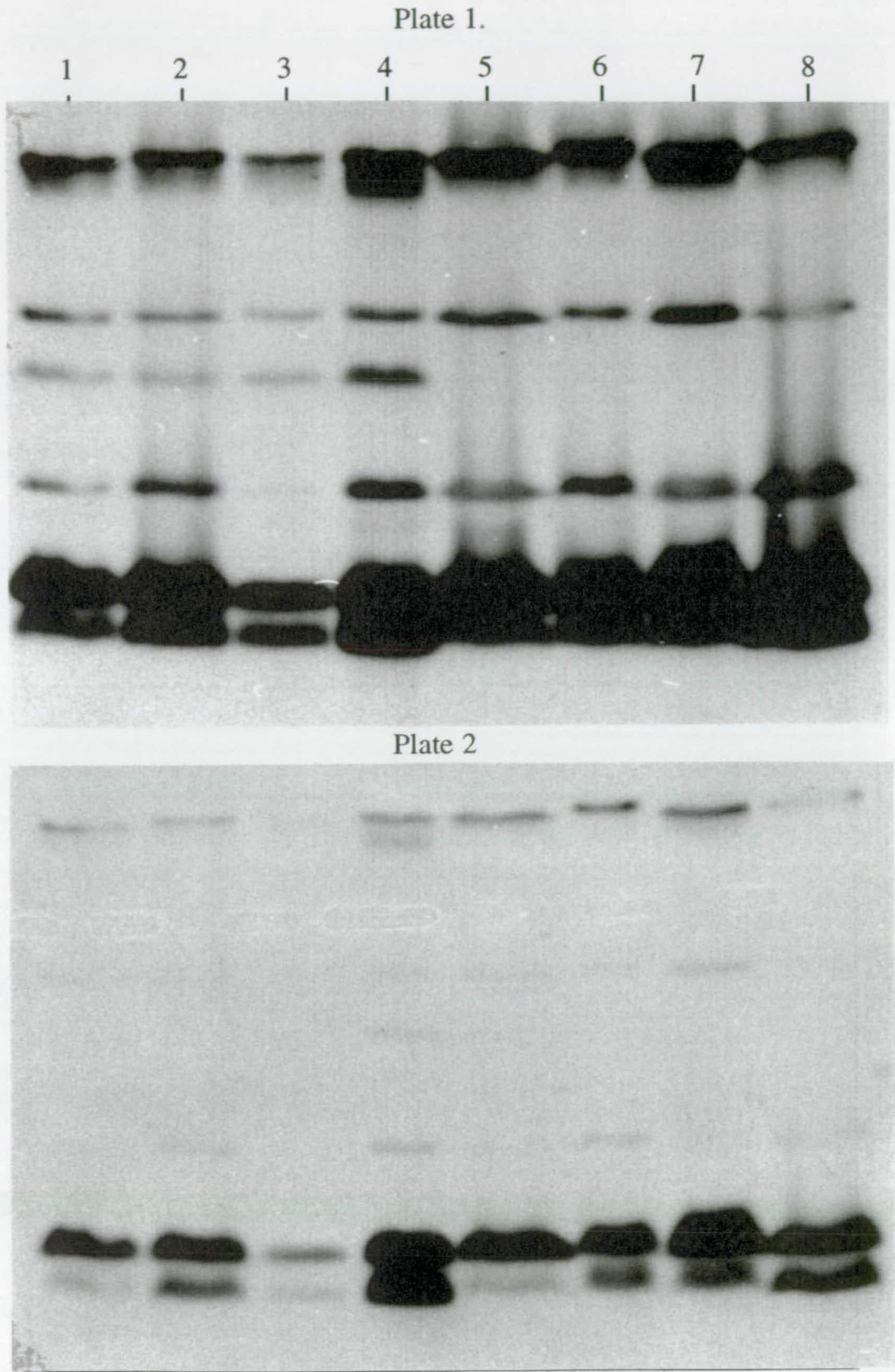


Table: 4.2 Relative Density of PBPs in the presence or absence of pAX607 (Figure:4.2).

Strain	Growth Temp.	PBP5 %of Total	PBP6 %of Total	PBP6 %of PBP5
AB2497	30	93	4	4
AB2497	42	95	3	3
AS 2497	30	85	15	18
AS 2497	42	52	46	90
TOE23	30	92	4	4
TOE23	42	76	17	23
TS 23	30	84	11	13
TS23	42	67	32	47

PBP5 and PBP6 formed over 90% of the image in all the tracks from the 24 hour exposure. Values expressed as either a percentage of total image or as a percentage of the PBP5 band are virtually identical. The PBP6 levels increased in TOE23 at the restrictive temperature, and whenever pAX607 was present. It could not be discounted that these increases represented a decrease in PBP5. However considering the original hypothesis it appeared unlikely that a decrease in DD-Carboxypetidases could result in suppression of *ftsI*23.

Increases in PBP6 levels have been reported in response to differences in media, growth rate or growth phase (Buchanan & Sowell, 1982; Toumanen *et al.*, 1986; Dreihuis & Wouters, 1987). In my experiments the medium was constant, and the culture reaching highest cell density; AB2497, showed no growth phase increase in PBP6. When considering growth rate it was evident the presence of pAX607 caused wild type cells to grow half as fast. By contrast TS23 cells were unaffected, except at the restrictive temperature when suppression enabled this strain to increase its growth in response to temperature. Slow growth has been reported to increase PBP6 (Tuomonen *et al.*, 1986) and therefore could be responsible for increased PBP6 in AS2497. However shifting of this culture to 42°C increased both growth rate and PBP6. This is the opposite effect to that which would be expected if growth rate was responsible for increasing PBP6 levels. Therefore stationary phase cells, media or growth rate did not significantly affect the levels of PBP6 in these experiments.

The presence of pAX607 caused a two to three fold increase in PBP6 when present in the TOE23 background and a four to ten fold increase when present in

AB2497. Change in temperature did not affect PBP6 levels in the wild type control but in TOE23 the reduction of PBP3 activity appeared to produce a two to three fold induction of PBP6 at 42°C. This induction was not sufficient to restore division, suggesting a critical level of PBP6 was required to suppress *ftsI*23. The mechanism by which PBP6 increases upon inactivation of PBP3 or overproduction of SufI is not known. However the situation was similar to the original observation that the amber mutation in *rodA*sui increased levels of PBP2 and PBP5 in KJB1 (Begg *et al.*, 1990). It was possible that some alteration of membrane structure could be responsible for altering the PBP binding profile. This could involve PBP3 inactivation or SufI overproduction affecting membrane topology and increasing accessability of Penicillin to PBP6. Alternatively the same changes may induce increased transcription or translation of *dacC* (PBP6).

4.2 Locating *dacC*.

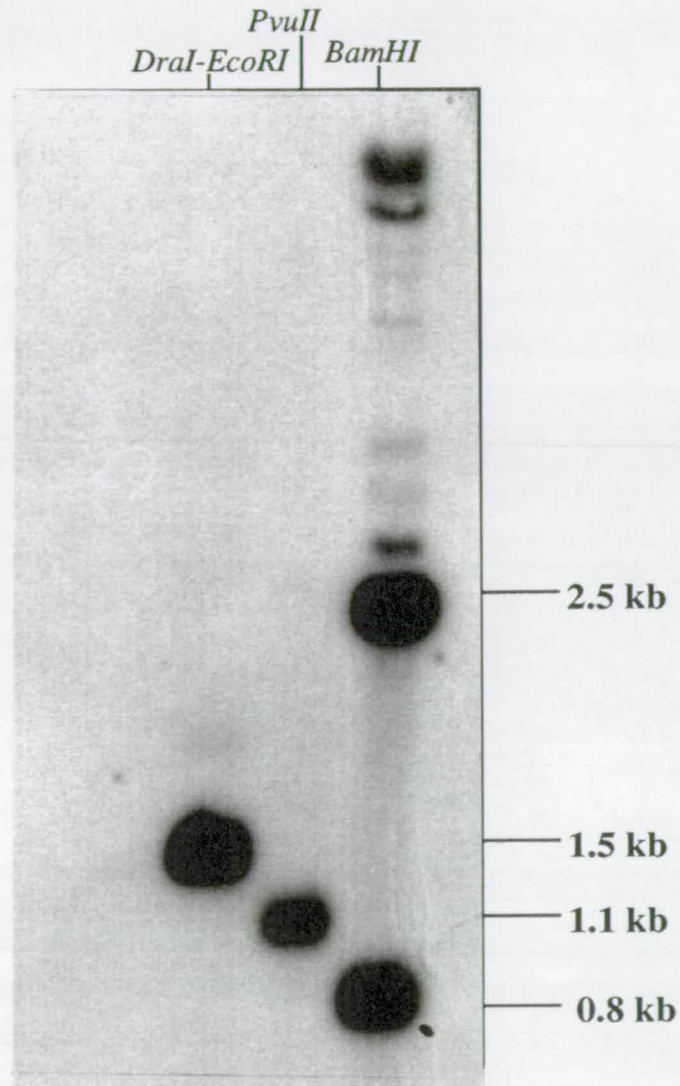
The location of the gene for PBP6; *dacC* was unknown. If transcriptional or translational control of PBP6 levels was responsible for suppression, it might result from location of *dacC* in an operon possibly with *sufI*. To discount such an association it was decided to locate the gene.

A 1.5 kb *DraI-SalI* fragment encoding *dacC* was liberated from pBS110 and purified by gel electrophoresis. This fragment was used to construct a probe by random-priming. The *dacC* probe was then hybridised, under high stringency, with a Kohara Genome Mapping Membrane. The Kohara Genome Mapping Membrane carries Kohara library phage DNA from all regions of the *E.coli* chromosome, laid out in an ordered grid. Once hybridisation was completed the membrane was exposed to pre-flashed film at -70°C overnight, and then developed.

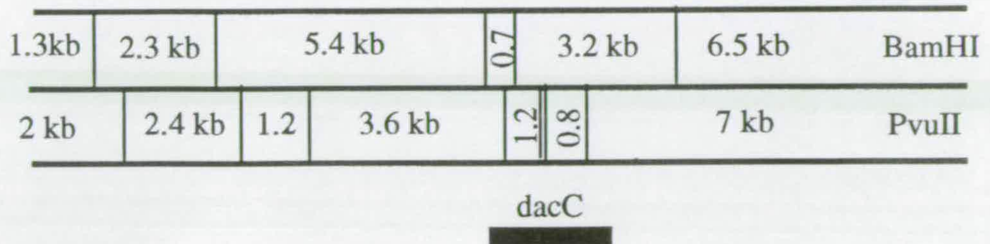
The resulting autoradiogram clearly showed hybridisation to two overlapping phages, 209 and 210. The appropriate phage were then recovered from the Kohara Phage Collection held by M.Masters. the phage was amplified on plates and then used to prepare a maxi-prep of phage DNA.

This DNA was quantified spectrophotometrically, and then subjected to restriction analysis. λ209 DNA was digested in three separate reactions. The first digest involved *BamHI*, the second *PvuII* and the third *DraI* and *SalI*. The resulting fragments were separated by gel electrophoresis and transferred to a Hybond-N nylon membrane. A new *dacC* probe was prepared by random-priming. This was hybridised with the membrane under high stringency, then the membrane was washed and exposed to photographic film (Figure: 4.3).

Figure: 4.3Probing for *dacC*



Restriction Map of the 19 minute region carried by Phage 209



The *dacC* gene has been sequenced (Broome-Smith *et al.*, 1988). Restriction with *DraI* and *SalI* should liberate a 1505 bp fragment carrying the entire *dacC* coding sequence. The probe was constructed using this 1.5 kb *dacC* fragment as a template. A 1.5 kb *DraI-SalI* fragment was released from λ209 DNA, but not λ210. This fragment hybridised with the *dacC* probe. *BamHI* cuts once within *dacC* at base 302. Two positive signals were obtained for the *BamHI* digest of λ209. These corresponded to 2.5 kb and 0.8 kb pieces of DNA. For the 19 minute region of the chromosome carried by λ209 digestion with *BamHI* is expected to liberate 5 products. One of these is predicted to be 0.7 kb and approximates to the 0.8 kb signal. The two *BamHI* fragments that lie adjacent to the 0.8 kb fragment have predicted sizes of 3.2 kb and 5.5 kb. The expected 3.2 kb probably corresponds to the 2.5 kb band observed. This places the *dacC* gene in the region shown in figure 4.3.

PvuII cuts twice in *dacC*, at bases 1094 and 1306. It would therefore be expected that 3 bands carrying part of the *dacC* coding region would be liberated by a *PvuII* digest of λ209. No 0.2 kb fragment was observed by restriction analysis, this was probably due to the band running off the gel during electrophoresis. A 1.1 kb fragment which overlaps the region covered by the 0.7 kb and 3.2 kb *BamHI* fragments did hybridise with the probe. The third band that was expected to hybridise with the probe had a predicted size of 0.8 kb. This band was not visible on the autoradiogram, but was visable on the original gel. It is not known why it did not hybridise with *dacC*.

Restriction analysis confirmed the inserts of λ209 and λ210 to correspond to the 19 minute region of the *E.coli* chromosome. Probing placed *dacC* on the insert carried by Kohara phage 209, but not 210. More precisely *dacC* was shown to overlap the 0.7 kb and 3.2 kb *BamHI* fragments at 19 minutes. The probing of a *PvuII* digest partially confirmed this, and the location was reported in the review of Holtje & Tuomanen (1991). Subsequent work mapping the *dacC* restriction pattern by computer analysis (K.Rudd) has placed the *dacC* gene at the same location.

The probing showed that *dacC* is not located adjacent to *sufI* and does not form a transcriptional unit with any known cell division or peptidoglycan gene.

4.3 DD-carboxypeptidase activity

The genetic studies and PBP assays provide evidence that an increased level of DD-Cpase activity was involved in SufI suppression. The next stage was to establish if strains carrying pAX607 actually exhibited increased membrane associated DD-Cpase activity. Therefore during two visits to the M.P.I. fur

Entwicklungsbiologie in Tubingen it was attempted to examine DD-Cpase activity and the murein synthesised in vivo by strains carrying pAX607.

Two test and two control strains were used to examine the affect of pAX607 on membrane-associated DD-Cpase activity. The control strains were, AB2497; the standard background, and D456. D456 is a derivative of SP1070 which has deletions of *dacA* and *dacC* (Broome-Smith, 1985). In addition, D456 carries a deletion in the third proposed DD-Cpase PBP4 (*dacB*). The construction of D456 is described in Chapter 5. Consequently this strain should lack all known DD-Cpase activity, and therefore provide an ideal background to examine the possibility that the 9 kb insert in pAX607 itself encoded a protein with DD-Cpase activity. The two test strains were pAX607 transformants of AB2497 and D456.

Table: 4.3 DD-Cpase assays in the presence and absence of pAX307.

Strain	Presence of pAX607 (SufI)	% Release of D-Alanine.
AB2497	-	35.73
AS2497	+	74.38
D456 (Δ*dacA*, Δ*dacB*, Δ*dacC*)	-	3.15
DS456	+	6.13
Control (No Membs)		2.68

Membrane bound DD-Cpase activity was assayed by A.Ursinus using two separate substrates. UDP-muramyl-pentapeptide, derived from the bacterial cell wall and the synthetic substrate bisacetyl-L-Lys-D-Ala-D-Ala. Only assays using UDP-muramyl pentapeptide demonstrated increased DD-Cpase activity.

PBP5 has been consistently reported to release D-alanine from both UDP-muramyl-pentapeptide and bisacetyl-L-Lys-D-Ala-D-Ala. PBP6 was twice demonstrated to have DD-Cpase activity in vitro by Amanuma & Strominger (1980 & 1984). However Glauner (1986) failed to show any alteration in peptidoglycan consistent with reduced DD-Cpase activity in a PBP6 deletion strain. Similarly Van der Linden *et al.* (1992) could not demonstrate a PBP6 overproducing strain to have any increased DD-Cpase activity. The substrate specificity observed in these assays would therefore be consistent with the problems reported demonstrating DD-Cpase activity for PBP6.

The increase in PBP6 previously observed in AS2497 was approximately 3 to 4 fold. The increase in membrane bound DD-Cpase activity was two fold. The presence of pAX607 in D456 also produced an increase in DD-Cpase activity.

However it is not clear whether the low levels of D-alanine released in this background was caused by any DD-Cpase activity, because the detected values were so close to the values for the substrate-only control. If this did represent an increase in DD-Cpase it appeared too small to be significant in suppression. Consequently the possibility of a DD-Cpase being encoded by pAX607 was discounted.

4.4 Murein Analysis.

Once it had been established that the presence of pAX607 was accompanied by both an increase in the levels of PBP6 and membrane bound DD-Cpase it was considered logical to examine its effect on the murein synthesised in vivo. HPLC of isolated sacculi degraded with muramidase was first performed by Glauner (1986) at the M.P.I. in Tubingen. During my two visits murein analysis was attempted on the four strains TOE23, AB2497, TS23, and AS2497 (Tables: 4.4–4.6). To complement the original PBP assays membranes were isolated from strains grown at the restrictive and permissive temperatures. Growth conditions and harvesting of samples were identical to those used for the PBP assays

4.4.1 Isolation of Peptidoglycan.

Murein from AB2497 could not be obtained during three separate attempts because of a failure to pellet enough material during the washing procedure. All the strains used were in the same background and for each the yield of murein was lower than expected. In the absence of figures for AB2497 it is impossible to accurately determine the effect of temperature shifts on overall murein composition, however some information exists in the thesis of Glauner (1986) where three wild type backgrounds were analysed at 30°C and 42°C. The composition of TOE23 murein at the permissive temperature also provided an indication of wild type values.

The presence of pAX607 enabled TOE23 cells to divide and form typical chains at the restrictive temperature. This was accompanied by a 3 fold increase in PBP6 and 2 fold increase in membrane bound DD-Cpase activity. Any analysis of the murein figures obtained had to consider that only two replicate assays were completed and that values for some components of the sacculus were not reproducible. This was particularly true of minor components, for which small changes in absolute values are amplified when expressed as a proportion. Therefore I considered only consistent changes in relatively abundant murein components as an indication of events in vivo.

Table: 4.4 Murein Analysis of T23 at the Permissive and Restrictive Temperature.

PEPTIDOGLYCAN	30°C	42°C	30°C	42°C	% Change
MONOMERS					
Total	48.38	40.02	52.02	45.99	**-14.3**
Tripeptides	10.50	5.17	6.86	4.56	**-44**
Tetrapeptides	33.62	26.32	38.16	30.30	**-21.2**
Pentapeptides	0.04	0.47	0.07	0.14	**+455**
Chain-ends	2.86	2.72	4.43	6.42	+25.3
anhydro	0.87	0.35	0.94	0.96	-27.6
DIMER					
Total	41.62	47.30	42.74	47.11	**+11.9**
Ala-Dap			39.44	43.10	
Dap-Dap			3.10	3.68	
Chain-ends	3.56	4.93	4.06	5.67	**+39**
Tripeptide acceptors	1.36	5.40	4.33	3.33	+53
anhydro	4.79	7.75	3.35	4.78	+54
TRIMER					
Total	9.82	11.99	5.05	6.48	**+24.2**
Ala-Dap	8.24	9.75	4.75	5.69	**+18.9**
Dap-Dap	1.85	2.92	0.30	0.79	**+72**
Chain-ends	1.14	1.45	0.19	0.42	**+40**
Tripeptide acceptors	1.80	1.80	0.42	0.24	**-8**
anhydro	2.91	4.64	1.64	2.17	**+49**
TETRAMERS					
Total	0.18	0.51	0.20	0.42	**+144**
Anhydro	0.18	0.51	0.10	0.22	+161
PENTAPEPTIDES					
Total	0.88	1.84	0.75	1.82	**+125**
Monomer	0.40	1.19	0.26	0.83	**+206**
Dimer	0.48	0.65	0.49	0.99	**+69**
Trimer					
CHAIN-ENDS					
Total	4.33	6.01	3.18	4.12	**+35**
Monomer	0.87	0.35	0.94	0.96	-27.7
Dimer	2.53	4.21	1.67	2.39	+57

PEPTIDOGLYCAN	30°C	42°C	30°C	42°C	Change
CHAIN-ENDS					
Dimer Ala-Dap	2.19	3.55	1.43	2.07	+55
Dimer Dap-Dap	0.61	1.34	0.24	0.32	+95
Trimer	1.16	2.00	0.55	0.72	+59
Tetramer	0.09	0.25	0.05	0.11	+157
LIPOPROTEINS					
Total	4.79	5.10	6.53	9.40	**+28**
Monomer	2.86	2.72	4.43	6.42	+25.4
Dimer	1.64	2.13	2.03	2.83	+35
Trimer	0.29	0.26	0.06	0.14	+14.3
GLYCINE					
Total	0.76	5.79	2.10	4.15	**+248**
Position 4	0.10	4.85	1.73	3.01	+329
Monomer	0.36	3.96	1.63	2.66	+233
Dimer	0.40	1.83	0.47	1.49	+282
TRIPEP Disacc					
Total	1.05	2.73	2.30	1.74	+33
Dimer	0.54	2.36	2.16	1.66	+49
Dimer Ala-Dap	0.21	2.07	2.11	1.62	+59
Dimer Dap-Dap	0.33	0.29	0.05	0.05	-11
Trimer	0.51	0.37	0.14	0.08	-31
CROSS-LINKAGE					
Total	27.49	32.03	24.88	28.19	**+15**
% Ala-Dap			23.13	25.92	
% Dap-Dap			3.30	4.48	
% Tri-Lys Arg	45.10	57.83	32.09	31.65	**+15.9**
% Anhydro			70.52	76.82	
% Tripeptide Acc			29.52	18.14	

Abbreviations; Monomer, GlcNAc-β-1,4 MurNAc peptide. Dimer, bis(GlcNAc-β-1,4 MurNAc peptide). Trimer, Tris(GlcNAc-β-1,4 MurNAc peptide). Tetramer, Tetrakis(GlcNAc-β-1,4 MurNAc peptide). Tripeptides, L-Ala-D-Glu-*m*-DAP. Tetrapaptides, L-Ala-D-Glu-*m*-DAP-D-Ala. Pentapeptides, L-Ala-D-Glu-*m*-DAP-D-Ala-D-Ala. Lys-Arg componds, muropeptides carrying L-Ala-D-Glu-*m*-DAP-L-Lys-L-Arg peptide moities. Anhydro Compounds, 1,6-anhydromuramic containing residues.

Table: 4.4 Murein Analysis of TS23 at the Permissive and Restrictive Temperature.

PEPTIDOGLYCAN	30°C	42°C	30°C	42°C	% Change
MONOMERS					
Total	44.66	43.32	48.89	46.76	**-3.7**
Tripeptides	6.52	6.76	4.89	4.17	-4.3
Tetrapeptides	33.25	30.37	33.92	33.22	**-5.3**
Pentapeptides	0.17	0.73	0.05	0.11	**+282**
Chain-ends	1.73	1.76	6.68	5.21	-17.2
anhydro	0.52	0.39	0.70	0.14	**-56**
DIMER					
Total	47.18	45.09	45.99	46.69	-0.1
Ala-Dap			40.89	42.33	
Dap-Dap			4.65	42.33	
Chain-ends	3.32	3.65	4.48	4.71	**+7.2**
Tripeptide acceptors	4.12	3.62	2.20	2.80	+1.5
anhydro	6.02	5.40	4.72	3.98	-12.7
TRIMER					
Total	8.16	11.24	4.91	6.20	**+33.4**
Ala-Dap	7.08	7.59	4.26	5.7	**+17.2**
Dap-Dap	1.35	4.05	0.65	0.5	+127.5
Chain-ends	0.68	0.93	0.24	0.29	+32.6
Tripeptide acceptors	0.91	2.62	0.42	0.55	+138
anhydro	2.36	5.25	1.53	2.04	+87.4
TETRAMERS					
Total	0	0.36	0.21	0.35	
Anhydro	0	0.29	0.12	0.14	
PENTAPEPTIDES					
Total	1.12	1.51	0.89	1.42	**+42.2**
Monomer	0.52	1.26	0.05	0.11	**+140**
Dimer	0.60	0.25	0.83	1.32	+9.8
Trimer					
CHAIN-ENDS					
Total	4.36	4.98	3.61	2.84	**-1.9**
Monomer	0.52	0.39	0.70	0.14	-56.6
Dimer	3.14	2.90	2.36	1.99	-11.1

PEPTIDOGLYCAN	30°C	42°C	30°C	42°C	Change
CHAIN-ENDS					
Dimer Ala-Dap	2.44	2.25	1.97	1.75	-9.3
Dimer Dap-Dap	0.98	1.05	0.39	0.24	-5.8
Trimer	0.97	2.02	0.51	0.68	+82
Tetramer	0	0.14	0.06	0.07	
LIPOPROTEINS					
Total	3.39	3.56	9	7.66	**-9.5**
Monomer	1.73	1.76	6.68	5.21	-17.1
Dimer	1.53	1.62	2.24	2.35	+5.3
Trimer	0.14	0.18	0.08	0.10	+27.3
GLYCINE					
Total	4.12	4.06	3.21	3.11	**-2.2**
Position 4	3.61	3.42	3.08	2.86	-6.1
Monomer	2.51	2.86	2.12	1.95	+3.9
Dimer	1.62	1.20	1.09	1.17	-12.6
TRIPEP disacc					
Total	2.14	2.35	1.24	1.58	**+16.8**
Dimer	1.93	1.61	1.10	1.40	-0.6
Dimer Ala-Dap	1.63	1.38	1.04	1.33	+1.5
Dimer Dap-Dap	0.30	0.23	0.05	0.07	-14
Trimer	0.21	0.74	0.14	0.18	+163
CROSS-LINKAGE					
Total	29.03	30.31	26.43	27.74	**+4.6**
% Ala-Dap			23.64	25.40	
% Dap-Dap			5.31	4.52	
% Tri-Lys Arg	55.64	59.96	25.78	31.96	**+12.9**
% Anhydro			80.46	95.21	
% Tripeptide Acc			19.91	23.90	

Abbreviations; Monomer, GlcNAc-β-1,4 MurNAc peptide. Dimer, bis(GlcNAc-β-1,4 MurNAc peptide). Trimer, Tris(GlcNAc-β-1,4 MurNAc peptide). Tetramer, Tetrakis(GlcNAc-β-1,4 MurNAc peptide). Tripeptides, L-Ala-D-Glu-*m*-DAP. Tetrapaptides, L-Ala-D-Glu-*m*-DAP-D-Ala. Pentapeptides, L-Ala-D-Glu-*m*-DAP-D-Ala-D-Ala. Lys-Arg componds, muropeptides carrying L-Ala-D-Glu-*m*-DAP-L-Lys-L-Arg peptide moities. Anhydro Compounds, 1,6-anhydromuramic containing residues.

Table: 4.5 Murein Analysis of AS2497 at the Permissive and Restrictive Temperature.

PEPTIDOGLYCAN	30°C	42°C	30°C	42°C	% Change
MONOMERS					
Total	48.04	49.42	52.39	50.28	**-0.8**
Tripeptides	6.55	7.93	6.07	5.77	+8
Tetrapeptides	31.66	31.83	39.96	37.41	+3.43
Pentapeptides	0.12	0.12	0.07	0.07	**0**
Chain-ends	4.14	4.19	5.98	3.74	-21.6
anhydro	0.48	0.68	0.99	1.08	+19.7
DIMER					
Total	42.89	41.75	43.38	44.21	**-0.04**
Ala-Dap			39.36	40.05	
Dap-Dap			3.70	3.73	
Chain-ends	2.35	2.40	3.61	3.16	-6.7
Tripeptide acceptors	4.89	5.10	3.81	5.10	+17.2
anhydro	4.03	3.18	3.28	3.65	-6.6
TRIMER					
Total	8.77	8.74	4.09	5.29	**+9.1**
Ala-Dap	7.15	6.50	3.84	4.94	+4.1
Dap-Dap	1.62	2.34	0.26	0.36	+43.6
Chain-ends	0.89	0.75	0.16	0.19	-10.1
Tripeptide acceptors	1.06	2.43	0.31	0.51	+114
anhydro	2.67	2.84	1.17	1.55	+14.3
TETRAMERS					
Total	0.30	0.09	0.13	0.22	**-6**
Anhydro	0.27	0.09	0.07	0.11	-41.2
PENTAPEPTIDES					
Total	0.74	0.97	1.31	1.45	**+18**
Monomer	0.54	0.57	0.46	0.63	+20
Dimer	0.20	0.41	0.85	0.82	+17
Trimer	-	-	-	-	-
CHAIN-ENDS					
Total	3.47	3.23	3.03	3.45	**+5.1**
Monomer	0.48	0.66	0.99	1.08	+18.4
Dimer	2.08	1.64	1.64	1.82	-7

PEPTIDOGLYCAN	30°C	42°C	30°C	42°C	Change
CHAIN-ENDS					
Dimer Ala-Dap	1.84	1.29	1.33	1.48	-12.6
Dimer Dap-Dap	0.37	0.45	0.31	0.34	+16.2
Trimer	0.98	1.01	0.39	0.52	+11.7
Tetramer	0.13	0.04	0.03	0.05	-44
LIPOPROTEINS					
Total	5.50	5.56	7.84	5.39	**-18**
Monomer	4.14	4.19	5.98	3.74	-21.6
Dimer	1.11	1.15	1.80	1.58	-6.2
Trimer	0.25	0.22	0.05	0.06	-6.7
GLYCINE					
Total	5.40	5.15	3.23	2.45	**-12**
Position 4	4.78	4.41	2.69	1.68	-18.4
Monomer	3.86	3.54	2.21	1.93	-9.9
Dimer	1.54	1.61	1.02	0.52	-16
TRIPEPTIDE disacc					
Total	2.69	3.28	2.01	2.72	**+27**
Dimer	2.38	2.50	1.91	2.55	+17.8
Dimer Ala-Dap	2.38	2.19	1.58	2.23	+11.6
Dimer Dap-Dap	0	0.31	0.32	0.32	
Trimer	0.31	0.78	0.10	0.17	+132
CROSS-LINKAGE					
Total	27.52	26.77	24.52	25.80	**+1**
% Ala-Dap			22.42	23.60	
% Dap-Dap			3.96	4.08	
% Tri-Lys Arg	26.75	26.07	23.68	30.56	**+12.3**
% Anhydro			67.50	68.64	
% Tripeptide Acc			30.33	32.60	

Abbreviations; Monomer, GlcNAc-β-1,4 MurNAc peptide. Dimer, bis(GlcNAc-β-1,4 MurNAc peptide). Trimer, Tris(GlcNAc-β-1,4 MurNAc peptide). Tetramer, Tetrakis(GlcNAc-β-1,4 MurNAc peptide). Tripeptides, L-Ala-D-Glu-*m*-DAP. Tetrapaptides, L-Ala-D-Glu-*m*-DAP-D-Ala. Pentapeptides, L-Ala-D-Glu-*m*-DAP-D-Ala-D-Ala. Lys-Arg componds, muropeptides carrying L-Ala-D-Glu-*m*-DAP-L-Lys-L-Arg peptide moities. Anhydro Compounds, 1,6-anhydromuramic containing residues.

4.4.2 Cross-linkage.

Upon shifting to the restrictive temperature, TOE23 shows a 14% reduction in monomeric uncrosslinked sub-units and a concomitant 12% increase in dimers.

Filaments formed by two strains with temperature sensitive *ftsI* alleles and one strain (PAT84) with the *ftsZ*84 allele were examined by Glauner (1986). The PAT84 filaments showed no shift from monomers to dimers, indicating that this change is not simply the result of filamentous growth. One of the *ftsI* mutant strains (SP63) showed a 4% reduction in monomers and 6% increase in dimers at the restrictive temperature. By contrast the second *ftsI* strain, JE5703, showed no increase in cross-linkage. This strain is from the heavily nitrosoguanidine mutagenised Hirota collection, and secondary mutations have been isolated from such backgrounds (Glauner, 1986). Therefore secondary mutations maybe responsible for its unexpected behavoir.

The shift from monomers to dimers in this study is of a higher magnitude than that reported by Glauner (1986). It was considered that this reflected differences between the two *ftsI* mutations, and indeed recent work has suggested that important differences do exist. SP63, like TOE23, exhibits, a temperature sensitive PBP3 in penicillin-binding assays, but, unlike, TOE23 it cannot be complemented by a plasmid carrying *ftsI*$^{+}$. SP63 can be suppressed by a plasmid carrying an unsequenced region of the *E.coli* chromosome around 12 minutes (M.Khattar *pers.comm.*). It therefore seems likely that SP63, the strain studied by Glauner, is not an *ftsI* mutant, but does affect penicillin-binding of PBP3.

4.4.3 Monomers & Dimers.

The shift from monomers to dimers in TOE23 was reflected in the 14% overall increase in cross-linkage. The change in the profile of monomers when PBP3 is inactivated is quite distinctive. Consistently tripeptides and tetrapeptides are depleted while pentapeptides accumulate. Monomeric pentapeptides are characteristic of newly inserted murein (Glauner & Holtje, 1990) and their accumulation can be considered to represent a reduction in DD-Cpase activity or transpeptidase activity. The occurence of decreased transpeptidase activity would not be consistent with the continued growth of the cell and the 14% increase in overall cross-linkage. It is more likely that the monomeric pentapeptides represent a decrease in DD-Cpase activity that accompanies PBP3 inactivation. Therefore it is proposed that these monomeric pentapeptides represent a sub-set of peptidoglycan dependent on PBP3 for their incorporation. Their location and accumulation would be determined at a stage in septum formation prior to or co-incident with PBP3 action. They could represent either donor side chains which would be hydrolysed during transpeptidation, or

potential acceptors hydrolysed to tripeptides prior to cross-linkage. In the latter situation a co-ordinated DD-Cpase activity would be required to produce tripeptides, and PBP6 would be a good candidate. This is an argument that will be returned to later.

When considering the accumulation of dimers, it is more difficult to draw any conclusions as only one clear analysis of constituent dimers was obtained. This analysis suggested that both tetra-tri and Dap-Dap bonds increased at the restrictive temperature. The Dap-Dap bonds are the product of a penicillin-insensitive LD-transpeptidase and are considered to increase as part of a rescue mechanism when the cells' integrity is threatened by a weakening of the cell wall (Kraus & Holtje, 1987). This has been demonstrated by using sub-inhibitory concentrations of penicillin, a situation in which division is blocked by inhibition of PBP3 but lateral cell wall synthesis continues. Such a situation is analogous to that in *ftsI*23 filaments at the restrictive temperature and it would therefore be reasonable to expect increased LD-transpeptidation to result from the same rescue mechanism.

It would be predicted from the preferred acceptor hypothesis that tripeptides involved in DD-cross-bridges would decrease upon inactivation of PBP3. Consequently the increase in DD-tetra-tri bonds was unexpected. The increase does not appear to be the result of increased lateral cell wall synthesis as it does not occur in *ftsZ* blocked filaments (Glauner, 1986). Pulse labelling of synchronised cultures with H^3-DAP has shown no increase in tetra-tri cross-bridges during division (de Jonge, 1989). Therefore it would appear that a temporary accumulation of tetra-tri cross-bridges occurs between the action of *ftsZ* and PBP3. The problem remains that this interpretation is dependent upon only a single analysis.

The difference between the effects of FtsZ and PBP3 inactivation suggests that an intermediate system of peptidoglycan synthesis exists. This maybe the same as Penicillin Insensitive Peptidoglycan Synthesis (PIPS) (Nanninga, 1991). PIPS was proposed to explain the continued incorporation of H^3-DAP at the leading edge of a cell constriction when PBP3 activity is blocked (Wientjes & Nanninga, 1989). This localised incorporation of peptidoglycan at constriction sites was first demonstrated by Woldringh *et al.* (1987) using autoradiography of H^3-DAP pulse labelled cells. Subsequent work using pulse labelling (de Jonge, 1989), showed that dividing cells accumulate tetra-tetra bonds between newly inserted peptidoglycan strands. This led to the suggestion that synthesis at the leading edge of a constriction involved the incorporation of double strands, or the insertion of single strands that are rapidly cross-linked together. This characteristic formation of tetra-tetra bonds also occurs

with filaments blocked by inactivation of *ftsA* (de Jonge, 1989). This indicated the insertion of double stranded peptidoglycan to be an early event in septation, concomitant with PIPs activity. PIPS might therefore explain the shift from monomers to dimers in TOE23 filaments, and the same effect in *envA* mutants (Romeis *et al.*, 1990). Implicit in this explanation is that *ftsZ* is required to initiate PIPS activity. Therefore continued PIPS activity and incorporation of rapidly cross-linked peptidoglycan was considered the most likely cause of the shift to dimers.

4.4.4 Pentapeptides.

The accumulation of monomeric pentapeptides, the characteristic sub-unit of newly inserted murein, is unexpected. The shift to dimers and a more cross-linked sacculus would be expected to involve increased depletion of donor pentapeptides. Normally pentapeptides face rapid decay with 90% being degraded during the first 60 seconds in the periplasm (Glauner & Holtje, 1990). The murein synthesised by ether permeabilised cells treated with the PBP3 specific antibiotic furazlocillin also show an increase in pentapeptide side chains (Kraus & Holtje, 1987).

In Penicillin G treated-cells, division is blocked by inactivation of PBP3 and cells ultimately autolyse. During this process pentapeptides transiently increase before being rapidly degraded to tripeptides (Kohlrausch & Holtje, 1991). This transient increase coresponds to PBP3 inactivation and is similar to the increase in ether permeabilised cells and *ftsI*23 filaments.

Therefore the accumulation of monomeric pentapeptides upon inactivation of PBP3 appears a consistent phenomena. A reduction in PBP3 mediated transpeptidation cannot be responsible because the total level of transpeptidation actually increases in *ftsI*23 filaments. The most likely cause of increased monomeric pentapeptides is reduced DD-Cpase activity. Reduced DD-Cpase activity has been demonstrated for filaments produced by either the addition of cephalosporin, or treatment with naladixic acid, or inactivation of *ftsZ* (Mirrelman *et al.*, 1977). In *ftsZ* filaments and amino acid starved cells (Mirelman *et al.*,1976; Harkness *et al.*, 1981) reduced DD-Cpase activity is associated with a fraction of the enzyme becoming cryptic. This could represent the same fraction of enzyme activity lost by PBP3 inactivation but released upon autolysis with Pen-G.

It would therefore appear that reduction in activity of a PBP3 dependent DD-Cpase is responsible for the build up of monomeric pentapeptides observed in *ftI*23 filaments. Such a link between DD-Cpase activity and PBP3 supports the connection proposed in the preferred acceptor hypothesis. Since PBP3 activity is spatially restricted to the septum it would be predicted that the accumulation of monomeric

pentapeptides would be localised. This would also be consistent with the observation that murein incorporation is localised in *ftsI* filaments, *ftsA* filaments and furazlocillin produced filaments (Woldringh *et al.*,1987).

Besides PBP5, PBP6 and PBP3, other PBPs have been inferred to share some degree of regulatory control. For example the levels of PBP2 and PBP5 (Begg *et al.*, 1990) increase in the presence of *rodA*sui, and a deletion in the *mre* locus can lead to increased PBP1B and PBP3 (Okada *et al.*, 1992). Circumstantial evidence exists in the literature that links PBP3 and PBP6. The first in vitro demonstration of purified PBP3 activity (Ishino & Matsuhashi, 1981) involved the isolation of a contaminating low molecular weight protein with penicillin-binding activity. Its weight corresponded to PBP6 but the authors reported a different β-lactam binding profile to that expected for PBP6, and considered this protein to be a break-down product of PBP3. An increase in PBP6 and a simultaneous reduction in PBP3 has been reported for slow growing (Dreihuis & Wouters,1982) and stationary-phase cultures (Buchanan & Sowell, 1982). It has been demonstrated in this work that the level of PBP6 increases when a temperature sensitive PBP3 was inactivated. In addition to its identification as a suppressor of *ftsI*23, PBP6 can be implicated in septation through work on a dispensible DNA binding protein; BolA.

BolA is a small 15 kd protein with characteristic helix-turn-helix motif which acts as a positive regulator of PBP6 (Aldea *et al.*, 1989). It has been demonstrated to increase the levels of PBP6 upon entry into stationary-phase. The *bolA* gene is itself under positive regulation by the stationary-phase sigma RpoS (Lange & Hengge-Aronis, 1991). When *bolA* is present uninduced on a multi-copy plasmid it causes stationary cells to become spherical, possibly through the action of PBP6. When *bolA* is induced in exponential phase cells it produces osmotically stable, mecillinam sensitive, spheres. It is reported that this shape change requires a functional FtsZ but not FtsQ, FtsA, PBP3, PBP5 or PBP6 (Aldea *et al.*, 1988). Therefore BolA is suggested to act on some intermediate process in early constriction, and PIPS is a good candidate. The link betwen *bolA* and septum synthesis is proposed to involve BolA recognising specific "gearbox" consesus sequences within promoters. Such sequences have been identified in the promoter region of *ftsQ* (Aldea *et al.*, 1989). Positive regulation of *bolA* by *rpoS* has been demonstrated upon entry into stationary-phase. This is co-incident with increased division at the end of exponential-phase growth. Therefore it has been suggested that increased BolA is responsible for increased transcription of several genes that are required for division and these include *dacC* (PBP6).

In *Bacillus subtilis,* two sporulation-specific PBPs have been identified. These PBPs are responsible for the construction of peptidoglycan in the spore cortex. This peptidoglycan forms a multilayered and hemispherical spore wall. The first sporulation-specific PBP is coded for by *spoVB*, which lies adjacent to the *pbpB* gene which encodes the septum-specific homologue of the *E.coli ftsI* gene (Yanouri *et al.*, in press). Its similarity in size and structure to *pbpB* has led to the suggestion that it may have arisen from duplication of *pbpB.* The second sporulation specific enzyme is PBP5*, a low molecular weight PBP with demonstrable DD-Cpase activity in vivo (Buchanan & Ling, 1992). PBP5*, like PBP6 in *E.coli*, is estimated to be responsible for a minor fraction of the total DD-Cpase activity observed in vivo. This is another situation in which DD-Cpase activity is found to be associated with a specialised transpeptidase activity (related to PBP3 of *E.coli.*).

A cryptic fraction of DD-Cpase activity that requires sonication to be released has been described (Harkness *et al.*, 1981; Mirelman *et al.*, 1977). PBP5 and PBP6 are the two major DD-Cpases in *E.coli* (Amanuma & Strominger, 1984). Overproduction and deletion of PBP5 causes the expected alterations in murein composition (Glauner, 1986). Deletion of PBP6 has no distinct affect on pentapeptide levels (Glauner, 1986) and its overproduction produces none of the morphological changes associated with high DD-Cpase activity in vivo (Van der Linden *et al.*, 1991). In these assays murein synthesised by membranes exhibiting increased PBP6 levels were enriched in pentapeptides. Therefore it would appear PBP6 is regulated at the functional level, an assumption which would explain the difficulty in demonstrating PBP6 DD-Cpase activity in vitro (Van der Linden *et al.*, 1991). Such a regulated DD-Cpase would be a good candidate for the cryptic DD-Cpase activity that has been reported for cells blocked in division (Mirelman *et al.*, 1977). This represents the final piece of evidence for suggesting PBP6 is involved in cell division.

4.4.5 Suppression.

In TS23 cultures the composition of the murein shows less change than T23 at the restrictive temperature. Small changes did occur in the levels of monomers, dimers and trimers but within the range of values exhibited by the control strain AS2497. In some cases (*eg.* dimers) these changes are not the same for the two replicates. The extent of overall cross-linkage did not indicate any gross increase in transpeptidation. Consequently SufI suppression appears to restore normal peptidoglycan synthesis.

The only distinct and clear difference between the behaviour of AS2497 and TS23 at 42°C was the accummulation of monomeric pentapeptides by the strain harbouring the temperature sensitive PBP3. Over the two membrane preparations this

accumulation of monomeric pentapeptides by TS23 was aproximately half that of the *ftsI23* filaments. This was consistent with the approximate 2-, to 4-fold increases in the levels of DD-Cpase and PBP6 levels.

AS2497 also exhibited an increase in PBP6 when shifted from 30°C to 42°C, and a demonstrably higher level of DD-Cpase activity than AB2497, but did not show the expected decrease in pentapeptides. This was identical to the results at the restrictive temperature for TOE23 (*ftsI23*), when an increase in PBP6 levels was accompanied by an increase in monomeric pentapeptides. Therefore increased levels of PBP6 do not automatically lead to increased DD-Cpase activity in vivo. This may be due to the enzyme activity remaining cryptic (Mirelman *et al.*, 1977; Harkness *et al.*, 1981.) in the presence of some secondary regulator. In these experiments the only increase in DD-Cpase activity in vivo required overproduction of SufI and inactivation of PBP3.

4.4.6 Summary

The development of a septum progresses through successive stages. These begin with the formation of an FtsZ ring (Lutkenhaus 1993), followed by induction of PIPS and the associated rapid incorporation of tet-tet crosslinked peptidoglycan. Septum formation then progresses through an FtsQ dependent stage to a point where PBP3-FtsA mediated synthesis begins.

Suppression of *ftsI23* by increased levels of PBP5, PBP6, and SufI have been shown in this work. It has also been demonstrated that increasing the level of cytoplasmic tripeptides with D-cycloserine can suppress *ftsI23* (Begg *et al.*, 1990). Consequently it is proposed that DD-Cpase and LD-Cpase activity is required to supply tripeptides for PBP3 mediated transpeptidation. This reaction would be expected to produce DD-tetra-tri cross-bridges. However murein analysis has shown no increase in DD-tetra-tri cross-bridges during septation (deJonge, 1989; Romeis *et al.*, 1991). The preferred acceptor hypothesis would also predict that cells with inactive PBP3 would have lower levels of DD-tetra-tri cross-bridges. However murein analysis of *ftsI23* filaments shows DD-tetra-tri cross-bridges to increase.

Despite these contradictory results there remains good evidence to suggest that DD-Cpases and tripeptides are involved in division (Pisabarro *et al.*, 1986; Schuster *et al.*, 1990; Goodell & Schwarz, 1977; Beck & Park, 1977; Mirelman *et al.*, 1977).

It has been reported that the contribution of PBP3 to the total murein synthesis of an exponentially growing culture is small (Schmidt *et al.*, 1981). However during division the contribution of PBP3 to total peptidoglycan synthesis is estimated to be as high as 50% (Nanninga *et al.*, 1989). Therefore the problem exists of how 50% of

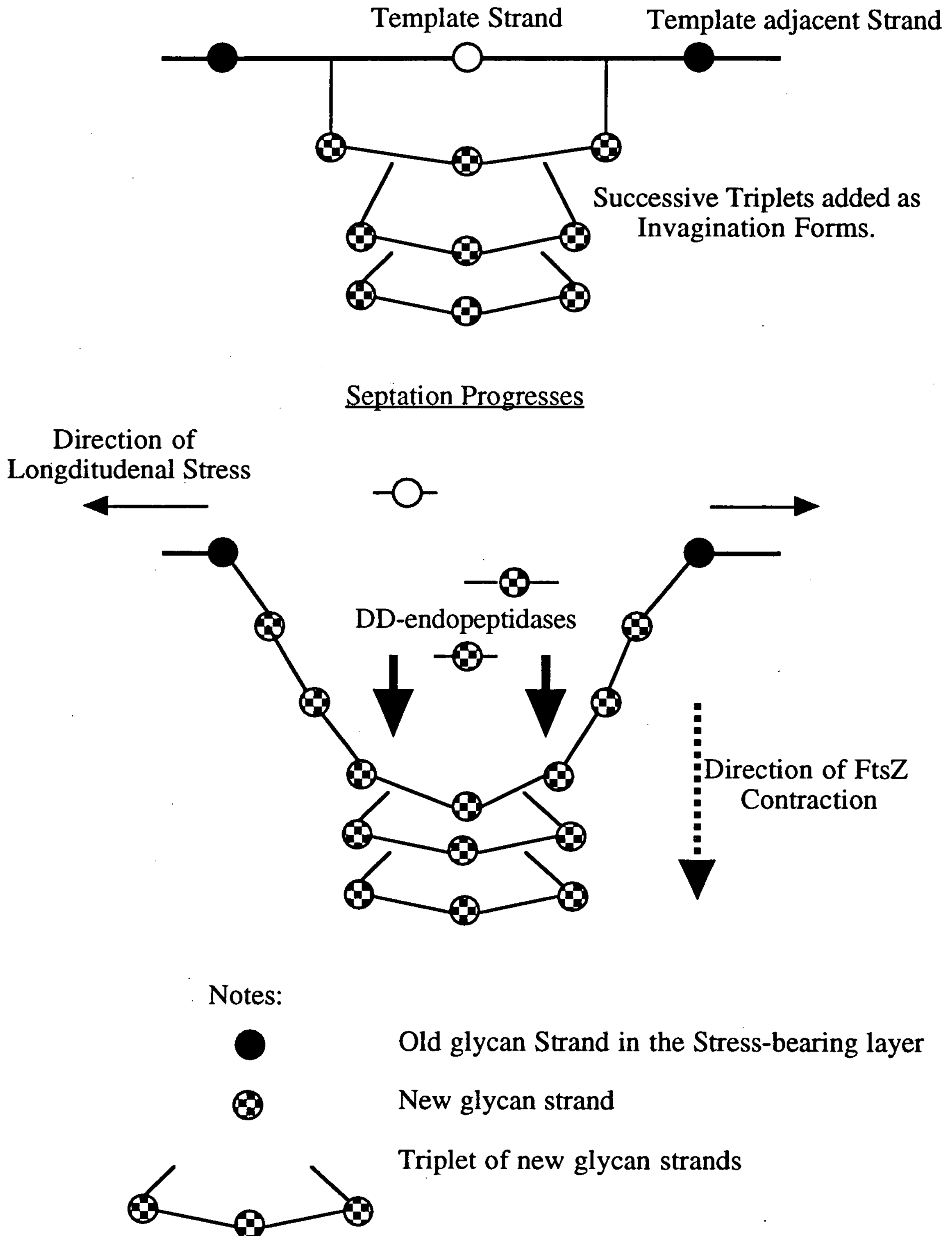
septal peptidoglycan synthesis can be dependent on PBP3 activity when there is no evidence for the increased formation of tetra-tri bonds during septation that is predicted by this work.

This apparent contradiction could be explained by PBP3 having a qualitative role that is essential for the normal incorporation of peptidoglycan during division. This qualitative role though essential for the normal incorporation of peptidoglycan during division may not cause any detectible change in peptidoglycan structure. The number of PBP3 and tripeptide lipid carriers per cell are estimated to be 50 (Spratt, 1975) and 100 molecules (Van Heijenoort *et al.*, 1993) respectively. In TOE23 the activity of this small number of PBP3 molecules is reduced at the restrictive temperature. Yet raising the level of cytoplasmic tripeptides, or periplasmic DD-Cpases, can rescue division at the restrictive temperature. This would be consistent with PBP3 having a qualitative role in divsion. To fit the tripeptide acceptor hypothesis such a qualitative role would have to involve the formation of a critical set of tripeptide containing cross-links.

In Holtje's 3 for 1 model of saccular growth (Holtje 1993) a critical qualitative role is ascribed to trimers for the insertion of new murein. These minor components of the overall peptidoglycan profile have a rapid turnover, and are temporarily enriched during septation (Kraus & Holtje, 1987). In the 3 for 1 model, normal replacement and insertion of peptidoglycan involves copying a template strand in the stress bearing layer (Figure: 1.7). This template strand is defined, at least at growth points, by its template defining cross-bridges. These cross-bridges are proposed to involve the tetrapeptide side chain of a template adjacent strand acting as a donor, and a tripeptide side chain of the template strand acting as an acceptor. A triplet of three cross-linked strands of new murein is synthesised beneath the template strand and hooked onto the stress-bearing layer by the formation of trimeric cross-bridges The formation of these trimers requires the *m*DAP in the tetrapeptide side chains of a template defining cross-bridge to act as an acceptor in transpeptidation. This results in the temporary formation of trimers on either side of the template strand. Hydrolysis of the cross-bridges between the template strand and the template adjacent strands in a trimer, generates a dimer and allows the template strand to be released. The remaining dimeric tetra-tetra cross-bridge between the triplet and template adajacent strands becomes stressed, and the triplet is lifted into the stress bearing layer. The template strand can be further degraded by hydrolases, and recycled.

Holtje envisages that, during invagination, successive packets of triplets are hooked beneath each other to form a temporarily three layered invagination (Figure:4.4). This successive addition of newly cross-linked triplets would be

Figure: 4.4. The 3 for 1 model of Septation.



responsible for the increased level of tetra-tetra cross-bridges observed during septation (de Jonge, 1989). It would also be responsible for the temporary increase in trimers observed during septation.

Each successive triplet is constructed beneath the central strand of a previous triplet. For these central strands to act as template strands they will require tripeptide side chains acting as acceptors in a dimeric cross-bridge. The formation of template defining cross-bridges does not represent a problem during elongation. This is because the action of LD-Cpases and endopeptidases will gradually produce tripeptide side chains during maturation. However for the rapid addition of new triplets during septation the normal action of LD-Cpases and endopeptidases will not be rapid enough to account for the formation of tripeptides in new peptidoglycan. This could be overcome by a specific tripeptide preferring transpeptidase and a co-ordinate supply of tripeptides during septation.

The template defining tetra-tri cross-bridges would only be required to define the point of trimer formation and consequently triplet attachment. These structures would be critical for the incorporation of new peptidoglycan during septation. By inhibiting formation of the tetra-tri crossbridge the 50% reduction in septal peptidoglycan synthesis produced by inactivation of PBP3 could be accounted for (Nanninga *et al.*, 1989). In this model trimers would represent a critical but minor component of the total peptidoglycan. Consequently low levels of residual PBP3 activity would be sufficient to restore division to a mutant. Such a situation is observed with the DD-Cpase suppression of *ftsI*23. During cleavage of the trimer the original tetra-tri cross-bridge would be degraded and only tetra-tetra cross-bridges would remain. Therefore this explanation would also account for the absence of tetra-tri cross-bridges in septal murein, by considering them critical but temporary structures that are rapidly turned over.

This explanation accounts for the observations that DD-Cpase activity is involved in septation, and the apparently contradictory evidence that septal murein is not enriched in tetra-tri cross-bridges. It incorporates the experimental data presented in this thesis with the most recent and complete model for saccular growth. This theory could accomodate the idea that PBP6 is a septum specific DD-Cpase whose activity is spatially restricted. It would also account for the suppression produced by PBP5 and D-cycloserine by considering it to be the result of a gross increase in the overall level of tripeptides.

4.5 The Involvement of a Stationary-phase Sigma in Suppression.

The plasmid pAX607 encoding SufI suppresses *ftsI*23 only in the presence of all three putative DD-Cpases. PBP profiles showed suppression was correlated with increased levels of PBP6, and membrane assays showed that DD-Cpase activity increased two-fold in strains transformed with pAX607. Murein analysis demonstrated that suppressed strains had normal cross-linkage although the proportion of monomeric pentapeptides was increased. Consistent with the observed alterations in DD-Cpase activity this accumulation was half that of an unsuppressed cell. The necessity for PBP4 and PBP5 that was found in the initial genetic studies, suggests that PBP6 overproduction alone is not responsible for SufI suppression. Therefore PBP6 appeared to have a central but not exclusive role in suppression.

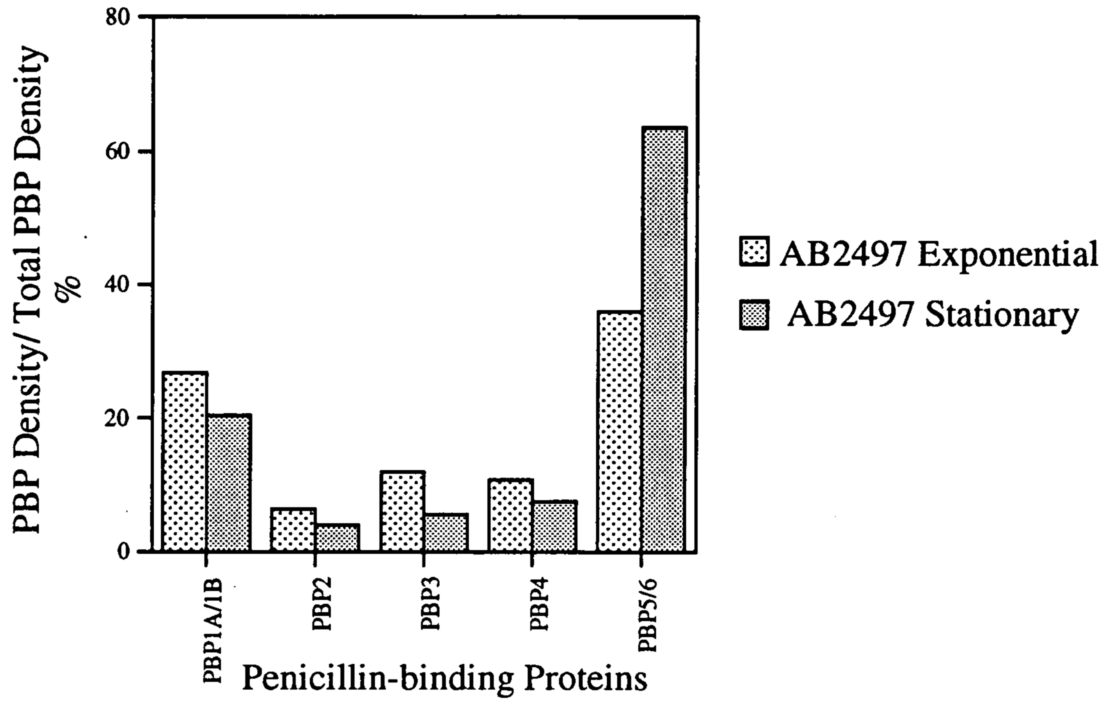
How PBP6 levels were affected by overexpression of SufI remains unexplained. The only known positive regulator of PBP6 is *bolA*, which is itself subject to positive regulation by the 'Stationary-phase Sigma' RpoS (Lange & Hengge-Aronis, 1991). To find out whether this sigma was involved in SufI suppression, strains deleted for *rpoS* were constructed.

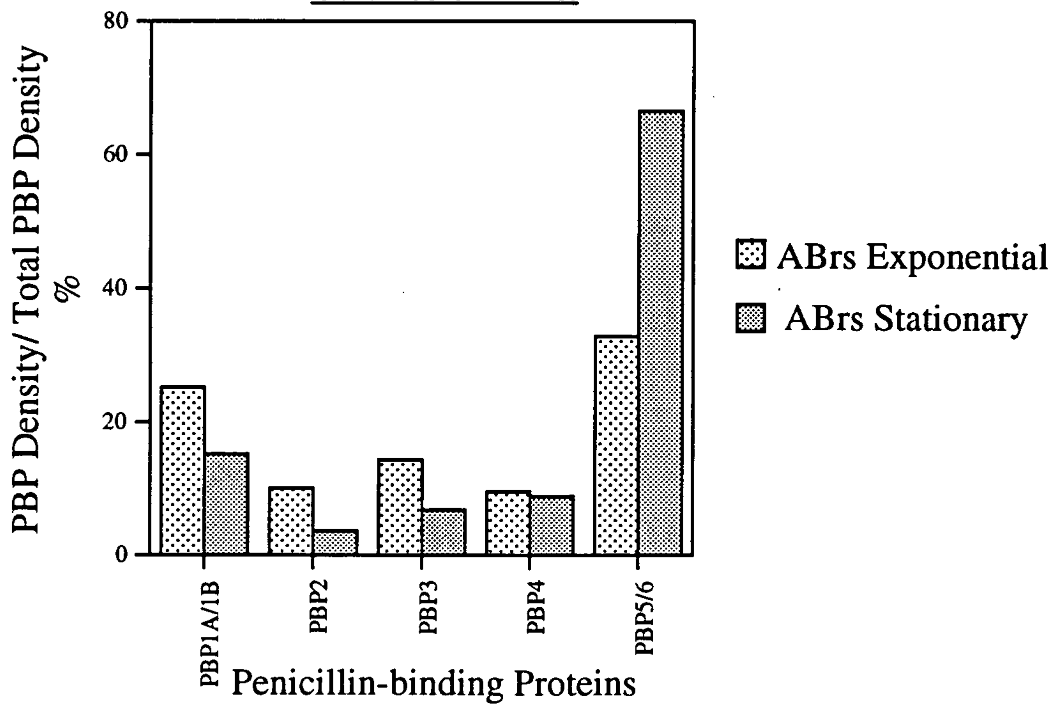
4.5.1 Examination of the role of rpoS in the Stationary-phase induction of PBP6.

The strain RH90 (Lange & Hengge-Aronis, 1991) carries the insertion *rpoS359* :: Tn*10*. A P1 lysate of RH90 was used to transduce the deletion into AB2497 and TOE23. Tetracycline resistant transductants were checked for catalase activity by exposure to H_2O_2. When *rpoS* is deleted stationary-phase cells do not produce catalase HPII (Lange & Hengge-Aronis 1991); consequently *rpoS*$^-$ colonies on old plates do not release O_2 when exposed to H_2O_2.

Microscopic analysis of stationary-phase cultures did not reveal the Δ*rpoS* strains to have the previously reported alteration in cell size and shape (Lange & Hengge-Aronis 1991). These cultures did however have reduced viability when stored in broth for more than five days. While it has been demonstrated that the increase of PBP6 co-incident with increased division in late exponential-phase was RpoS and BolA dependent, it had only been inferred that the same mechanism was responsible for the gradual increase of PBP6 during stationary-phase (Aldea *et al.*, 1989). Therefore PBP assays were carried out on stationary-phase and exponential-phase cultures of AB2497 transduced to Δ*rpoS*. These assays were performed with two separate aliquots of the same cultures, one harvested at an OD_{540} of 0.4 and the second 8 hours after the cells first entered stationary-phase, the optimum time period for an increase in PBP6 according to Buchanan and Sowell (1982).

Figure: 4.5. Changes in the PBP profiles of Stationary-phase cultures.





4.5.2 The involvement of *rpoS* in the stationary-phase induction of PBP6.

PBP5 and PBP6 could not be separated sufficiently for separate densities to be obtained by scanning densitometry. The proportion of PBP5 plus PBP6 for the control strain increased from approximately 36% in exponential phase to 63% in stationary-phase. This is consistent with Buchanan and Sowells' observations, but not as distinct as the four fold increase in PBP6 they described. This stationary-phase increase has been reported to be strain specific, and not to occur in AB1157 (de la Rosa *et al* 1983). AB2497 is derived from AB1157 and therefore the two-fold rather than four-fold increase PBP6 is probably the result of strain variations.

The deletion of *rpoS* had no effect on the stationary-phase increase in the PBP5/PBP6 band (Figure: 4.5). It was possible changes in PBP5 could obscure changes in PBP6. Therefore, to confirm this result the individual behavoiur of PBP5 and PBP6 in stationary-phase was examined in deletion strains E6c and A5.

4.5.3 Examination of *rpoS* in stationary-phase cultures of Strain.A5 (Δ*dacA*)

These gels showed no significant difference between proportions or absolute amounts of PBP5 and PBP6 in the absence of a functional *rpoS*. (Figure:4.6 & 4.7) Therefore the stationary-phase increase in DD-Cpase levels is *rpoS* independent.

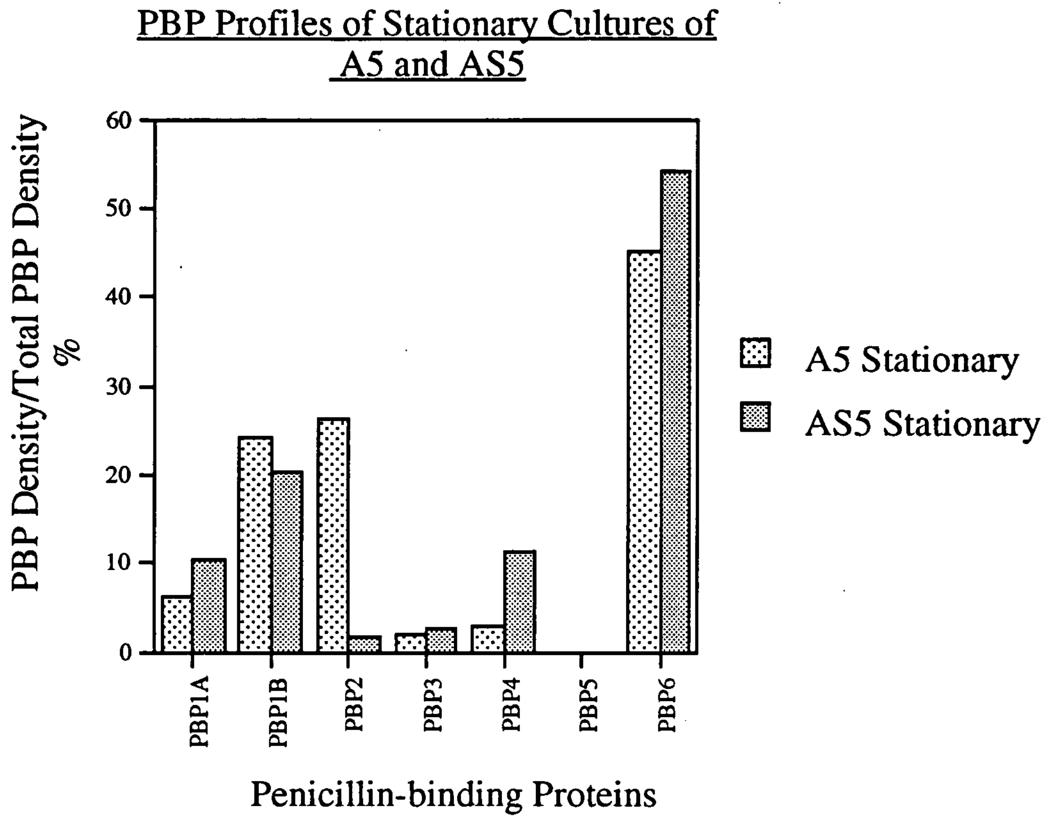
In the absence of either PBP5 or PBP6 the remaining DD-Cpase appeared to increase. A similar situation has been reported for PBP1A and PBP1B mutants (Tameki *et al.*, 1977). The increased density of the remaining PBP5 or PBP6 band corresponded approximately to their cumulative value in the AB2497 background. This suggests a mechanism exists to compensate for the loss of one by increasing the level of the other, and would be consistent with the two proteins having a common function. This increase was also independent of a functional *rpoS*.

4.5.4 The involvement of *rpoS* in SufI suppression.

Trs23 contains the *ftsI*23 allele and *rpoS*::359 insertion. It was transformed separately with pAX607 (*sufI*) and pBS110 (*dacC*) and purified transformants checked for suppression.

The plasmid pBS110, but not pAX607 suppressed Trs23. This was interpreted as indicating that the sigma factor was required for SufI suppression. This requirement could be either for induction of PBP6 or for overexpression of SufI. In both cases PBP6 induction would not be expected in strains deleted for *rpoS*. To test this hypothesis *dacA* deletion strains were used to examine the affect of pAX607 in strains deleted for *rpoS* (Figure: 4.8).

Figure: 4.6 RpoS dependent Changes in the Stationary-phase PBP profiles of dacA and dacC deletion Strains.



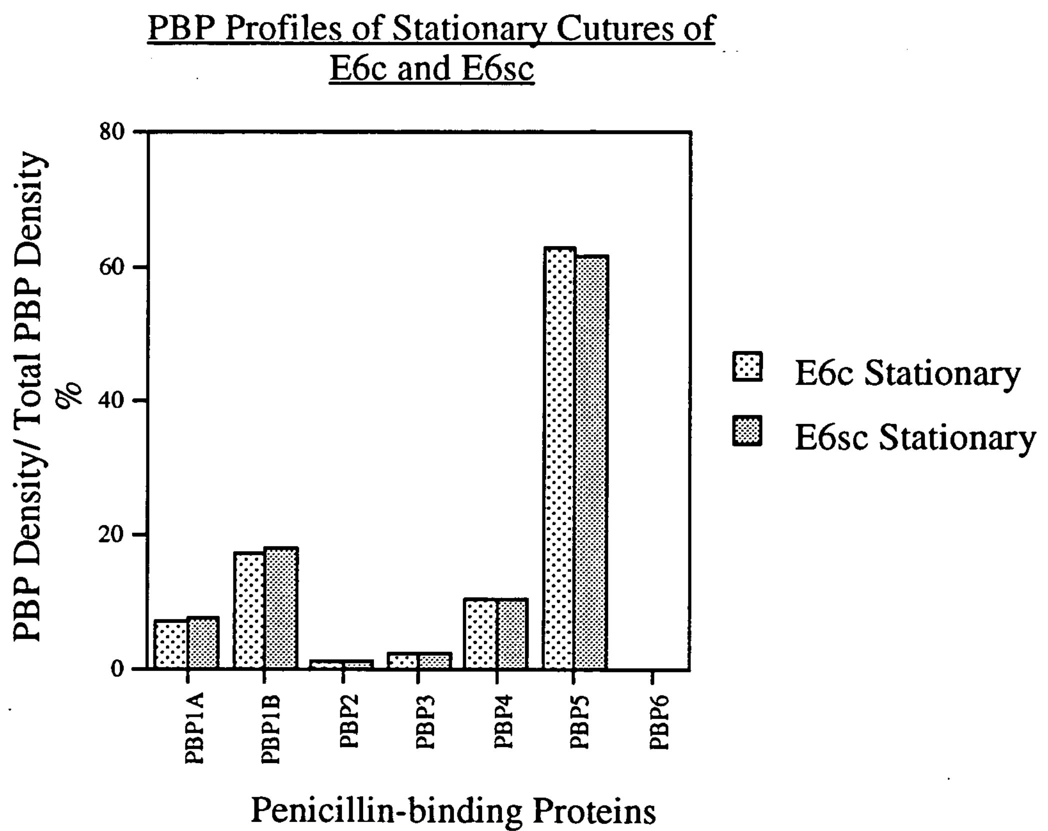


Figure: 4.7 PBP Assay of Stationary phase Cultures of *dacA* and *dacC* deletion strains without a functional RpoS

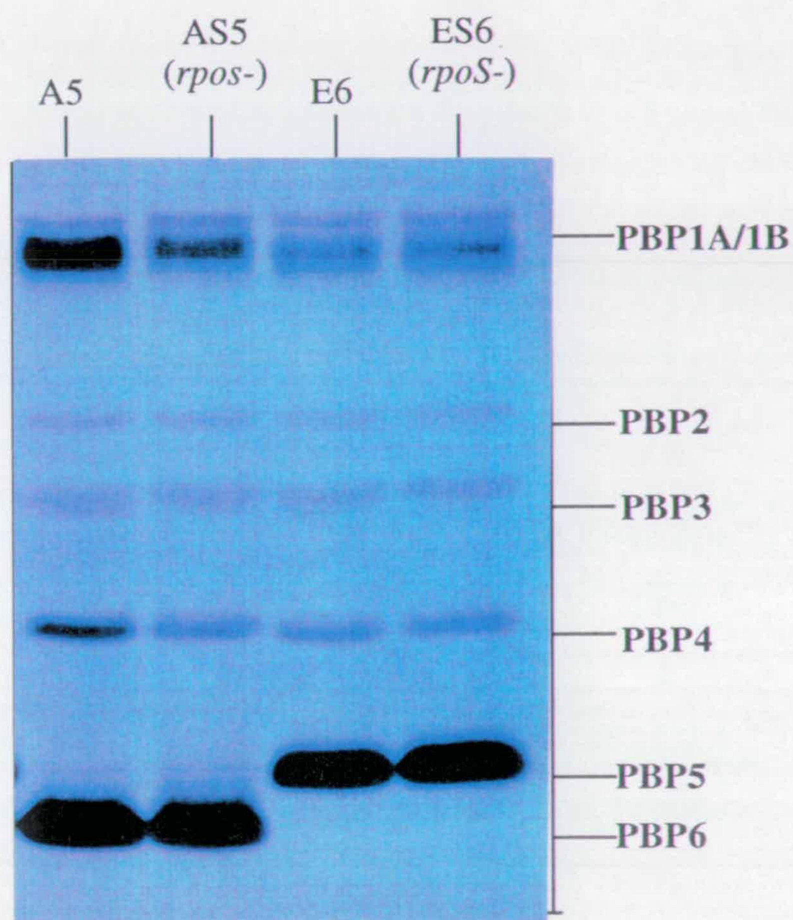
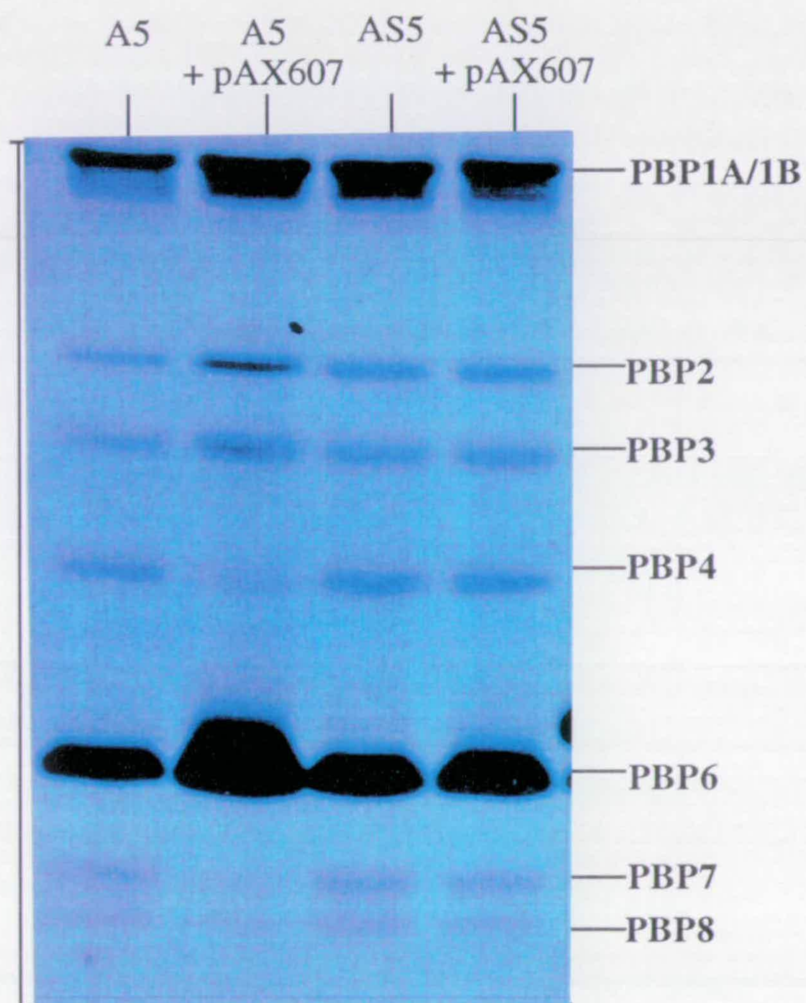
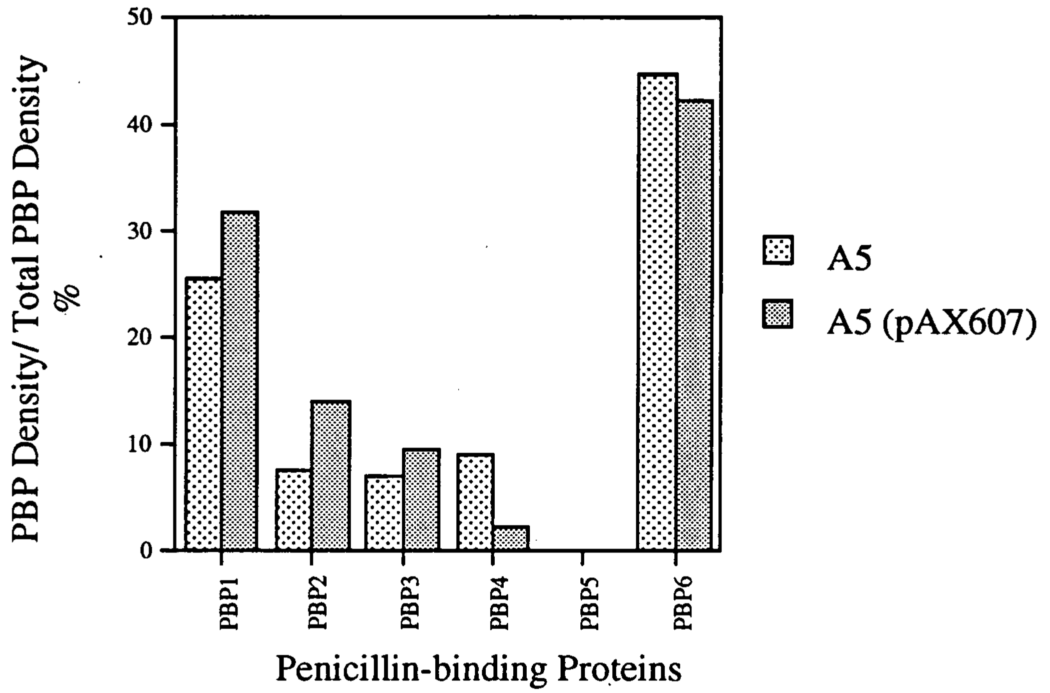
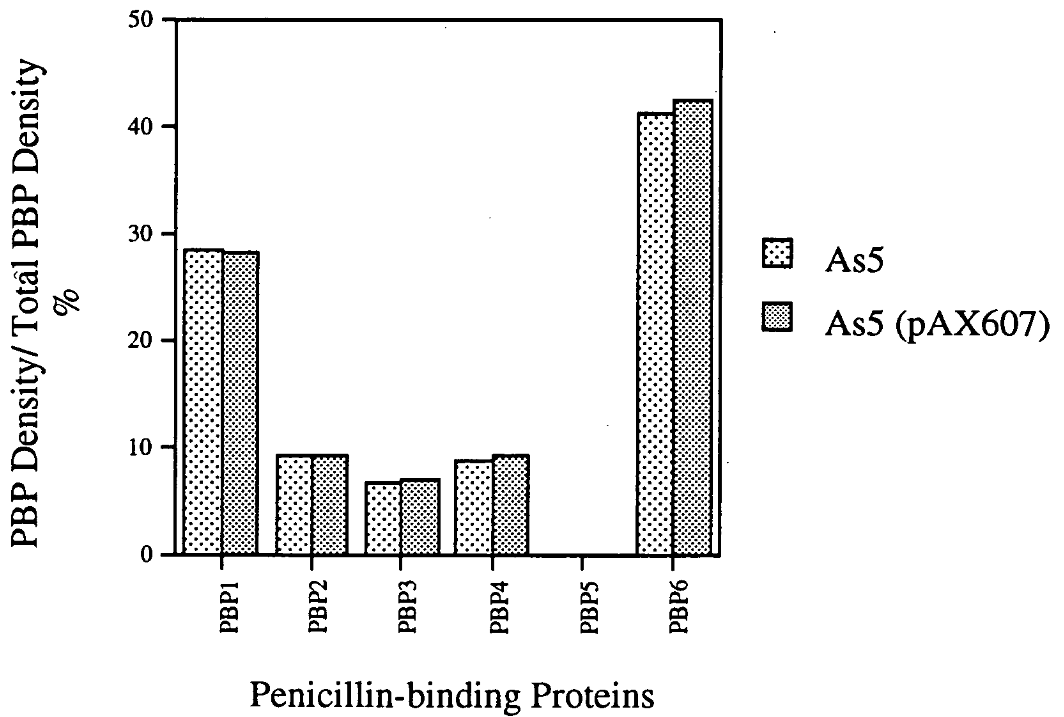


Figure: 4.8 PBP Assay of *dacA rpoS-* Strain Transformed with pAX607 (*sufI*)




PBP Proportions of A5 & A5 (pAX607)
PBP Density/ Total PBP Density
%
50
40
30
20
10
0
PBP1
PBP2
PBP3
PBP4
PBP5
PBP6
Penicillin-binding Proteins
A5
A5 (pAX607)


PBP Proportions of As5 & As5 (pAX607)
PBP Density/ Total PBP Density
%
50
40
30
20
10
0
PBP1
PBP2
PBP3
PBP4
PBP5
PBP6
Penicillin-binding Proteins
As5
As5 (pAX607)

In the PBP5 deletion strain the level of PBP6 increased (Figure: 4.8). This is consistent with the increase in PBP6 observed when A5 was assayed in stationary-phase. It had previously been shown that deletion of *dacA* and overproduction of SufI do increase PBP6. The deletion of *dacA* increased the level of PBP6 in the absence of a functional *rpoS*. However overproduction of SufI had no significant affect on PBP6 levels in either, A5 or AS5. This could be caused by deletion of *dacA* interfering with the mechanism of SufI induction. It is also possible that deletion of *dacA* or overproduction of SufI increase PBP6 by the same mechanism. In this case deletion of one membrane protein or overproduction of another would not have a cumulative effect. This would explain why pAX607 does not increase PBP6 in the A5 background.

A cryptic fraction of DD-Cpase activity has been demonstrated in *E.coli* (Harkness *et al.*, 1981). If this corresponded to PBP6, overproduction of SufI or deletion of *dacA* could be responsible for releasing this fraction. It could be imagined that the absence of one membrane protein or overproduction of another would disturb the structure of the cytoplasmic membrane and release the cryptic PBP6. However Tuomanen *et al.* (1991) demonstrated the release of cryptic DD-Cpase activity by deletion of *ampD* was not accompanied by an increase in the levels of PBP5 or PBP6. This suggests that the cryptic DD-Cpase activity is not the product of PBP5 or PBP6. Therefore it is more likely that the increase in PBP6 is the result of transcriptional regulation.

4.5.6 Does a pathway exit to alter the levels of PBP5 and PBP6 in vivo?

A mechanism for sensing general alterations in peptidoglycan and altering the levels of chromosomally encoded enzymes has been described. These general alterations can be produced by β-lactam antibiotics or the incorporation of certain D-amino acids into the sacculus (Martin & Schmidt, 1993). The experimental work for describing this system has involved the expression of the *ampC* and *ampR* genes of *Citrobacter freundii* and *Enterobacteria cloacae* in *E.coli* (Honore *et al.*, 1986; Lindberg *et al.*, 1989). This is because *E.coli* no longer contains the inducible chromosomally encoded β-lactamase (*ampC*) possessed by these other organisms. However *E.coli* does maintain some of the regulatory elements associated with an inducible *ampC ie. ampD* and *ampE.* The *amp* system includes a signal transducer located in the cytoplasmic membrane; AmpE. This signal transducer is proposed to sense changes in the peptidoglycan via an active site that includes a Ser-X-X-Lys motif (Honore *et al.*, 1989). A second membrane spanning protein, AmpG, is required to establish the transmission of a signal by AmpE. This signal then relieves transcriptional repression of *ampC* (serine β-lactamase) by AmpD (Lindquist *et al.*

1989). The induction of chromosomally encoded β-lactamases is dependent upon the presence of a functional FtsZ and PBP2 (Ottolenghi & Ayala, 1991; Oliva *et al.*, 1989). However induction does not require PBP5, PBP6, FtsQ or FtsA (Bennett & Chopra, 1993).

Although no inducible *ampC* is present in *E.coli*, overexpression of AmpD has been reported to repress DD-Cpase activity in the presence of exogenous DAP (Tuomanen *et al.*, 1991). Like repression of DD-Cpase activity observed with *ftsI* filaments, *ftsZ* filaments or amino acid-starvation, this is not the result of reducing the levels of PBP5 and PBP6.

A system similar to the *amp* system may exist to sense alterations in the sacculus and change the level of murein associated enzymes. This system may involve *ampD* which represses DD-Cpase activity, or even SufI which increases the level of PBP6. In this explanation deletion of *dacA* or overproduction of SufI would cause sufficient alterations to induce the DD-Cpase PBP6. The previous increase in PBP5 produced by deletion of *dacC* could be by the same mechanism.

4.5.6 A critical amount of PBP6 is required to suppress *ftsI*23.

The proportion of PBP6 in cells lacking PBP5 are higher than that found in wild type cells carrying pAX607 (SufI) (Figure:4.8). However the presence of pAX607 is sufficient to suppress *ftsI23*, but deletion of *dacA* is not. It has also been shown that *ftsI*23 Δ*dacC* cells carrying pAX607 remained temperature sensitive. This could be interpreted as indicating that a functional PBP5 is required for suppression of *ftsI*23. This is not the case because T5 (*ftsI*23 Δ*dacA*) can be suppressed by pBS110 (*dacC*).

Therefore in the absence of PBP5, a 2 to 4 fold increase in PBP6 (produced by either pAX607 or Δ*dacA*) is not sufficient to suppress *ftsI*23, but a 10 fold (Glauner 1986) increase produced by pBS110 is. These results suggest a critical amount of DD-Cpase activity is required for suppression.

4.6 Sub-cloning *sufI*.

All the biochemical and genetic studies presented were carried out using pAX607. This plasmid carries a 9 kb insert that has *sufI* and approximately 7 kb of unsequenced DNA downstream. The presence of this uncharacterised DNA was undesirable. However numerous attempts at sub-cloning failed, and due to lack of time caused by visits to Smith Kline Beecham and the Max-Plank Institute no other plasmid was available for the initial biochemical work. Subsequently it was decided to continue using pAX607 while attempting to construct a better plasmid.

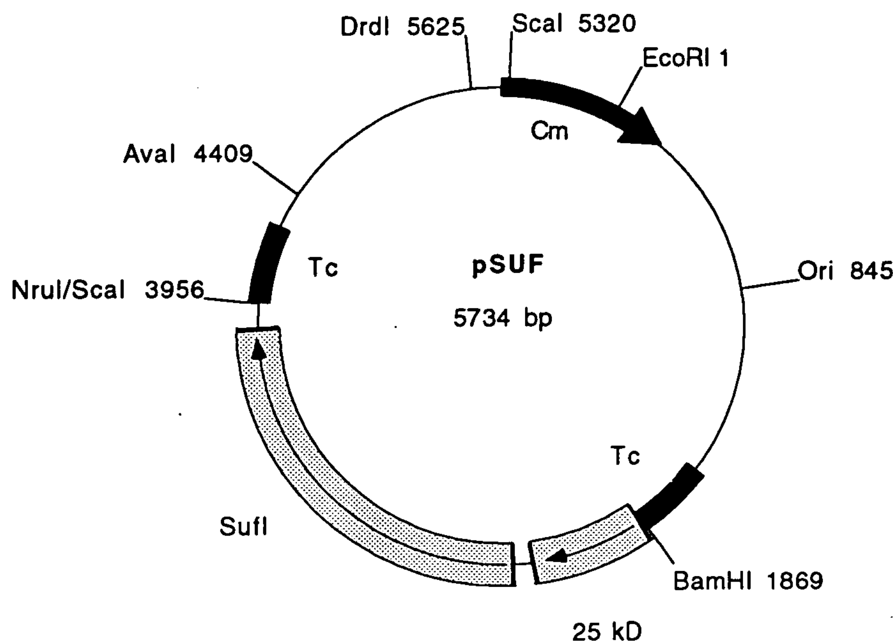
Early attempts at sub-cloning involved restricting pAX607, gel-purifying a 2.5 kb *ScaI-BamHI* fragment and blunt end ligating it into pJF118EH. pJF118EH carries a lac promoter and was chosen to provide controllable over-expression. This cloning was unsuccessful and it was decided to attempt sub-cloning into the low copy number vector already carrying the 9 kb insert, *ie.*pACYC184. Again this was unsuccessful.

In early work with pAX607 it had been noticed that when antibiotic selection was relaxed that a high proportion of transformants would lose the plasmid. Even under selection some TOE23 (*ftsI23*) transformants would lose their suppressed phenotype. Upon restriction analysis these strains were shown to be carrying a version of pAX607 which had lost the insert. The instability of pAX607 may be due to the uncharacterised downstream DNA, or to deleterious effects of SufI overproduction.

Some stable forms of pAX607 were isolated from transformants that were consistently suppressed. The yield of plasmid DNA from these isolates was consistently lower than that obtained from freshly transformed cultures. It was therefore considered that high levels of SufI may cause a problem to the cell and that mutations lowering gene dosage had been selected for. This again would explain the difficulty in sub-cloning. Therefore the procedure of sub-cloning into pACYC184 was repeated in a strain with a *pcn-* genotype.

The *pcn* product is similar in structure to the tRNA nucleotidyl transferase and promotes the rapid turnover of RNAI (Lin He *et al.*,1993). RNAI controls the rate of ColEI plasmid replication by inhibiting primer formation. A *pcn-* strain increases the stability of RNAI and inhibits ColEI plasmid replication, reducing the plasmid copy number to 10% of its normal level (Masters *et al.*, 1993). Sub-cloning of the 2.5 kb *ScaI-BamHI* fragment of pAX607 into pACYC18 and transforming it into a *pcn-* strain proved successful (Figure: 4.9). Subsequent transformation of TOE23, and curing, established that the 2 Kb fragment would suppress *ftsI*23 on a multi-copy plasmid.

Figure: 4.9 Plasmid map of pSUF.



4.7 Summary.

Overproduction of the 54 kd periplasmic protein SufI can suppress *ftsI*23. Suppression requires the presence of all three proposed DD-Cpases and is accompanied by an increase in PBP6 and membrane bound DD-Cpase activity. Murein analysis showed inactivation of a temperature sensitive PBP3 to result in a shift from monomers to dimers and an increase in monomeric pentapeptides. This increase in monomeric pentapeptides occured in the presence of elevated levels of PBP6. The monomeric to dimeric shift is considered to result from PIPS. The accumulation of monomeric pentapeptides is suggested to result from inactivation of a PBP3 associated DD-Cpase. This reduction in DD-Cpase activity is proposed to be overcome sufficiently by SufI and PBP6 overproduction to increase the level of tripeptides. Tripeptides acting as prefered acceptors for PBP3 mediated transpeptidation are then proposed to rescue septation. This explanation is supported by the literature and was incorporated into the most recent model for saccular growth and division.

The mechanism by which PBP6 is increased by SufI overproduction remains unknown. The only reported positive regulator of PBP6; BolA, was examined using *rpoS* deletion strains. RpoS positively regulates *bolA* and has been postulated to be responsible for the stationary-phase increase in PBP6. In this work no evidence was found for *rpoS* being involved in the stationary-phase increase in PBP6. However a

functional *rpoS* was necessary for SufI suppression but PBP assays gave no indication of altered PBP levels being responsible.

It was also observed that deletions of PBP5 and PBP6 increased the amount of the remaining DD-Cpase. To explain this and the other alterations in PBP profiles produced by *rodA*sui and other deletion strains, I have suggested the existence of a system sensing alterations in peptidolglycan. This would be similar to the suggested involvement of a peptidoglycan sensing system in β-lacatamse induction (Martin & Schmidt, 1993; Ottolenghi, 1993).

CHAPTER 5

CONSTRUCTION OF A TRIPLE DELETION STRAIN

The periplasmic control of levels of tetrapeptides and tripeptides through the action of DD-Cpases and LD-Cpases respectively has been proposed to have a role in septation (Beck & Park, 1977; Markiewicz *et al.*,1982; Pisabarro *et al.*, 1986; Begg *et al.*, 1990). Three PBPs have been attributed DD-Cpase activity in *E.coli.* (Tamura *et al.*,1977; Amanuma & Strominger, 1980.). PBP5 is considered the major DD-Cpase (Amanuma & Strominger, 1984) and studies involving its inactivation and overproduction have proved consistent with this (Markiewicz *et al.*,1982; Glauner, 1986). PBP6 DD-Cpase activity has proved more difficult to demonstrate (van der Linden *et al.*,1992), but extragenic suppression of PBP3 and sequence similarity suggests that PBP5 and PBP6 have the same enzyme activity in vivo (Broome-Smith *et al.*, 1988; Begg *et al.*, 1990). The third protein PBP4 has been demonstrated to exhibit both DD-Cpase and endopeptidase activity in vitro and in vivo, but cannot extragenically suppress *ftsI*23 (Iwaya & Strominger, 1977; Begg *et al.*, 1990; Koret *et al.*,1990).

In the model of PBP3 preferred tripeptide acceptors DD-Cpases would be predicted to have an essential role. The existence of a PBP5-PBP6 double deletion strain (Broome-Smith, 1985) would therefore be accommodated by considering the three low molecular weight PBPs as redundant, with any one capable of compensating for loss of the other two. Such a redundancy can be considered analogous to the suggested redundancy of the major peptidoglycan synthesizing enzymes PBP1A and PBP1B (Yousif *et al.*, 1985).

Therefore it was predicted that, in the absence of all three low molecular weight PBPs, depletion of tripeptides would block septation as effectively as PBP3 inactivation. To test this hypothesis it was intended to construct a PBP4 deletion and introduce it into the original PBP5, PBP6 double deletion background of SP1070 (Broome-Smith, 1985).

5.1 Construction of a Disrupted *dacB*.

The plasmid pBK18-1 carrying a 1.9 kb *SmaI-EcoRI* chromosomal fragment encoding the PBP4 gene *dacB*, was kindly supplied by W.Keck. Three characteristic amino acid fingerprints are common to penicillin-interacting enzymes. In PBP4 the active site serine penicillin-binding domain; SXXK lies between 40 and 60 amino acids from the amino terminus, SXN lies approximately 80 amino acids terminal to SXXK, and KTG is located 60 amino acids from the carboxyl-terminus (Joris *et al.*, 1988). All three motifs appear from tertiary structure studies to form part of the active site cleft (Spratt & Cromie, 1988). A *ClaI* site situated approximately 0.5 kb from the

N-terminus of the *dacB* coding sequence separates the SXXK motif from the other conserved regions of PBP4. It was decided to disrupt the gene at this *ClaI* site by introduction of the Ω fragment. The Ω fragment is a 2 kb DNA segment consisting of a antibiotic resistance gene flanked by short inverted repeats that encode a polylinker and transcriptional and translational termination sequences (Prentki & Krisch, 1984). Its introduction at the *ClaI* site was expected to result in an effective deletion.

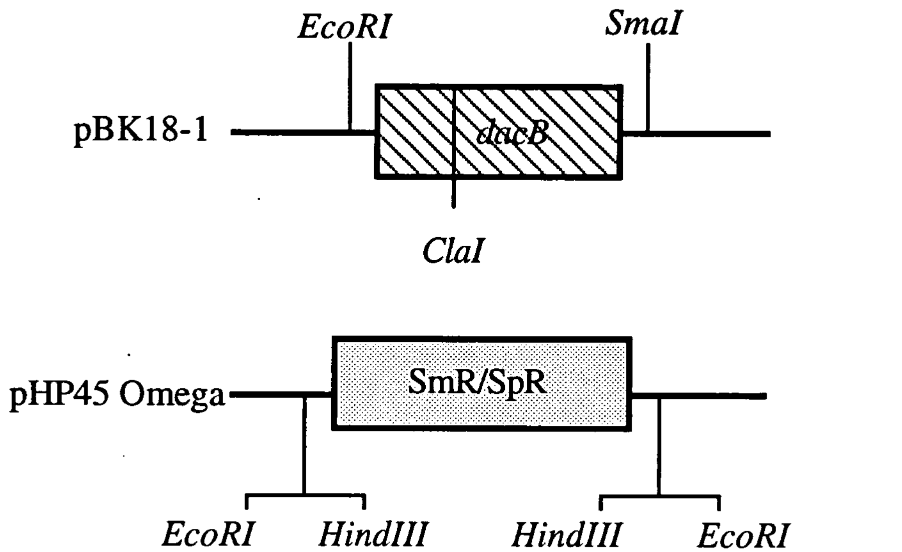
5.1.1 Cloning and Linear Transformation of *dacB* deletion.

The Ω fragment was excised from the vector pHP45 by *SmaI* digestion and gel purified. The PBP4 plasmid was restricted with *ClaI* and the overhang filled in with *Klenow*. Equimolar amounts of the Ω fragment and pBK18-1 were then blunt end ligated and transformed into strain DH1. Transformants were obtained and DNA isolated for restriction analysis. Two clones with the appropriate restriction patterns were identified, and after further diagnostic restriction analysis maxiprep DNA was prepared from both.

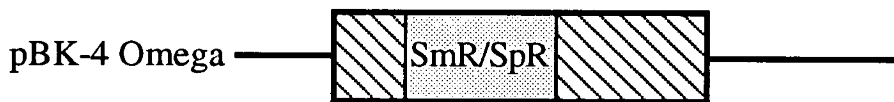
Two strains deleted for PBP4 had previously been constructed by nitrosoguanidine mutagenesis (Iwaya & Strominger, 1977; Matsuhashi *et al.*, 1977). Neither had been extensively characterised or removed from their heavily mutagenised backgrounds and one was subsequently discovered to carry a mutant penicillin-insensitive transpeptidase (Glauner, 1986). The Ω disrupted *dacB* therefore repesented the best characterised PBP4 deletion. The *SmaI-EcoRI* Ω *dacB* disruption was gel purified and used for linear transformation of the *recD* $^-$ strain DL301 (Russell *et al.*, 1989). Spectinomycin resistant linear recombinants were then selected (Figure: 5.1). Three transformants were obtained and one exhibited ampicillin reisistance which suggested the pBK18-1 plasmid had been carried over. P1 lysates were prepared from the other two transformants and after being titred these were used to transduce the deletion into the AB2497 background.

The Ω *dacB* deletion was sub-cloned from the pBK18-1 plasmid into the temperature sensitive replicon pMAK705. The intention was to use this for the deletion of *dacB* in the SP1070 (Δ*dacA*, Δ*dacC*) background by the method of Hamilton *et al.* (1989) Transduction of SP1070 with the lysates prepared from the linear transformants produced spectinomycin transductants at the same frequency as the wild type control. The antibiotic markers and auxotrophies were confirmed and representive transductants streaked to purity. Microscopic analysis revealed a small but

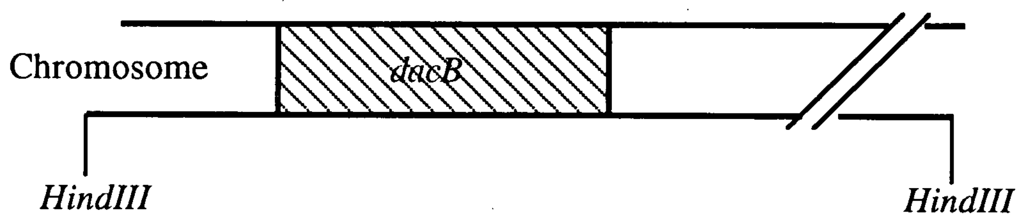
Figure: 5.1 Construction of a *dacB* deletion.



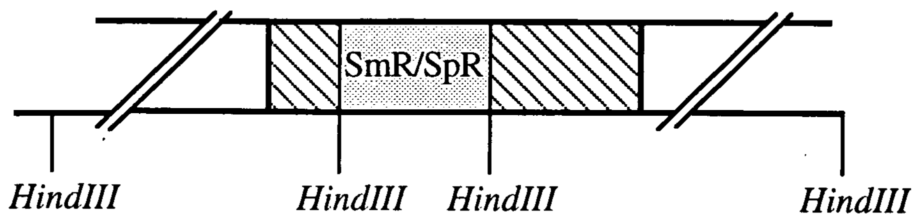
The *EcoRI* Omega fragment was blunt end ligated into the *ClaI* site of *dacB*



Recombination between linearised plasmid and chromosomal *dacB*



Select for Chromosomal Replacement by Selecting for SmR/SpR



consistent proportion of aberrantly shaped cells. These were most frequent in early log-phase of liquid cultures where approximately 5% of cells exhibited a forked or "Y" shaped phenotype (Figure: 5.2). 'Branches' occured either at the end or centre of the cell, and appeared to correspond to potential division sites. This phenotype appeared to indicate that a triple deletion of all three putative DD-Cpases had been constructed and further work on the temperature sensitive replicon replacement system was therefore considered unnecessary.

5.1.2 Confirmation of Chromosomal Deletions by Southern Probing.

Chromosomal DNA was prepared by the mini-preparation method from the original backgrounds of DL301 and SP1070, and the corresponding spectinomycin resistant transductants. The chromosomal preparations were restricted with *HindIII* which cuts in the Ω polylinker but not the *dacB* coding sequence. Therefore the wild type background was expected to liberate a single fragment of approximately 30 kb, and by contrast the Ω disrupted *dacB* to release two bands of 24 kb and 6 kb carrying portions of the coding sequence. These predicted sizes were obtained from the Kohara map.

Restricted chromosomal DNA was separated by gel electrophoresis and then transferred by the method of Southern to Hybond-N membranes. A probe was constructed from the *SmaI-EcoRI dacB* fragment of pBK18-1 by random-priming. Probing was then performed on the prepared membrane under high stringency, and the membrane exposed to photographic film.

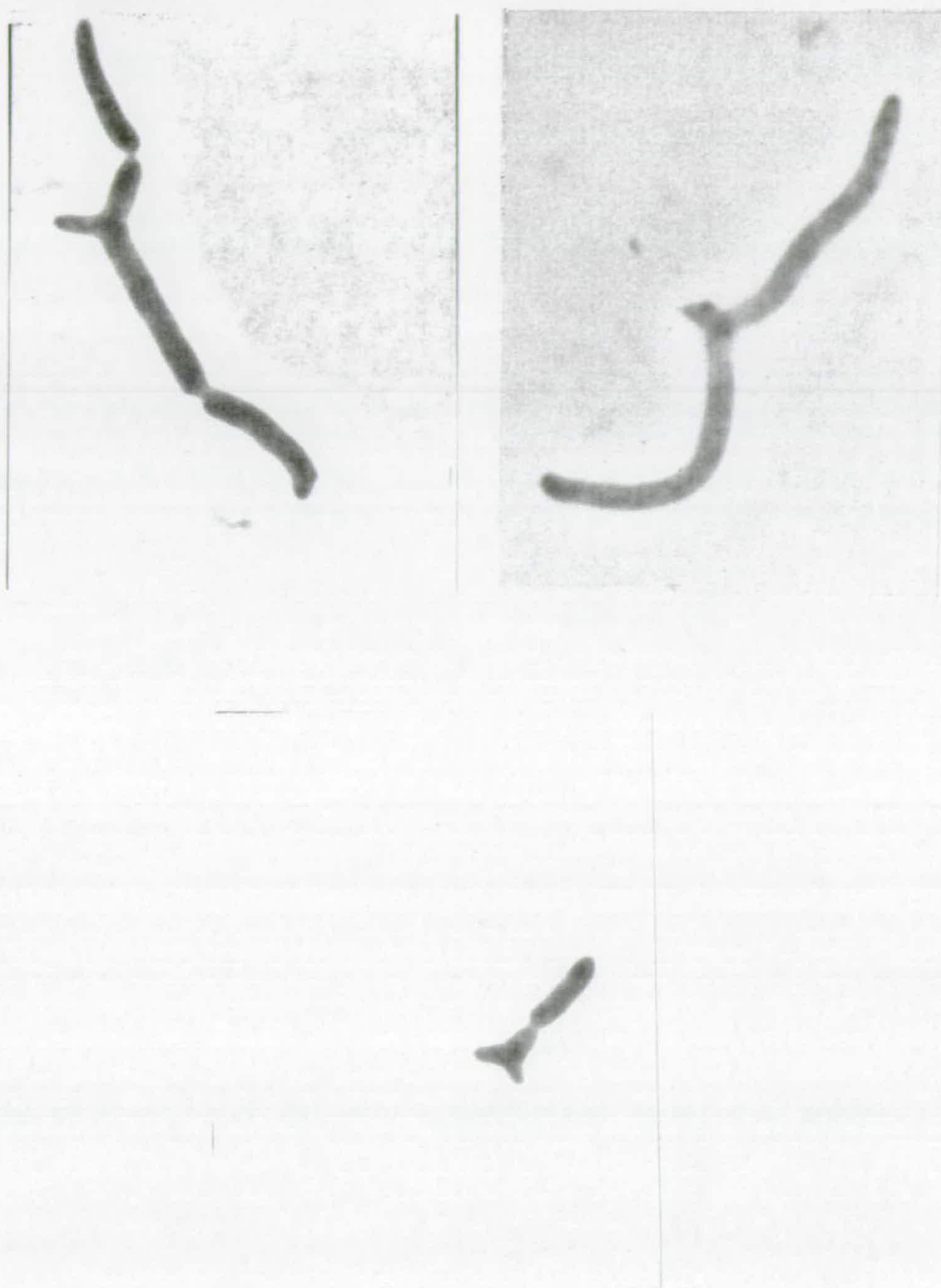
Probing confirmed the introduction of a *HindIII* site into *dacB* that was co-transducible with spectinomycin resistance. No band corresponding to an intact *dacB* was detected except in the wild type controls (Figure: 5.3).

5.1.3 PBP assays of Deletion Strains.

Penicillin-binding assays were performed to confirm that PBP4 had been deleted, and that PBP5 and PBP6 were not present in either SP1070 or D456. The same D456 transductants analysed by chromosomal probing were used for these PBP assays (Figure: 5.4).

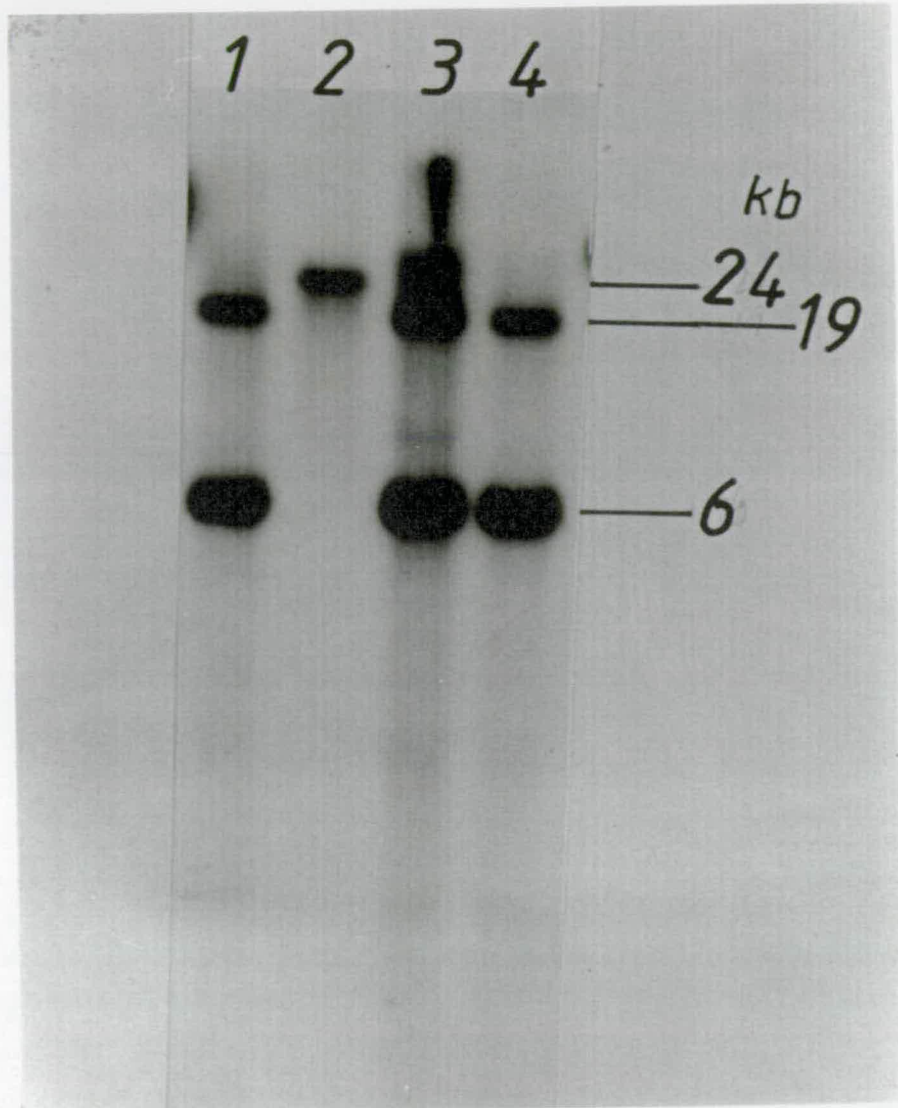
Assays confirmed that the spectinomycin resistant transductants did not contain a penicillin-binding protein that corresponded to PBP4. Penicillin acts as a functional analogue (Tipper & Strominger, 1965) of the protein's substrate and therefore the loss

Figure:5.2 "Y" Shaped cells in exponential cultures of D456.



Notes: Cells are from exponential-phase cultures of D456 grown in L-broth at 37°C.

Figure:5.3 Southern analysis of deletion strains.



Key: 1; D456a, 2; SP1070, 3; D456b, 4; A4.

Notes: Chromosomal DNA restricted with *HindIII*, separated by agarose gel elcetrophoresis, transferred to Hybond-N and probed. The probe was constructed by random-priming the 1.7 kb fragment of pBK18–1 carrying the *dacB* gene.

Genotypes;	
A4	(Δ*dacB*,)
SP1070	(Δ*dacA*, Δ*dacC*)
D456a & D456b	(Δ*dacA*, Δ*dacB*, Δ*dacC*)

of binding activity is considered a good indication of the enzyme's inactivation. The absence of PBP5 and PBP6 in the appropriate strains was also confirmed.

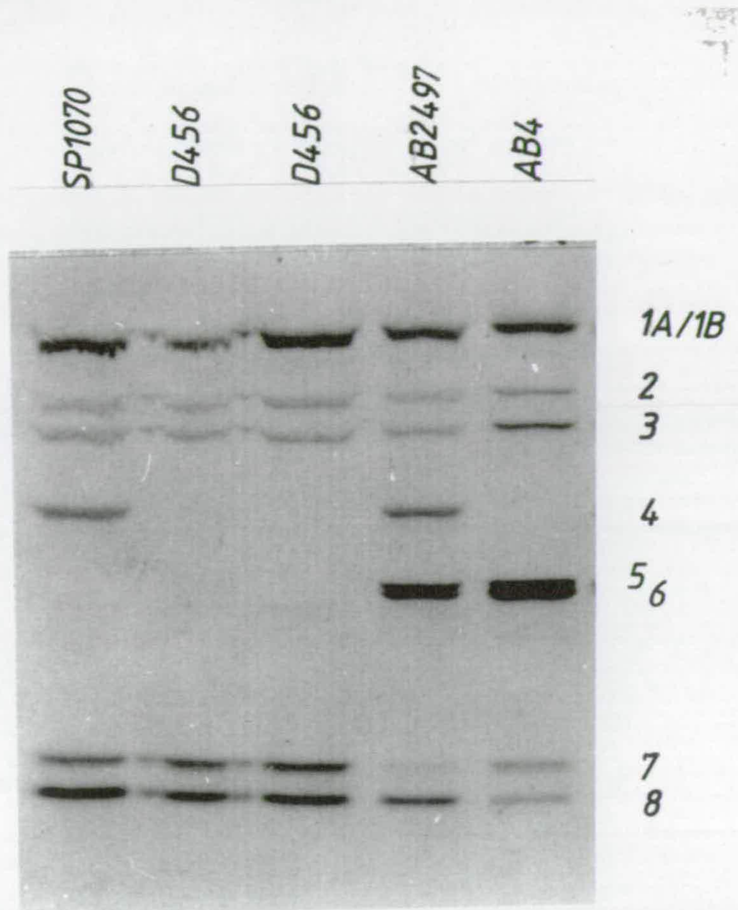
Scanning densitometry was performed on the autoradiograms as previously described. There appeared little difference between the A4 and AB2497 strains (Figure: 5.5). Only PBP3 showed a clear increase from 5% to 14%. Separate analysis of AB2497 in earlier *rpoS* studies had established that PBP3 could reach a level as high as 12% of the total amount of PBPs. So the increase to 14% was not considered significant. Interpretation of PBP levels was difficult in the absence of the three DD-Cpases, however the minor PBPs, PBP7 and PBP8, appeared more distinct in the deletion strains: an observation confirmed by scanning densitometry (Figure: 5.4). Again this could represent increased levels of protein, or altered affinity to radiolabelled penicillin. It was also evident that a minor band appeared just below PBP6. This was apparent in the SP1070 and both D456 strains. It may represent a minor penicillin-binding component that is obscured by PBP6 or a product from one of the *dacA*, or *dacC* disruptions. In the latter case the PBP6 deletion is the least characterised (Broome-Smith & Spratt, 1982) and represents the best candidate for producing a truncated protein.

5.1.4 PBP assays of Deletion Strains in Stationary-Phase.

PBP7 and PBP8 are poorly characterised and have been considered breakdown products of higher molecular weight PBPs. However peptide mapping has shown PBP7 to be distinct and it has been suggested it may be responsible for synthesis of autolysin resistant peptidoglycan (Tuomanen & Schwartz, 1987). Consistently PBP7 and PBP8 are found in exponential but not stationary cultures. As their levels appeared higher in the absence of all three DD-Cpases, it was decided to examine stationary cultures to see if they were maintained.

None of the preparations of stationary-phase single, double or triple mutants exhibited any PBP7 or PBP8. Therefore if PBP7 and PBP8 do increase in the absence of DD-Cpase activity, this occured only in exponential phase and is not essential for cell viability.

Figure: 5.4 Penicillin-binding assay of deletion strains.

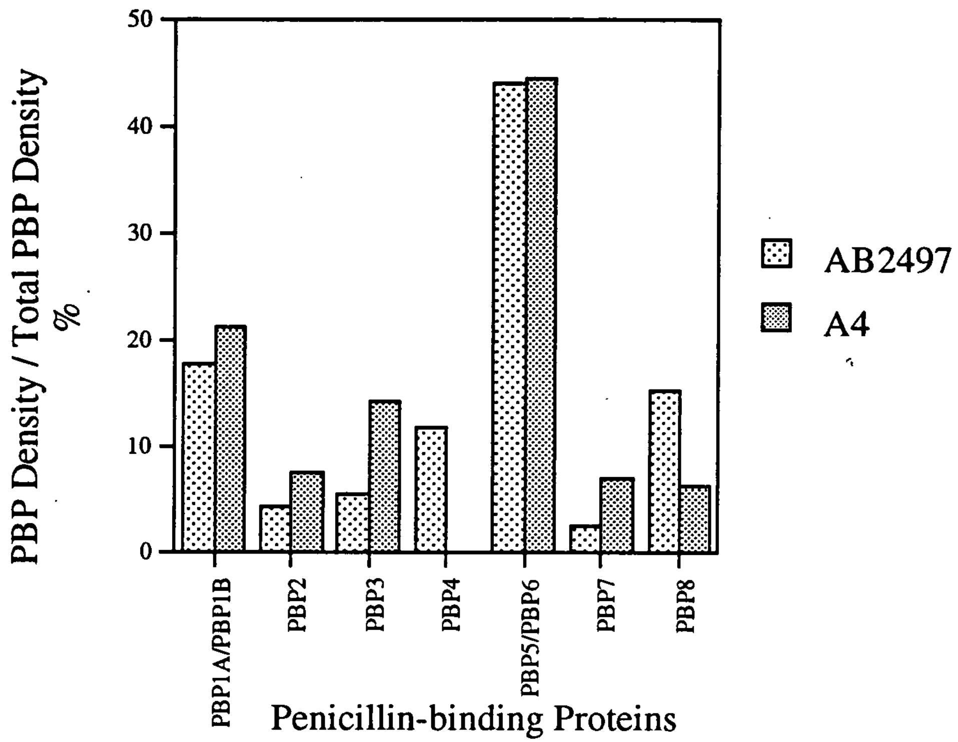


Lanes: 1; SP1070, 2; D456a, 3; D456b, 4; AB2497, 5; A4.

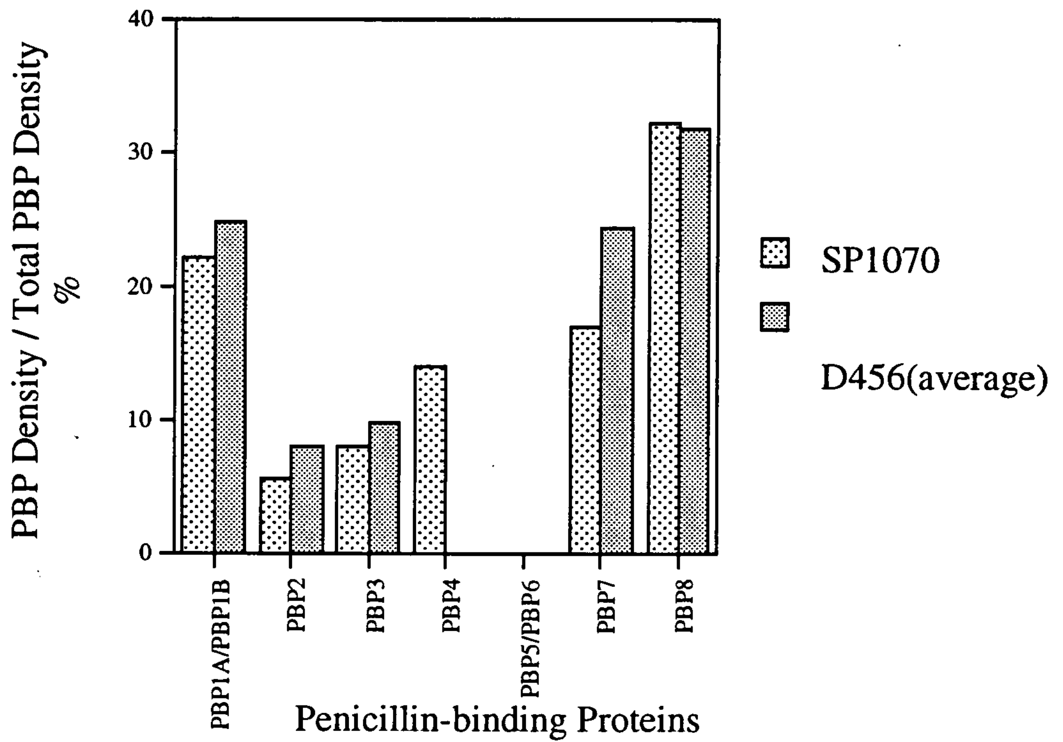
Notes: All strains were grown to mid-exponential phase in L-broth at 37°C. Membranes were harvested and PBPs labelled by the standard procedure.

Genotypes;	
A4	(Δ*dacB*,)
SP1070	(Δ*dacA*, Δ*dacC*)
D456a & D456b	(Δ*dacA*, Δ*dacB*, Δ*dacC*)

PBP Proportions of AB2497 and A4



PBP Proportions of SP1070 and D456



5.1.5 DD-Cpase activity in Deletion Strains.

Overproduction of PBP4 in vivo has been shown to produce a decrease in pentapeptides (Koret *et al.*, 1990). However these assays (Table: 5.1) showed deletion of PBP4 in either the AB2497 or SP1070 backgrounds has no significant effect on the membrane bound DD-Cpase activity.

Table: 5.1 Assay of DD-carboxypeptidases activity.

Strain	Replicate 1	Replicate 2	Replicate 3	Average
AB2497	35.73	30.00	42.53	36.09
A4	50.53	24.83	25.05	33.47
SP1070	3.15	12.33	11.21	8.9
D456a	1.58	10.61	9.52	7.24
D456b	2.58	11.55	11.21	8.45
Control				6.37

Note: DD-Cpase assay of isolated membranes. Activity expressed as the percentage of D-alanine released from 50 μg of muramyl-pentapeptide at 37°C.

PBP4 exists both as a membrane associated and as a soluble enzyme (Mottl, 1991; Iwaya & Strominger, 1977). The protein lacks any obvious membrane anchor (Mottl, 1992) but is consistently visualised in PBP assays of cytoplasmic membranes (Spratt, 1975; Spratt, 1977). The reported biphasic binding of penicillin by PBP4 in PBP assays (Spratt, 1975) may be due to the presence of two forms of protein. Koret *et al.* (1990) demonstrated that gross overproduction of PBP4 produced changes in the murein sacculus that were consistent with increased DD-endopeptidase and DD-Cpase activity *ie.* a 30–50% decrease in free pentapeptides, and a 20% decrease in overall cross-linkage. This overproduction of PBP4 resulted in 80% of the enzyme accumulating as a soluble form. It may be that DD-Cpase activity is only associated with PBP4 when it is in a soluble form. The failure to detect any change in the membrane bound DD-Cpase activity of PBP4 deletion strains, suggests membrane bound PBP4 functions solely as a DD-endopeptidase in vivo. This would be consistent with the failure of PBP4 to extragenically suppress PBP3 mutations (Begg *et al.*, 1990).

Low levels of D-alanine were still released by membranes prepared from D456 and SP1070. These values corresponded closely to the spontaneous D-alanine released from the substrate-only control. If they represented residual activity, it would suggest the existence of a third or fourth DD-Cpase. It is also possible that any residual activity may have resulted from an altered PBP6. This is because PBP6 is the least characterised of the three deletions (Broome-Smith & Spratt, 1982).

5.1.6 Murein Analysis of Deletion Strains.

Murein analysis was attempted for the same four strains that had been examined for enzyme activity. Despite harvesting twice, the normal volume of culture the AB2497 background strains failed to provide enough material for analysis. Therefore due to the time constraints upon working at the Max-Plank only figures for two repetitions on SP1070 and D456 could be obtained.

All cultures were harvested as mid-exponential-phase cultures grown at 37°C. In the absence of a wild type background average, murein profiles figures from the work of Glauner (1986) are presented for comparison.

Both the double and triple mutants exhibited a large increase in pentapeptides (Table: 5.2). In the absence of the major DD-Cpases the level of pentapepides will be regulated by transpeptidation and possibly the activity of a D-Ala-D-Ala removing enzyme. Glauner (1986) reported a wild type average for total pentapeptides of 0.51%, which rose to 4.24% in SP1070. In my analysis on cells grown under identical conditions the level of pentapeptides in SP1070 was 8.46%. The level of pentapeptides in the first analysis of D456 was 7.95% and in the second 11.77%. These wide variations in the percentage of pentapeptides in replicate experiments suggests regulation of pentapeptides in the absence of PBP5 and PBP6 is not efficient.

The increased availability of pentapeptides in SP1070 may have produced the observed increase in overall cross-linkage when compared to the wild type average. This was reflected in a small shift from monomers to dimers. Larger increases in cross linkage would be expected if transpeptidation was normally limited by the availability of donor pentapeptides. Consequently these results suggested that cross-linkage is regulated by enzyme activity and not substrate availability. The absence of any significant difference between the overall cross-linkage of D456 and SP1070 suggests that PBP4 does not limit the level of cross-linkage.

Tripeptides are approximately half the wild type level in both deletion strains. Consistent with this, the one clean analysis of D456 showed that Dap-Dap crossbridges account for only 1.34% of all peptide side chains. This is lower than the wild type average of 4.73% and the figure of 1.79% determined for SP1070 by Glauner (1986). Although the difference between SP1070 and D456 is small it does support the view that a portion of Dap-Dap bonds are derived from tripeptides released by PBP4 DD-endopeptidase activity (Glauner & Holtje, 1990). This is also supported by the observation that PBP4 overproduction increases the formation of Dap-Dap cross-bridges (Koret *et al.*,1990).

The only other significant difference in the murein of SP1070 and D456 was an increase in Tri-Lys-Arg muropeptides. The attachment of lipoprotein to produce Tri-Lys-Arg muropeptides requires an LD-transpeptidation. (Glauner, 1986). This LD-transpeptidation has a preference for tetrapeptides already participating in a peptide cross-bridge (Glauner & Holtje, 1990). Both SP1070 and D456 have more tetrapeptide containing dimeric cross-bridges than the wild type murein. This increased substrate is probably the reason for the increased lipoprotein attachment.

Table: 5.2 Murein Analysis of the D456 and Sp1070

PEPTIDOGLYCAN	SP1070	D456	D456	WT
MONOMERS				
Total	51.22	50.37	50.49	54.52
Tripeptides	4.99	4.93	4.25	
Tetrapeptides	30.97	31.36	32.55	
Pentapeptides	2.86	3.05	5.30	
Chain-ends	8.25	7.66	4.91	
anhydro	0.37	0.42	0.78	
DIMER				
Total	44.16	44.62	43.60	40.74
Ala-Dap			41.58	
Dap-Dap			2.19	
Chain-ends	5.11	4.60	3.80	
Tripeptide acceptors	1.99	1.90	1.85	
anhydro	4.54	5.24	3.77	
TRIMER				
Total	4.53	4.91	5.82	4.58
Ala-Dap	3.92	4.20	4.54	
Dap-Dap	0.66	0.81	1.45	
Chain-ends	0.41	0.51	0.37	
Tripeptide acceptors	0.38	0.31	0.34	
anhydro	1.51	1.83	2.29	
TETRAMERS				
Total	0.10	0.11	0.09	0.16
Anhydro	0.10	0.11	0.09	
PENTAPEPTIDES				
Total	8.46	7.95	11.77	0.13
Monomer	4.43	4.05	6.71	
Dimer	4.03	4.05	4.68	
Trimer	-	-	0.37	
CHAIN-ENDS				
Total	3.18	3.70	3.48	3.43
Monomer	0.37	0.42	0.78	
Dimer	2.30	2.67	1.97	

PEPTIDOGLYCAN	SP1070	D456	D456	WT
CHAIN-ENDS				
Dimer Ala-Dap	1.66	2.02	1.73	
Dimer Dap-Dap	0.70	0.76	0.42	
Trimer	0.54	0.68	0.88	
Tetramer	0.05	0.05	0.05	
LIPOPROTEINS				
Total	10.89	10.04	6.78	5.10
Monomer	8.25	7.66	4.91	
Dimer	2.53	2.25	1.81	
Trimer	0.12	0.13	0.06	
GLYCINE				
Total	4.51	4.23	4.16	3.16
Position 4	1.98	2.32	1.66	2.78
Monomer	3.40	2.68	2.64	
Dimer	1.10	1.55	1.52	
TRIPEPTIDE dissach				
Total	1.07	0.97	0.89	1.5
Dimer	0.97	0.90	0.84	
Dimer Ala-Dap	0.81	0.80	0.76	
Dimer Dap-Dap	0.16	0.10	0.08	
Trimer	0.11	0.07	0.05	
CROSS-LINKAGE				
Total	25.17	25.66	25.75	23.55
% Ala-Dap			24.37	21.89
% Dap-Dap			3.64	1.66
% Tri-Lys Arg	24.70	24.60	29.82	
% Anhydro			76.68	
% Tripeptide Acc			9.19	

Abbreviations; Monomer, GlcNAc-β-1,4 MurNAc peptide. Dimer, bis(GlcNAc-β-1,4 MurNAc peptide). Trimer, Tris(GlcNAc-β-1,4 MurNAc peptide). Tetramer, Tetrakis(GlcNAc-β-1,4 MurNAc peptide). Tripeptides, L-Ala-D-Glu-*m*-DAP. Tetrapaptides, L-Ala-D-Glu-*m*-DAP-D-Ala. Pentapeptides, L-Ala-D-Glu-*m*-DAP-D-Ala-D-Ala. Lys-Arg componds, muropeptides carrying L-Ala-D-Glu-*m*-DAP-L-Lys-L-Arg peptide moities. Anhydro Compounds, 1,6-anhydromuramic containing residues.

5.2 The Viability of D456.

The action of DD-endopeptidases in splitting peptidoglycan crossbridges is considered essential to the recycling of old murein (Goodell, 1985) and insertion of new murein (Koch, 1990; Holtje, 1993). Nevertheless deletion of PBP4 proved viable, even in the absence of the two major DD-Cpases. The existence of two DD-endopeptidases, MepA and PBP4, in *E.coli*, apparently provides redundancy (Keck *et al.*,1990; Koret *et al.*, 1990). A strain has been constructed with an uncharacterised PBP4 deletion and an impaired MepA protein (Iida *et al.*,1983). This double mutant was estimated to have only 15% residual DD-endopeptidase activity, but grew and divided normally. Therefore it would appear that DD-endopeptidase activity is normally present at much higher levels than are essential for growth. The presence of two DD-endopeptidases and probably a third specialised DD-endopeptidase (T.Romeis *pers. comm*), represents a degree of enzyme redundancy analogous to the situation with DD-Cpases. Further investigation requires the construction of a strain deleted in all three DD-endopeptidases.

Murein analysis of D456 revealed slight alterations in overall cross-linkage, chain length, lipoprotein attachment, and DD-peptide cross-bridges. The only major feature was the accumulation of pentapeptides. These analyses gave no insight into the cause of "Y" shaped cells.

It had been predicted from the PBP3 preferred tripeptide acceptor model that deletion of DD-Cpase activity would result in septation being blocked. However it was clear that D456 was viable and that tripeptides continued to form cross-bridges. This suggests that periplasmic DD-Cpase activity is not essential for the production of tripeptide acceptors. Alternative sources of these side chains include recycled material (Goodell, 1985; Goodell & Schwarz, 1985), a putative D-ala-D-ala removing enzyme, or tripeptides transported across the cytoplasmic membrane (van Heijenoort *et al.*, 1992). Besides overproducing periplasmic DD-Cpases the only other mechanism demonstrated to suppress *ftsI*23 is the addition of D-cycloserine. D-cyloserine increases the level of cytoplasmic tripeptides (Neuhaus & Hammes, 1981), which may be transported across the cytoplasmic membrane (van Heijenoort *et al.*, 1992). Therefore of the alternative sources of tripeptides only increased transport of tripeptides across the cytoplasmic membrane has been demonstrated to be capable of rescuing division (Begg *et al.*, 1990).

Two other explanations for the viabilty of D456 cannot be discounted. The first would be that a residual DD-Cpase activity from an altered PBP6 would supply sufficient tripeptides for the synthesis of a septum. In the double and triple deletions

used, the PBP6 mutation was the least characterised. PBP4 and PBP5 were inactivated by insertion of antibiotic cassettes into the *dacA* and *dacB* coding sequences (Broome-Smith, 1982; Broome-Smith & Spratt, 1985). By contrast the PBP6 deletion was obtained by the aberrant excision of λ phage, and identified by penicillin-binding profiles. Iwaya and Strominger (1977) have reported a nitrosoguanidine mutated PBP4 allele that failed to bind radiolabelled Penicillin-G but was sensitive to it, and Mottl (1992) deleted extensive internal regions of PBP4 without significantly effecting its activity. A penicillin-binding D-alanine carboxypeptidase in *Bacillus stearothermophilis* has been reported to lose penicillin-sensitivity, without concomitant loss of enzymic activity (Barnett, 1973). Similarly in KJB1, PBP3 can fail to exhibit penicillin-binding yet the strain remains sensitive to the specific inhibitor of PBP3, furazlocillin (Begg *et al.*, 1990). A *C*-terminal disruption of the PBP3 protein of *Streptococcus pneumonia* has been constructed which although not detectable in the cell membrane or soluble fraction, can be isolated from the culture media (Severin *et al.*, 1992).

These observations indicate it is possible that an altered PBP6 which is not detectable in PBP assays may be responsible for some residual DD-Cpase activity. This PBP6 may have an altered active site, or simply be soluble. A faint band of approximately 39 kD is apparent in PBP assays of SP1070 and D456. This may be a truncated form of PBP6 or another PBP. Rather than characterising the existing *dacC* allele further it would be more desirable to construct a better deletion of *dacC* with a selectable marker.

The second alternative explanation for the viability of D456 is based upon the initial in vitro studies on PBP3. These studies established that PBP3 can utilise pentapeptides as both a donor and acceptor molecule (Ishino & Matsuhashi, 1981). Therefore an increase in pentapeptides might compensate for the loss of tripeptides in vivo and this would only affect septation in a relatively small proportion of cells.

The occurence of "Y" shaped cells in D456 was the only evidence of impaired division (Figure: 5.2). Similar cells have been reported for a PBP4, PBP5 double mutant (de Pedro & Schwarz, 1981), a *rodA* mutant treated with furazlocillin (Schmidt *et al.*, 1981), some *ftsZ* alleles (Bi & Lutkenhaus, 1992) and some Min mutants (Akerlund *et al.*, 1993). The altered polar formation and production of branches observed with FtsZ mutants is thought to be linked with a geometrically altered FtsZ ring. The increased frequency of "Y" shaped cells when the two major DD-Cpases and the major endopeptidase are deleted would suggest disturbed peptidoglycan synthesis at potential division sites. DD-endopeptidases and DD-Cpases have been implicated in division by the demonstration that hydrolases peak at the time of increased cell

division that occurs after chromosome replication (Hackenbeck & Messer, 1977). It has also been reported that minicell membranes have twice the 'carboxypeptidase and endopeptidase' activity of normal cell membranes (Goodell & Schwarz, 1977). Since all the cells which were observed to form aberrant branches are to some degree filamentous, it may be inferred that the formation of a branch is at the expense of a division event. As the cultures enter late exponential growth the number of "Y" shaped cells decreases. It is not known whether a cell that forms such a branch is destined to lyse or if it can recover.

Despite the formation of "Y" shaped cells it is clear that in the absence of PBP4, PBP5 and PBP6 a cell is still viable. Therefore it would appear that the sacculus is a highly resilient structure which is relatively insensitive to changes in the amounts of several murein hydrolases.

5.3 Antibiotic Studies.

The single and double deletions of PBP5 and PBP6 had been reported to exhibit a slightly increased sensitivity to Ampicillin (Broome-Smith, 1982; Broome-Smith & Spratt, 1985) and the original PBP4 mutation had been described as slightly more stable to penicillin-induced lysis (Iwaya & Strominger 1977). It was decided to construct a complete set of mutants carrying all the possible combinations of the *dacA*, *dacB*, and *dacC* deletions. These could then be used to examine the affects of different β-lactams on specific deletions. It was predicted that deletions of *dacA* (PBP5) and *dacC* (PBP6) would increase sensitivity to PBP3 specific antibiotics.

The single, double and triple deletions were constructed by P1 transduction. The *dacB* and *dacA* deletions were 100% co-transducible with linked selective markers. The *dacC* deletion was not selectable and any attempts to score transduction of this deletion would have required repeated PBP assays. Therefore strains carrying the PBP6 deletion were constructed using SP1070 or KJB33. KJB33 was constructed by K.Begg using SP1070. He had made this strain *ftsI*23 and had replaced the Δ*dacA* with a wild type allele. E6c was made by replacing the *ftsI*23 with a wild type allele that cotransduced with *leu*::Tn*9*. This strain was then transduced with a *dacB*::Spec/Strep lysate to produce D46. The SP1070 and D456 strains had already been constructed (Broome-Smith & Spratt, 1985). Due to unavailability of ED3184 (the parental strain of SP1070 and D456) the single and double PBP4 and PBP5 deletions were constructed in AB2497.

5.3.1 General Observations.

The use of two strain backgrounds complicated interpretation of antibiotic MICs. This was unavoidable due to the nature of the PBP6 mutation and the problems encountered while trying to obtain ED3184. Towards the end of the project Tom Henderson in North Dakota supplied the ED3184 strain and its antibiotic sensitivities were then determined separately.

5.3.2 Benzyl-PenG & Azlocillin.

From these assays it is evident that ED3184 is generally more resistant than AB2497 to the broad spectrum β-lactams (Figure: 5.5). Benzyl-Penicillin has a broad penicillin-binding profile with highest affinity for PBP1A, PBP2, PBP3 and PBP4. The MIC.s that I obtained fitted the general pattern previously reported (Broome-Smith, 1982; Broome-Smith & Spratt, 1985). Single and double deletions of PBP4, PBP5 and PBP6 all increased sensitivity. Of the three low molecular weight PBPs deletion of PBP5 had the most consistent and significant effect.

Azlocillin has a similar PBP affinity pattern to that of Benzl-Penicillin except for a higher specificity for PBP3. It would be predicted that a strain depleted for tripeptides by deletions of PBP5 or PBP6 would be more sensitive to PBP3 inhibition. Therefore as expected the deletion of *dacA* produced the greatest increase in Azlocillin sensitivity.

Sensitivity to broad spectrum antibiotics is probably due to general weakening of the cell wall in the deletion strains resulting in increased sensitivity to autolysis. Increased sensitivity was observed for all three PBP deletions, and suggests that, though not essential PBP4, PBP5, and PBP6, have significant roles in the synthesis of a fully functional sacculus.

5.3.3 Aztreonam & Furazlocillin.

Induction of autolysis by β-lactam antibiotics has been proposed to follow one of three patterns. Total saturation of PBP1A and PBP1B, binding combinations involving PBP1A or PBP1B and PBP2 or PBP3, or inhibition of both PBP2 and PBP3 (Spratt, 1983; Gutmann *et al.*,1989). Aztreonam and furazlocillin show high specicifity for PBP3 but bind to different secondary targets. Furazlocillin is relatively specific for PBP3 and PBP1A, while Aztreonam binds to PBP3 and PBP2. Thesensitivity of the deletion strains to Furazlocillin and Aztreonam was different

Figure:5.5 Sensitivity of Deletion Strains to Broad Spectrum β-lactams.

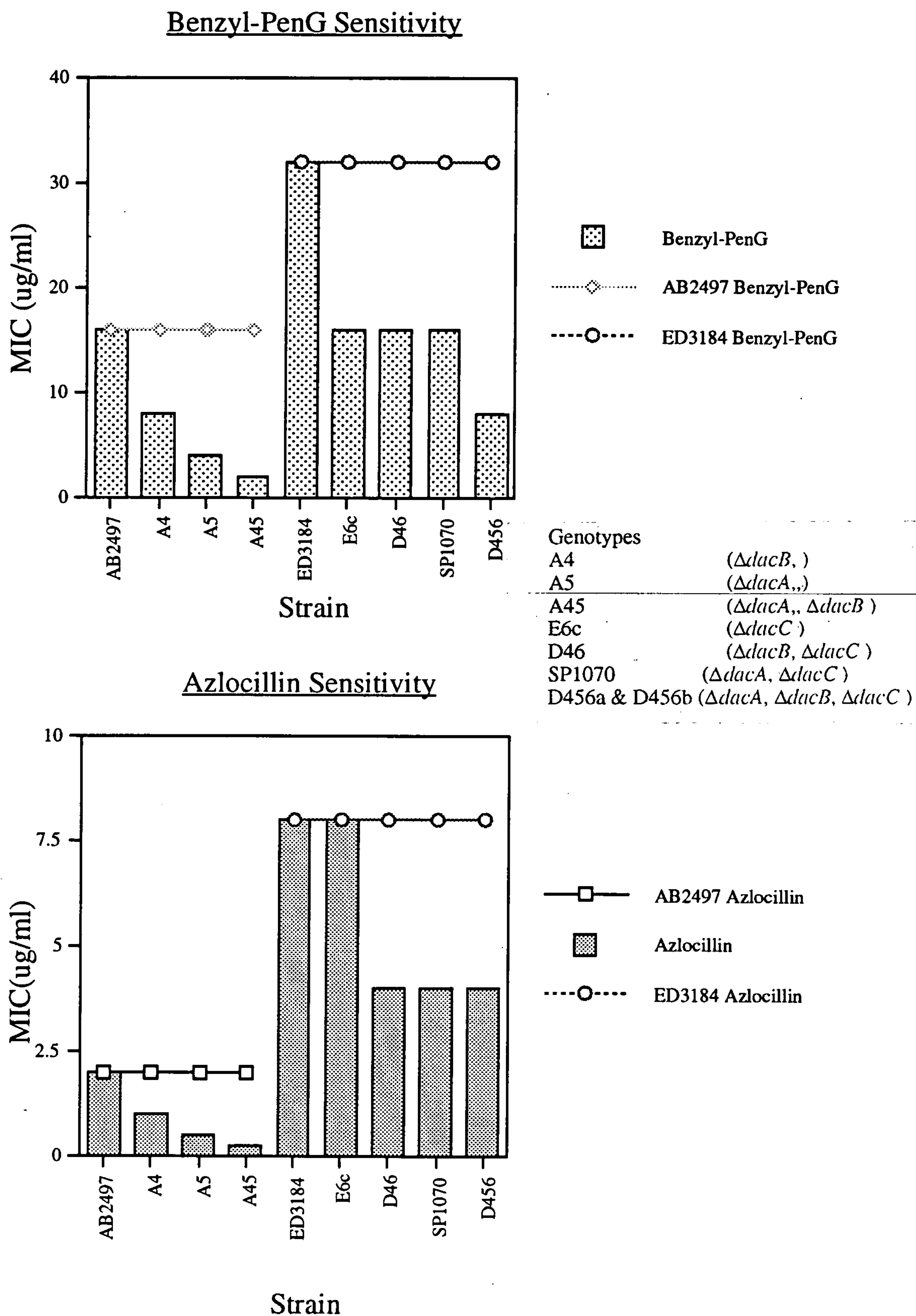
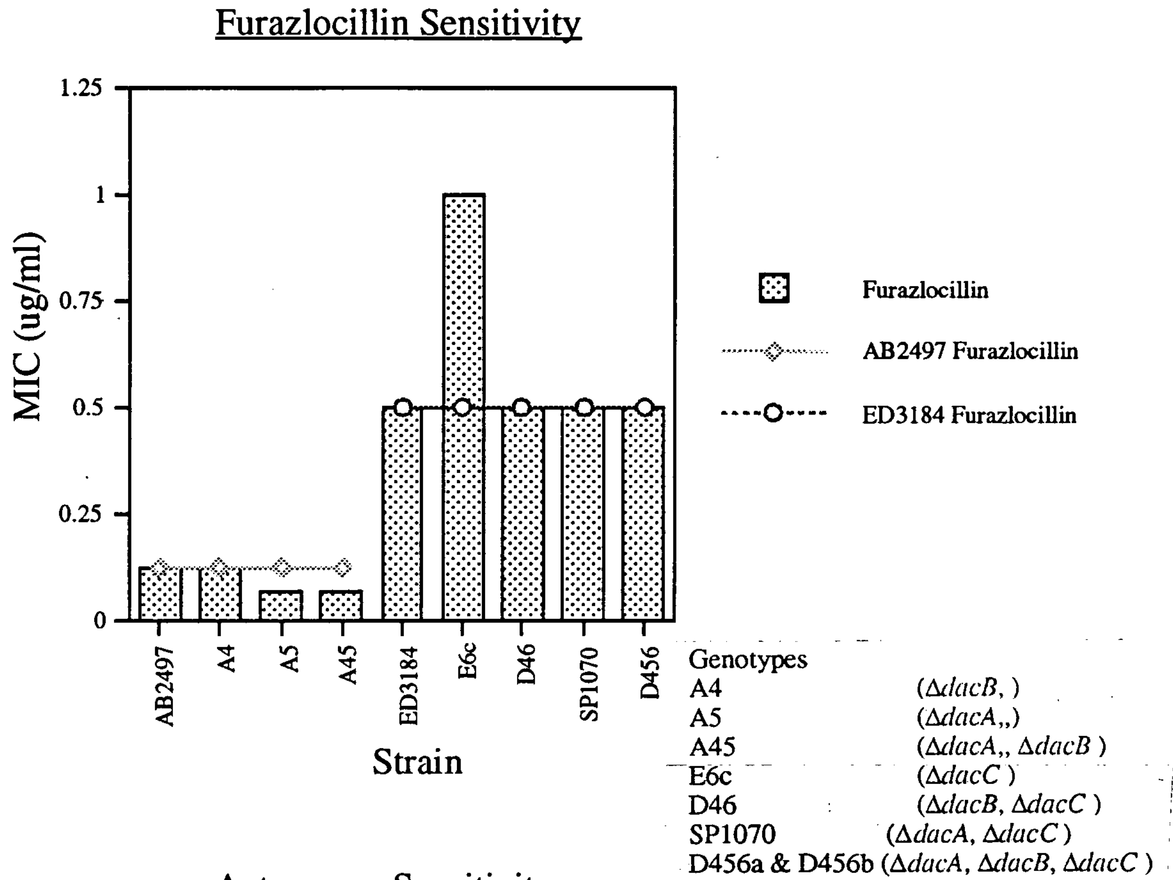
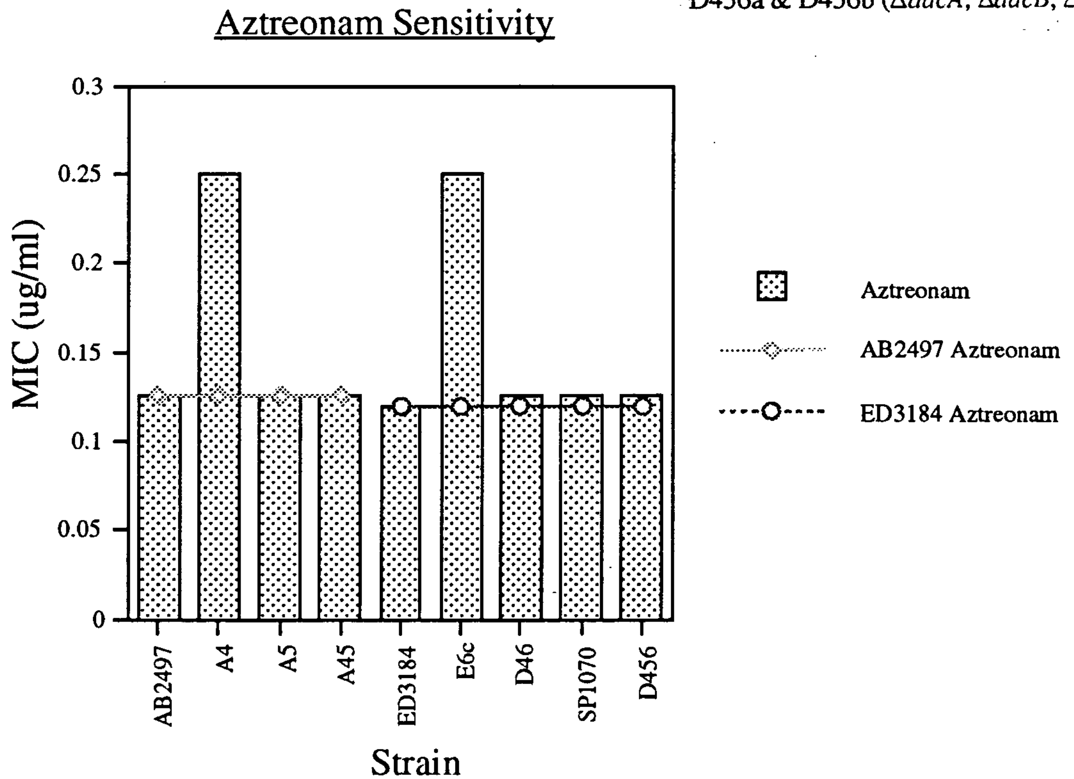


Figure:5.6 Sensitivity of Deletion Strains to PBP3 Specific Antibiotics





(Figure:5.6). Resistance to Aztreonam increased in the absence of PBP4 or PBP6. However the strain with multiple PBP mutations showed wild type sensitivity. Sensitivity to Furazlocillin increased in the absence of PBP5 or PBP4 and PBP5. The PBP6 mutant strain E6c was also slightly resistant to furazlocillin.

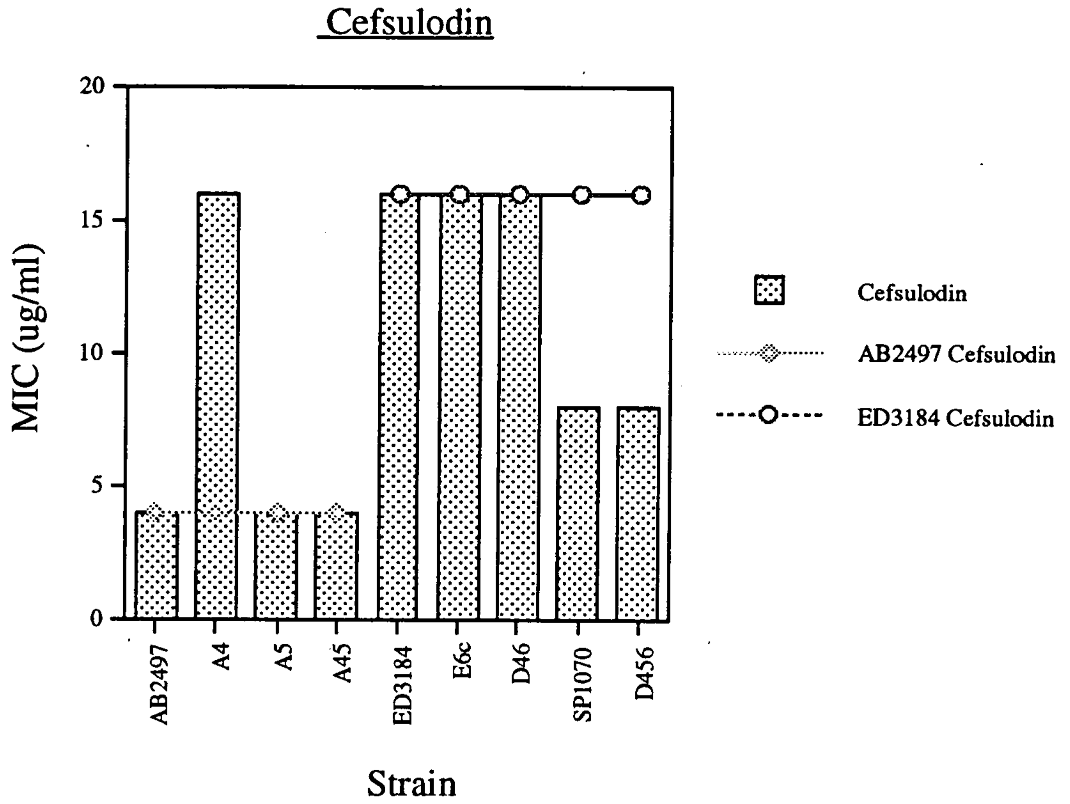
For both these PBP3 specific antibiotics the changes in MICs were marginal, and provided no insight into the roles of the low molecular weight PBPs. It would be predicted from the tripeptide acceptor hypothesis that a strain with reduced DD-Cpase activity, and consequently reduced levels of PBP3 preferred acceptor side-chains, would be more sensitive to inactivation of PBP3. Apart from the sensitivity of A4 and A45 to furazlocillin no significant increase in sensitivity did occur. I have proposed that PBP6 has a specific role in supplying PBP3 with tripeptides. In the absence of PBP6 resistance to these specific antibiotics increased. This is the complete opposite to what would be expected if PBP6 did have the specific role I have proposed.

5.3.4 Cefsulodin.

Cefsulodin is specific to PBP1A and PBP1B, the principal targets of β-lactam induced autolysis (Kitano & Tomasz, 1979). Cefsulodins' bacteriolytic action is limited by its affinity for PBP1B (Youssif *et al.*, 1985) and is characterised by lysis without the characteristic block to division of PBP3 inhibition. This lysis occurs co-incident with but independent from division and is suggested to involve a major autolysin (del Portillo *et al.*, 1990). The four fold increase in cefsulodin resistance found in the single PBP4 deletion strain suggests this is the major autolysin responsible (Figure: 5.7) PBP4 has been inferred to be a major autolysin from the observation that some β-lactams at high concentrations inhibit the rate of wall degradation at the same high concentrations that they saturate PBP4 (Kitano & Tomasz, 1979).

Increased cefsulodin resistance by deletion of *dacB* (PBP4) was abolished by the deletion of *dacA* (PBP5), or *dacA* (PBP5) and *dacC* (PBP6). It was also evident that deletion of *dacA* (PBP5) and *dacC* (PBP6) alone did not affect sensitivity to Cefsulodin. The differences between the murein synthesised by A4, A45, and D46 is not known. It can be assumed reduced DD-Cpase activity will increase the level of pentapeptides and decrease the level of tetrapeptides and tripeptides. An increased amount of pentapeptides capable of acting as donors might be expected to produce an increased level of cross-linkage. However murein analysis of SP1070 and D456 showed no large increase in the level of DD-peptide crossbridges (Glauner, 1986).

Figure: 5.7 Sensitivity of Deletion Strains to Cefsulodin.



Genotypes
A4 (Δ*dacB*,)
A5 (Δ*dacA*,,)
A45 (Δ*dacA*,, Δ*dacB*)
E6c (Δ*dacC*)
D46 (Δ*dacB*, Δ*dacC*)
SP1070 (Δ*dacA*, Δ*dacC*)
D456a & D456b (Δ*dacA*, Δ*dacB*, Δ*dacC*)

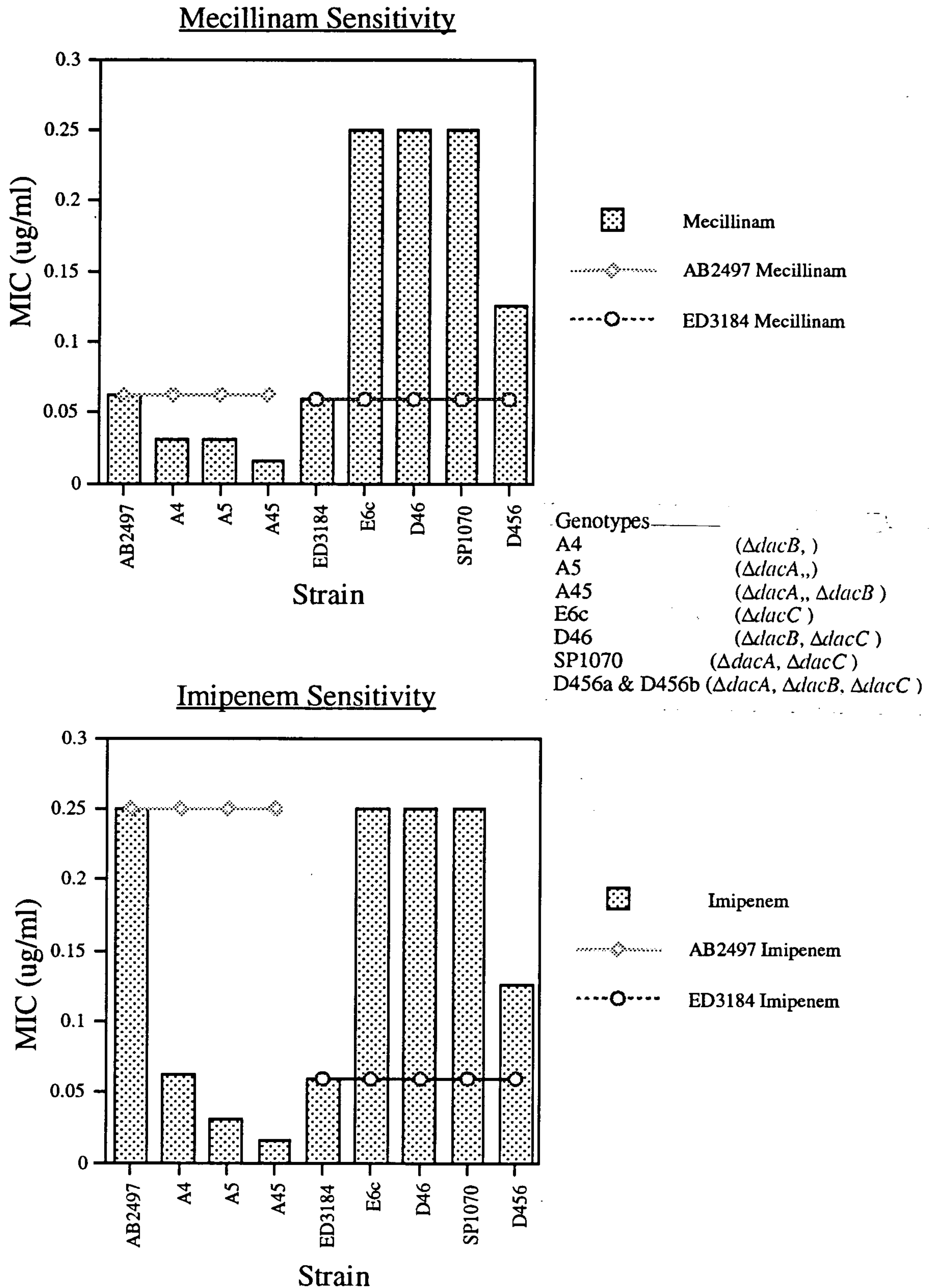
Reduced DD-Cpase activity will lead to a decrease in nascent tetrapeptides. This is because DD-Cpase activity normally degrades 89% of new peptidoglycan within 60 seconds in the periplasm (Glauner & Holtje, 1990). Tetrapeptides provide the substrate for LD-transpeptidation, a process which increases when the cell is challenged with penicillin. This increase in LD-transpeptidation has been considered part of a rescue mechanism which restores saccular integrity. Therefore the restoration of cefsulodin sensitivity to a *dacB* deletion strain by the deletion of *dacA* or *dacC* could involve reduced tetrapeptides and reduced LD-transpeptidation. It is also possible that the sacculi of A45 and D46 are simply too comprimised to exhibit the resistance normally associated with deletion of *dacB*. Without information on the difference between the peptidoglycan of A4, A45 and D46 these explanations remain speculative.

5.3.5 Mecillinam.

Mecillinam specifically binds to PBP2 (Spratt, 1975) and is reported to inhibit 50% of peptidoglycan synthesis (Burman & Park, 1973). PBP2 has been demonstrated to be essential for the maintenance of the cell's rod shape (Spratt, 1975) and is required for the recovery of cells from stationary phase (Pisabarro *et al.*, 1987; Roriguez & de Pedro, 1990). However PBP2 is inessential for cell survival when ppGpp, the effector of the stringent response is induced (Vinella *et al.*, 1992).

Deletion of *dacB* and *dacA* increased mecillinam sensitivity (Figure: 5.8). By contrast, deletion of PBP6 produced resistance to mecillinam. Previous reports of mecillinam resistance have been associated with mutations in *rodA* and *pbpA*, slow growth (Bourman *et al.*, 1981), and mutations of either aminoacyl tRNA synthetases, *cya*, or *crp* (Ogura *et al.*, 1989). The most recent studies have suggested that high levels of the nucleotide ppGpp are responsible for mecillinam resistance. This nucleotide is the effector of the stringent response. The stringent response has been demonstrated to shut down murein synthesis by blocking phospholipid synthesis (Ishiguro & Ramey, 1976; Ishiguro, 1983) and to produce phenotypic tolerance in amino acid-starved bacteria. At the same time ppGpp is also responsible for reducing DD-transpeptidase and DD-Cpase activity (Hartman *et al.*, 1981). The reduction in DD-Cpase activity involves a fraction of the enzyme becoming cryptic, a process that can be reversed by brief sonication of ether treated cells. This is similar to DD-Cpase activity becoming cryptic in *ftsZ*84 filaments (Mirelman *et al.*, 1977).

Figure: 5.8 Sensitivity of Deletion Strains to Mecillinam & Imipenem



The role of ppGpp in mecillinam resistance and suppression of PBP2 deletions has been suggested to indicate a link between ribosome activity and cell wall growth (Vinella *et al.*, 1992; Bouloc *et al.*, 1993). However it has not been discounted that ppGpp confers resistance to PBP2 inactivation by the same mechanism by which it stabilises amino acid-starved cells in the presence of ampicillin *ie.* by stopping phospholipid synthesis, and reducing DD-Cpase and transpeptidase activity (Pisabarro *et al.*, 1990). The tolerance of cells to PBP2 inactivation in the presence of elevated ppGpp (Vinella *et al.*, 1992), and its associated reduction in DD-Cpase activity (Hartman *et al.*, 1981) is similar to E6c resistance to mecillinam. Deletion of another murein hydrolase, the Soluble lytic transglycosylase, produces super-sensitivity to mecillinam (Templin *et al.*, 1992). It would therefore appear that murein hydrolases are closely associated with the action of PBP2 in vivo.

5.3.6 Imipenem.

Imipenem has a relatively high specificity for PBP2, PBP4 and PBP7, but binds to all PBPs with the exception of PBP3 at relatively low concentrations (Tuomonen & Schwartz, 1987). Its ability to lyse non-growing bacteria has been correlated with its specificity for PBP7 (Tuomonen & Schwartz, 1987), and its ability to induce a specific set of autolysins (Cozens *et al.*, 1989). The MICs for Imipenem were almost identical to the results obtained for mecillinam (Figure:5.8). Deletion of PBP4 or PBP5 increased sensitivity, while deletion of PBP6 produced a four fold increase in resistance. Increased levels of PBP6 occur in response to slow growth (Dreihaus & Weuters, 1987) or stationary phase growth (Buchanan & Sowel, 1982). These are situations when cells exhibit degrees of phenotypic tolerance (Tomasz, 1979) to most β-lactams but not to Imipenem (Tuomonen & Schwartz, 1987).

The only common feature of the two antibioics is their high affinity for PBP2. The resistance of E6c to both Mecillinam and Imipenem may be interpretated as indicating that PBP6 is involved in the inhibitory action of both these antibiotics. This explanation would also be consistent with the sensitivity of stationary-phase cells to imipenem, because stationary phase cells have increased levels of PBP6 (Buchanan & Sowell, 1982). Increased levels of PBP6 are also produced by overexpression of *bolA* (Lange & Hengge-Aronis, 1991). However these cells are not more sensitive to mecillinam

How inactivation of PBP6 confers resistance can only be speculated. From the effect of PBP6 mutations on the sensitivity to mecillinam and imipenem would apear to be linked to PBP2. It is not known whether this could be through a direct

interaction, or an indirect effect such as the elevated levels of PBP5 associated with a *dacC* deletion.

5.3.7 Discussion of Antibiotic Results.

The examination of the response of the deletion strains to various β-lactam antibiotics was intended to give an insight into the roles of the DD-Cpases in vivo, and to possible interactions between them. PBP interactions have been suggested from several areas of research. These include;

i.) Studies on the altered PBP profiles of deletion strains (Tameki *et al.*, 1977).

ii.) Studies with cleavable cross-linkers (Mohamaid-Said & Holtje, 1983).

iii)Experiments with membrane vesicles (Leidenix *et al.*, 1989).

iv) Studies of extragenic suppressors and regulatory proteins (Wachi & Matsuhashi, 1989; del Portillo *et al.*, 1991).

Some of the conclusions drawn from this work have included that PBP3 and PBP5 interact (Mohammed-Said & Holtje, 1983), that PBP1B and PBP3 interact (Wachi & Matsuhashi, 1989; del Portillo *et al.*, 1991), and that PBPs1-3 and PBPs 6-8 form membrane associations (Leidenix *et al.*, 1989). In addition the functions of PBP1A and PBP1B have been considered redundant (Kato *et al.*, 1985; Youssif *et al.*, 1985) and the construction of deletion strains has shown only PBP3 to be absolutely essential (Hara & Park, 1993).

The MIC studies combined the use of relatively specific antibiotics with PBP deletion strains with the intention of obtaining an insight into the roles of these low molecular weight PBPs. The resilience of the sacculus structure and the flexibility of its metabolism, combined with the presence of groups of redundant enzymes made interpretation difficult. Only three firm conclusions could be drawn from this work. As expected the sensitivity to broad spectrum antibiotics of strains deleted for the three low molecular weight PBPs increased. It was also apparent that cefsulodin-induced autolysis normally involves the action of PBP4. The third conclusion was that mecillinam and imipenem were both more active in the presence of a functional PBP6. The increased sensitivity to PBP3 specific antibiotics expected of *dacA* and *dacC* deletion strains was not observed. This may have been due to the PBP3-specific antibiotics interacting with their secondary targets. Because of the complexity of interactions between enzymes involved in construction of the sacculus it was decided to re-examine the affect of PBP3 inhibition using the *ftsI*23 allele instead of specific β-lactams.

5.4 Salt Sensitive Suppression.

The *ftsI23* allele was transduced into the deletion strains using co-transduction with either Tn*10* or Tn*9* disruptions of the *leu* gene. Chloramphenicol or tetracycline transductants were selected and colonies patched at 30°C and 42°C to screen for temperature sensitivity. Temperature sensitive patches were streaked to purity at the permissive temperature, and re-screened at 42°C.

Approximately 60% of A5, E6c, D46, SP1070 and D456 transductants were temperature sensitive. However no D4 or D45 transductants were temperature sensitive on LB agar. The transduction of *ftsI*23 into D4 and D45 was repeated. Again no temperature sensitive transductants were isolated. Of the D45 transductants obtained, some exhibited variable growth at 42°C. This variable growth involved transductants growing on LB at 42°C for one plating but not the next. This did not appear to be due to reversion because a transductant which grew at 42°C during one experiment could recover temperature sensitivity during the next. The variable temperature sensitivity disappeared when these transductants were plated upon Nutrient Broth agar (NB). Under these conditions none of the D4 transductants were temperature sensitive.

The efficiency of transducing tetracycline resistance into D4 and D45 using a lysate of TOE23 (*leu*::Tn*10* *ftsI*23) was identical. However an expected class of temperature sensitive transductants had not been isolated. Therefore it was decided to construct the same strains in reverse. Both T23 and T5 had previously been characterised as carrying the temperature sensitive *ftsI*23 allele. TOE23 and T5 cells were transduced with a lysate of A4 (*dacB*::Spec/Strep).

Spectinomycin resistant TOE23 and T5 transductants were obtained at a frequency similar to the wild type control. When screened at the permissive and restrictive temperatures the Δ*dacA* Δ*dacB* strain again appeared to suppress *ftsI*23 completely. The majority of *ftsI*23 Δ*dacB* transductants were initially temperature sensitive on LB plates. When streaked on NB agar their temperature sensitivity disappeared. The continued presence of the *ftsI*23 allele in T5 and T45 strains was confirmed by transducing the temperature-sensitive mutation back into the wild type background. Therefore it appeared that on LB plates a deletion of *dacA* and *dacB* could suppress *ftsI*23 at the restrictive temperature.

The problem remained of the inconsistent nature of the suppression apparent with the single deletion of PBP4. During screening the original transductants for temperature sensitivity the suppression had appeared clearest on Nutrient Broth plates.

The use of Nutrient Broth has revealed the phenotypes of a number of division mutants to be salt sensitive (K.Begg *pers. comm.*). The role of salt in suppression was examined by screening deletion mutants carrying *ftsI*23 for growth at 30°C and 42°C on LB plates containing different concentrations of salt.

Table: 5.3 Salt sensitive suppression on solid media.

Strain	1%	0.5%	0.25%	0%
T23	-	-	-	-
T4	-	+	+	+
T5	-	-	-	-
T6	-	-	-	-
T45	+	+	+	-/+
T56	-	-	-	-
T46	-	-	+	+
T456	-	-	-	-

+ ; Suppression demonstrated by growth on solid media at 42°C.
+/- : Inconsistent growth at 42°C on solid media. - ; temperature sensitive division at 42°C on LB media.

Analysis of growth on plates showed that salt sensitive suppression accompanied deletion of *dacB*. Temperature sensitivity of T456 indicates the presence of a functional DD-Cpase was required for suppression. This implied either specific involvement of DD-Cpase in suppression or simply that the suppression cannot be sustained when the structure of the sacculus is so generally compromised.

5.4.1 Growth Curves.

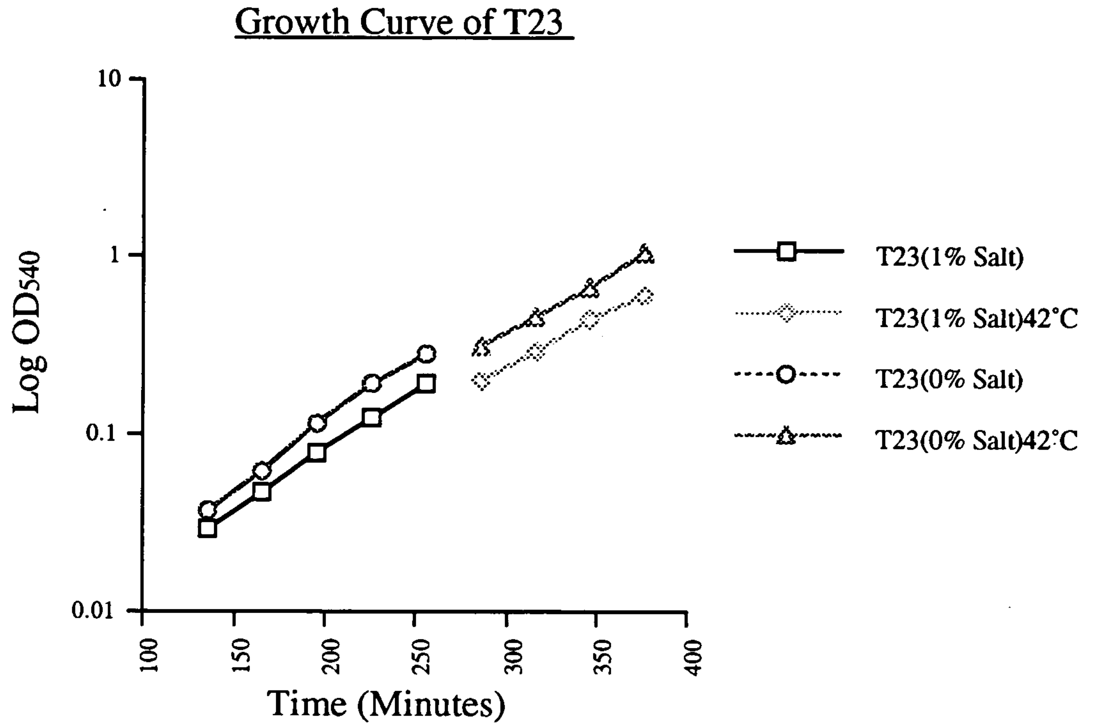
Gowth curves and microscopic analysis of liquid cultures were used to analyse suppression by deletion of PBP4 in liquid cultures shifted to the restrictive temperature (Figures 5.9–5.14). Coulter counts were also attempted but due to debris from lysis even in the suppressed strains no clear figures could be obtained.

At the restrictive temperature it was clear that autolysis occured most rapidly in the T56 and T456 strains. The presence of salt appeared to potentiate lysis of T456. In

theory T456 represents the strain with the most compromised cell wall. Therefore increased sensitivity to PBP3 inhibition would be expected. It was also clear that autolysis of T456 proceeded more rapidly in the presence of salt. This was analogous to the sensitivity of T4 and T46 to 1% salt media.

T45 exhibited a normal growth rate and cell morphology in the presence and absence of salt. The clearest demonstration of salt sensitivity occured for T4. In 1% salt LB T4 formed characteristic filaments at 42°C (Figure: 5.13). In 0% salt T4 grew and divided normally at the restrictive temperature.

Figure: 5.9 Growth of TOE23 and T456 shifted from the permissive to the restrictive temperature, in the presence and absence of Salt.



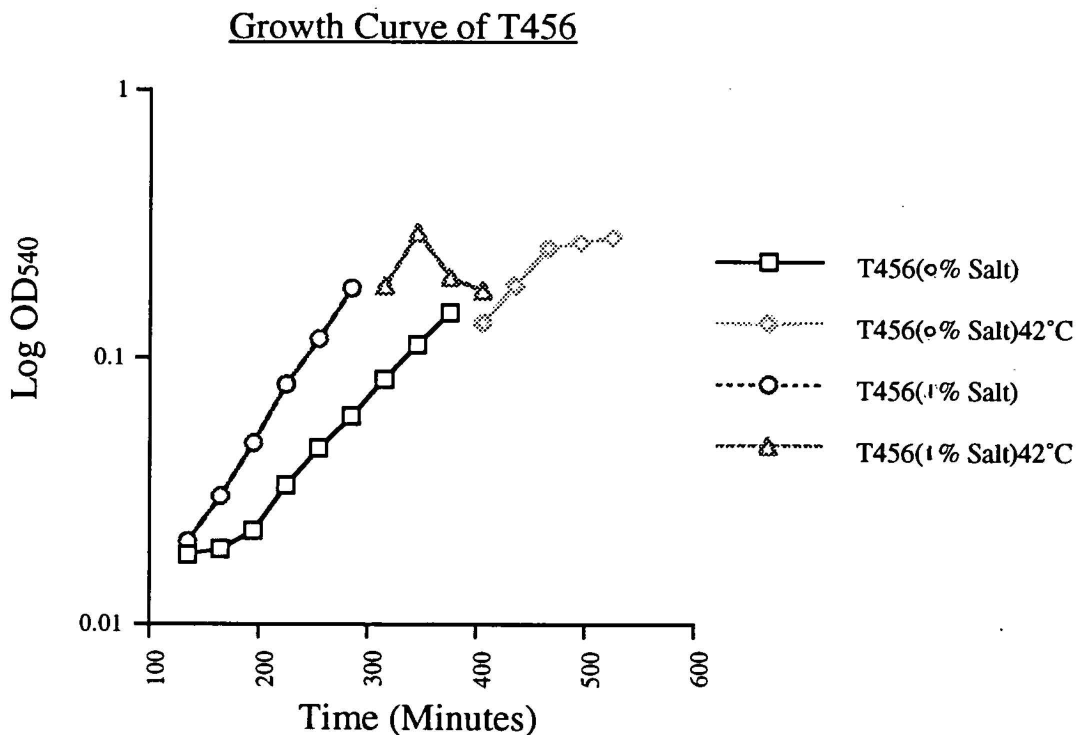
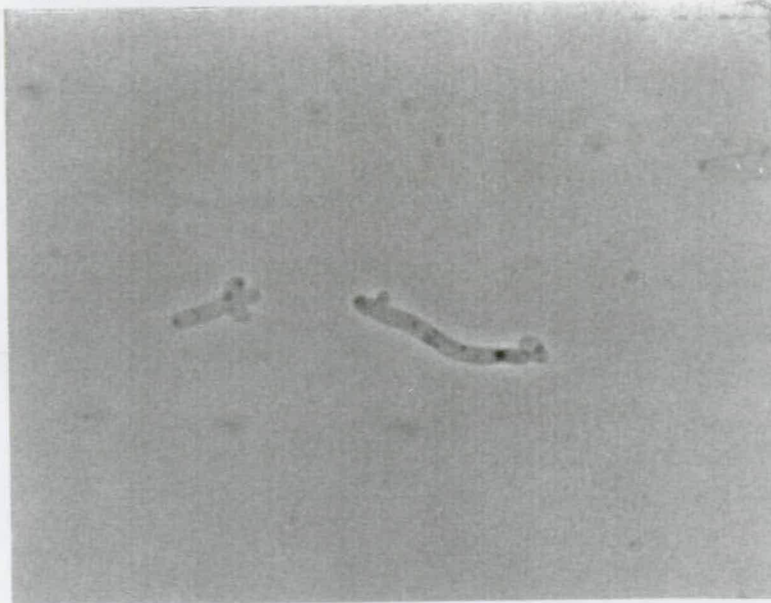


Figure: 5.10 Morphology of T456 in different salt concentrations.

Liquid culture grown at 42°C in 1% Salt L-broth for 60 minutes.



Liquid culture grown at 42°C in 0% Salt L-broth for 60 minutes.

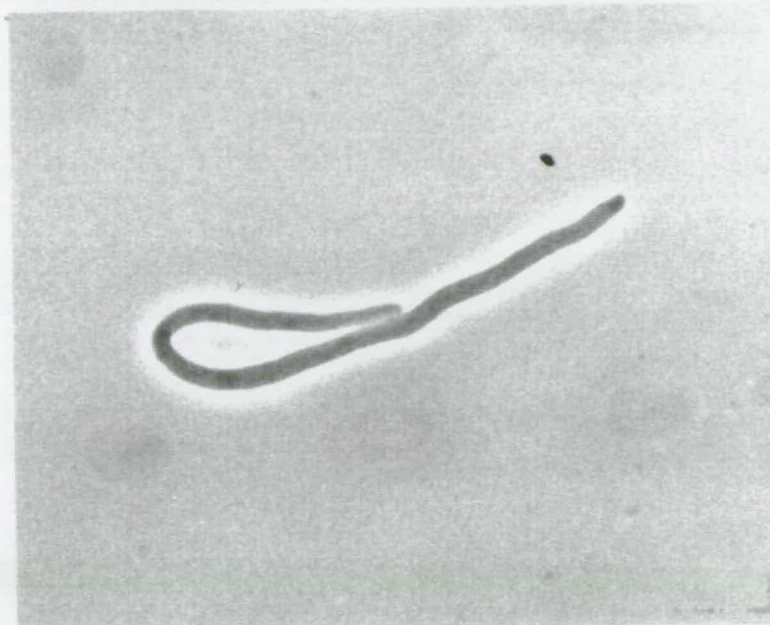
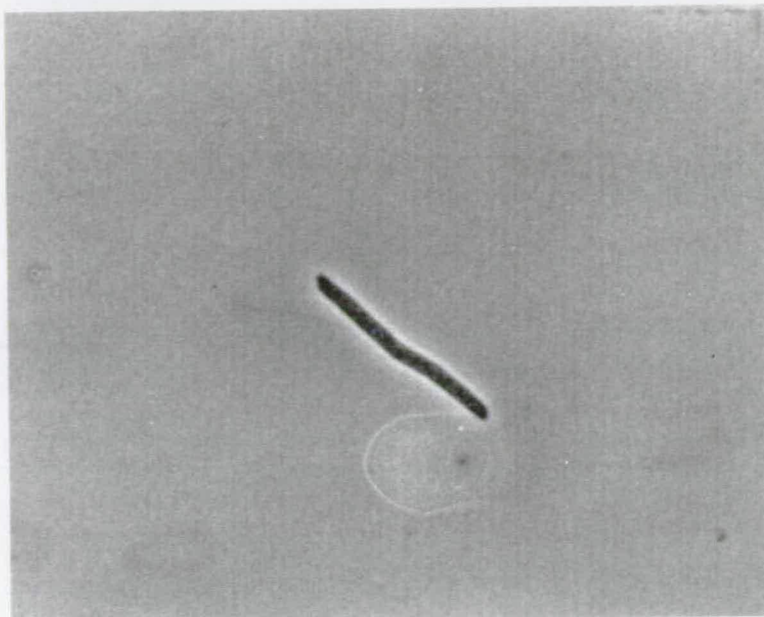


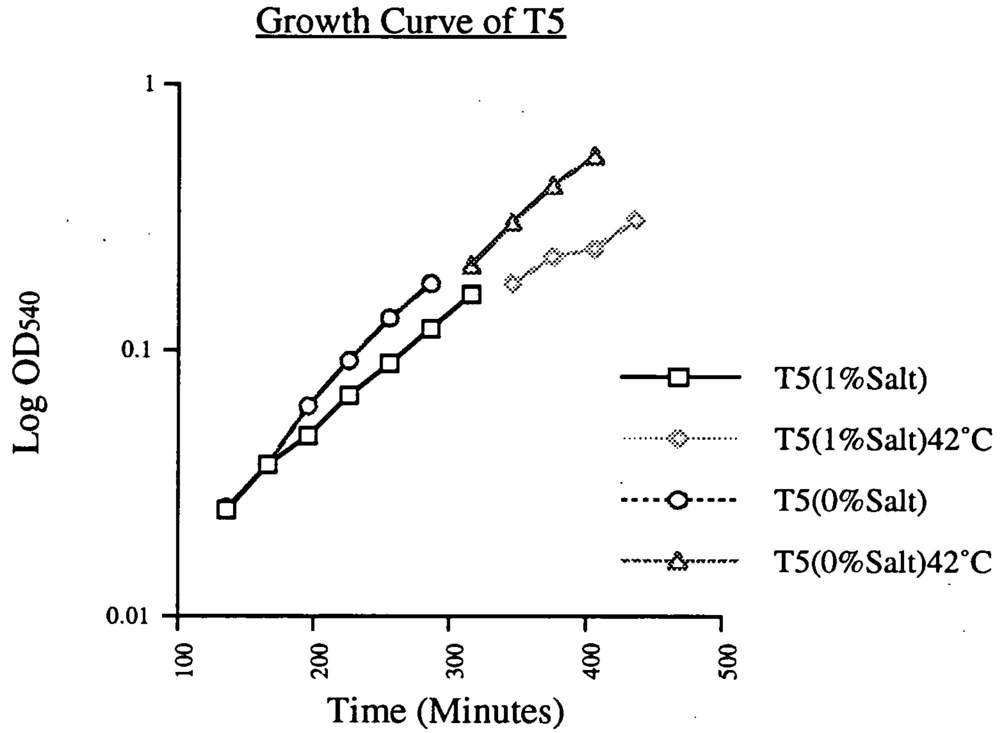
Figure: 5.11 Morphology of TOE23 in different salt concentrations.

Liquid culture grown at 42°C in 1% Salt L-broth for 60 minutes.



Liquid culture grown at 42°C in 0% Salt L-broth for 60 minutes.

Figure: 5.12 Growth of T5 and T56 shifted from the permissive to restrictive temeperature, in the presence and absence of Salt.



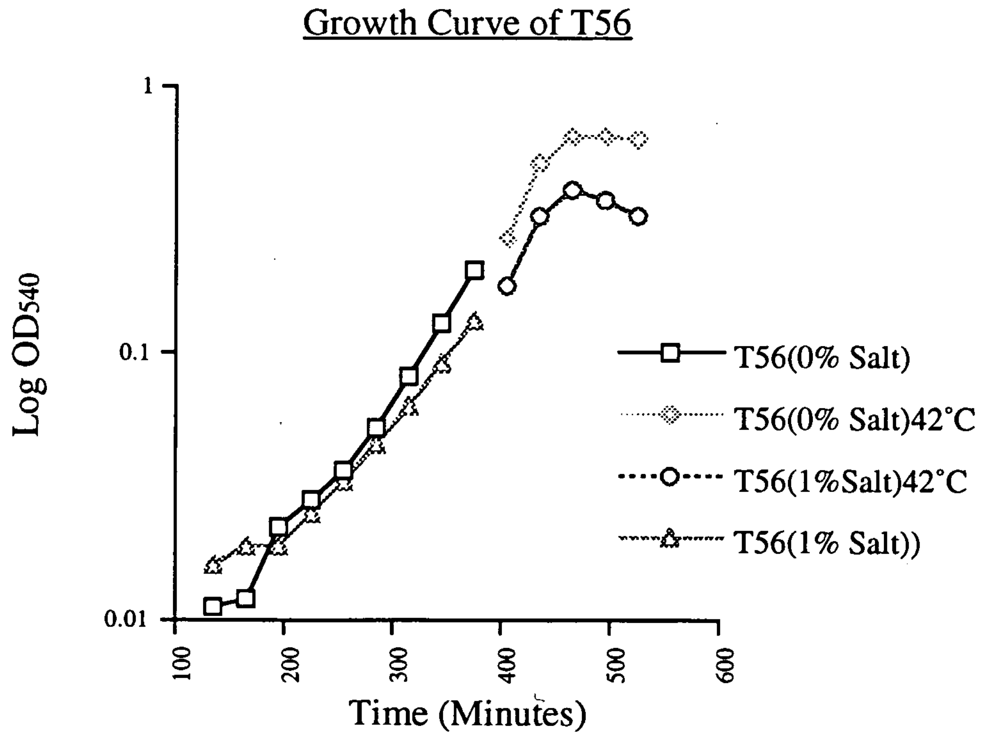
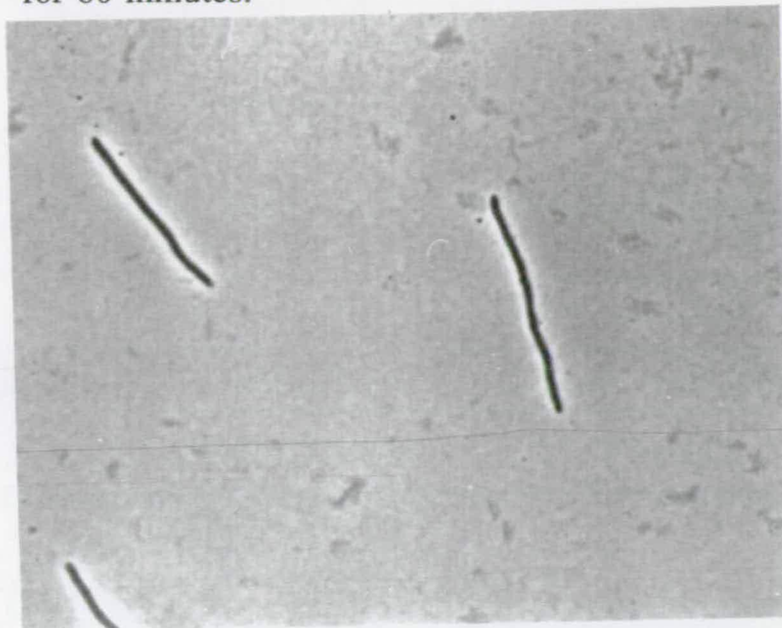


Figure: 5.13 Morphology of T4 in different salt concentrations.

Liquid culture grown at 42°C in 1% Salt L-broth for 60 minutes.



Liquid culture grown at 42°C in 0% Salt L-broth for 60 minutes.

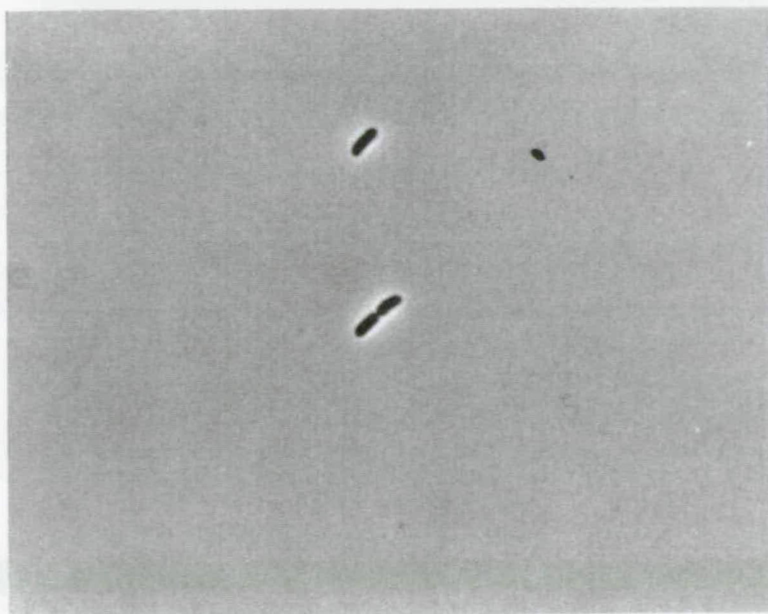
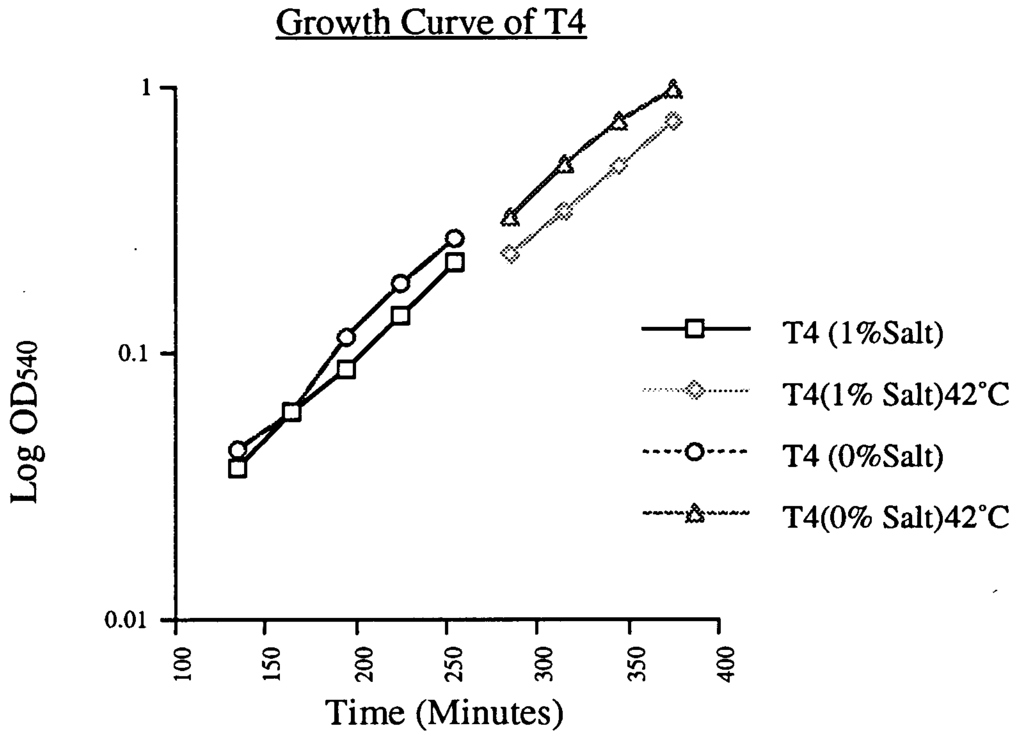


Figure: 5.14 Growth of T4 and T45 at the premissive and restrictive temperature, in the presence and absence of salt.



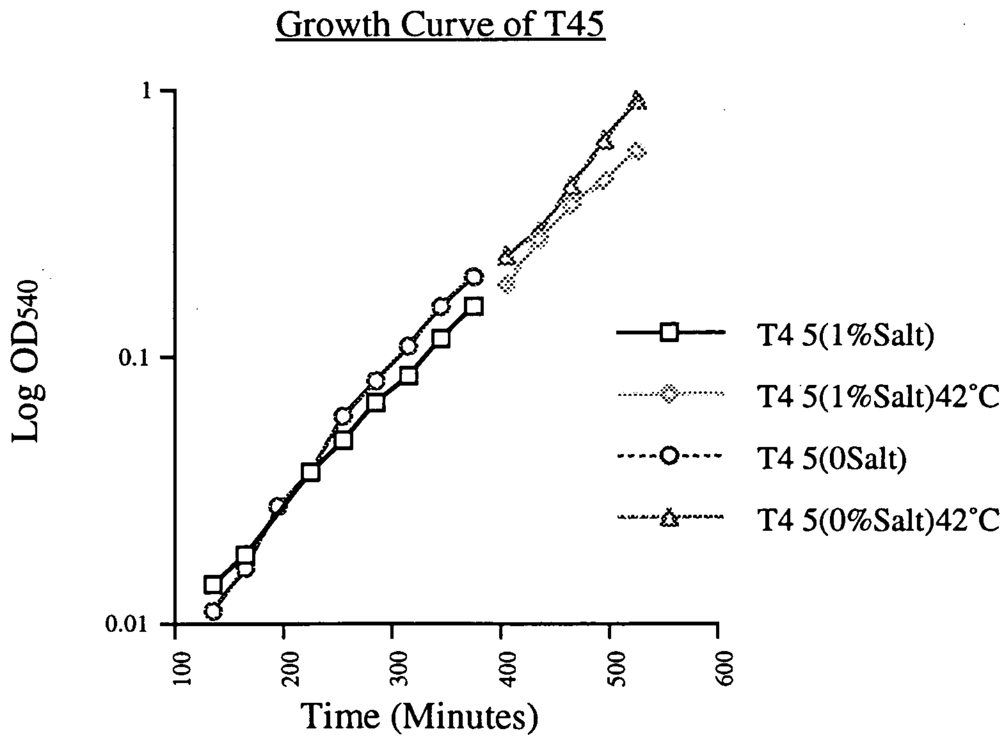
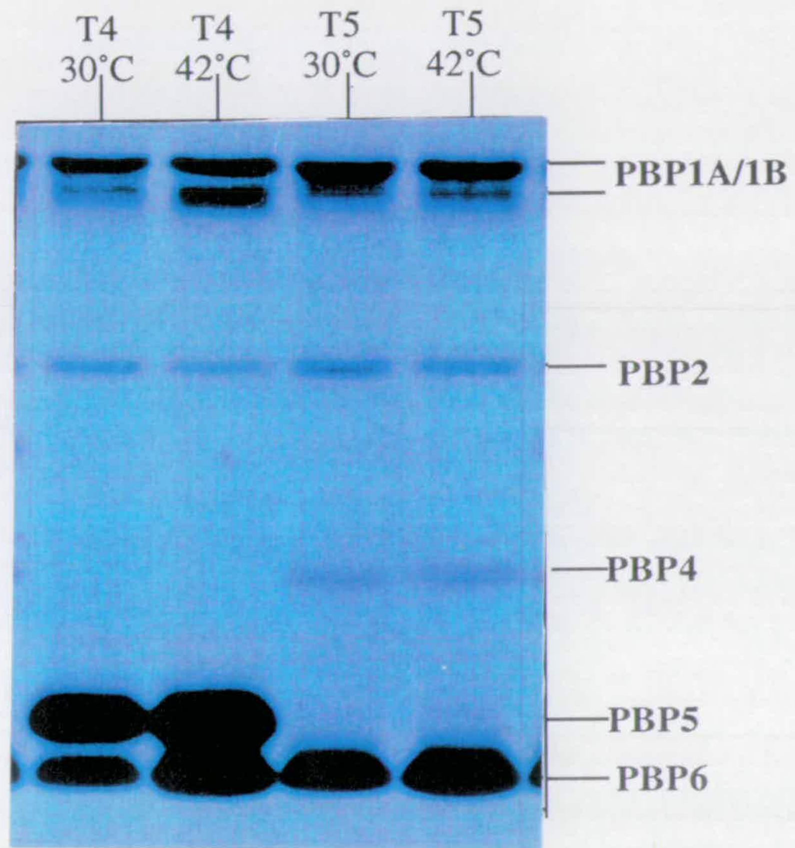


Figure:5.15 PBP Assay of T4 and T5 at the Permissive and Restrictive temperatures



5.4.2 The *dacB* deletion does not suppress by altering DD-Cpase levels.

It was known *ftsI*23 could be suppressed by increasing DD-Cpase activity or altering the levels of tripeptides (Begg *et al.*, 1990). This suppression did not display salt sensitivity. Deletion of PBP4 in a wild type background had not produced any alterations in the levels of PBP5 and PBP6, or changes in membrane bound DD-Cpase activity. To confirm PBP5 and PBP6 were not responsible for suppression PBP assays were performed on T4 and T5 at permissive and restrictive temperatures. The strains were grown in 0.5% NaCl LB to enable suppression of the *ftsI*23 allele in T4.

Table: 5.4. PBP assays of T4 and T5 (Figure:5:15).

	T4 (30°C)	T4(42°C)	T5(30°C)	T5(42°C)
PBP1	9.97	8.56	28.4	22.57
PBP2	1.24	0.51	5.27	2.6
PBP3	-	-	-	-
PBP4	-	-	1.7	2.08
PBP5	69.16	50.98	-	-
PBP6	19.62	39.94	64.6	72.74

Note; Individual PBP levels expressed as a % of the total level of PBPs in a single track. All PBP levels determined as described previously.

Both strains examined (T4 and T5) were derived from the isogenic parent strain for TOE23, AB2497. Two exposures of 10 and 30 days were scanned and the 10 day exposure chosen for interpretation because by 10 days PBP5 and PBP6 had not become saturated.

The PBP assays confirmed the deletions of PBP4 and PBP5, and the presence of the *ftsI*23 allele. Microscopic analysis of the cultures during preparation had revealed the suppressed phenotype of T4 and the expected filamentation of T5. The T5 culture did show evidence of some cell lysis after 90 minutes at the restrictive temperature, but this was not considered to have a significant effect on the PBP assays as only intact cells should be harvested by the initial centrifugation.

Levels of PBP2, PBP3 and PBP4 were considered too low to determine from the 10 day exposure. Temperature shifting T4 to the restrictive temperature produced

an increase in both the proportion and absolute amount of PBP6. This increase in PBP6 was responsible for reducing the proportion of PBP5.

The level of PBP6 in T5 was elevated at both the permissive and restrictive temperatures. This increase had previously been observed when Δ*dacA* (PBP5) was present in a wild type background (AB2497). Temperature shifting T5 also increased the level of PBP6.

Throughout this work thermal inactivation of the *ftsI*23 allele, or deletion of *dacA* has increased the proportion of PBP6 in the cell membrane. The increase in the the absolute amount of PBP6 produced by shifting T4 to 42°C was from 1,638,620 to 4,793,374. The increase in PBP6 produced by shifting T5 to the restrictive temperature was from 2,102,756 to 3,569,705. Therefore it was possible that suppression by deletion of *dacB* could be the result of elevated levels of PBP6. Two previous experiments argued against PBP4 deletion suppressing by altering PBP6 levels. First the salt sensitivity of suppression. Second the failure of pAX607(*sufI*) to suppress in the absence of PBP4. To check the pAX607(*sufI*) result T4 was transformed with pBS110(PBP6) and pAX607.

The standard procedure of transforming at the permissive temperature, streaking to purity, checking mini-preparations of the plasmid DNA and then curing to screen out revertants was followed. All these procedures were carried out using standard LB with a salt concentration of 1%.

Neither the plasmid pBS110 nor pAX607 could suppress *ftsI*23 in the absence of PBP4. This result clearly indicates that increased PBP6 was not responsible for suppression by the PBP4 deletion. Therefore it was concluded that a second mechanism of *ftsI*23 suppression was responsible.

5.5 Discussion

PBP4 is a major DD-endopeptidase responsible for hydrolysis DD-peptide cross-bridges between murein side chains. The deletion of *dacB* caused a salt sensitive suppression of *ftsI*23. This suppression was unaffected by deletion of either of the major DD-Cpases but did not suppress in the absence of both PBP5 and PBP6. It has been established that increased levels of DD-Cpase activity can suppress *ftsI*23 (Begg *et al.*, 1990). The deletion of *dacB* (PBP4) in a wild type background does not increase PBP6 or membrane bound DD-Cpase activity. However T4 (*ftsI*23 Δ*dacB*) does exhibit increased levels of PBP6 when grown at the restrictive temperature for 90 minutes in the presence of 0.5% salt. To test if this was responsible for suppression T4 was transformed with plasmids which suppress *ftsI*23 by increasing DD-Cpase

activity. Neither pAX607 (*sufI*), or pBS110 (*dacC*) suppressed T 4 on 1% LB. In fact deletion of PBP4 abolished the suppression of *ftsI*23 produced by pBS110 and pAX607. It was therefore concluded that increased DD-Cpase activity was not responsible for suppression by the *dacB* deletion.

The best suppression of T4, and T46, occured in no salt media, when the sacculus is expected to be under greatest osmotic stress. It is not clear why salt should interfere with suppression. Divalent cations are involved in DD-endopeptidase (Montilla *et al.*, 1983) and general hydrolase activity (Holtje & Tuomanen, 1991). It has been demonstrated that the chelation of divalent cations can produce premature division in synchronised cultures (Nanninga *et al.*, 1983). However there is no evidence for NaCl having similar effects.

NaCl has been demonstrated to protect against chaotropic agents that disturb the structure of the cell membrane, and to inhibit division and induce autolysis (Ingram, 1981). Salt concentrations between 0.5% and 1.8% have been hypothesised to rescue some division mutants by restoring the confirmation of temperature sensitive proteins (Ricard & Hirota, 1973). However rather than requiring high salt concentrations for suppression by the *dacB* deletion, low salt concentrations were required.

High concentrations of salt have been suggested to increase the level of hydrolase activity in the periplasm (Hartman *et al.*, 1974). NaCl has also been demonstrated to induce the release of DD-endopeptidase activity when used for osmotic upshift (Iida *et al.*, 1983). In *Staphylococcus aureus* the major autolysin *N*-acetyl-L-alanine amidase is activated by NaCl (Gilpin *et al.*, 1972). This process involves NaCl positively regulating transcription of the *lyt* gene (Mani *et al.*, 1993). In turn the *lyt* gene product positively regulates autolysin production. As would be expected of a mutation which abolishes positive regulation of the major autolysin, the *lyt* mutation confers resistance to autolysis. In *E.coli* an L-form-derived revertant with a rigid cell wall has been isolated, which can spontaneously degrade its sacculus when NaCl is added to the media (Onoda *et al.*, 1987). LD-cross-bridges have been shown to increase in response to overproduction of autolysins (Holtje & Glauner, 1990) and may be an indicator of increased autolysin activity in normal cells. Increased osmolarity has been reported to produce more LD-cross-bridges (Glauner & Schwarz, 1990).

From this literature it would appear autolysin activity responds positively to increasing salt concentrations. The extragenic suppression of *ftsI*23 by the deletion of a major autolysin is sensitive to high concentrations of salt. The concentrations of salt to which suppression is sensitive fall within the range demonstrated by Hartman *et al.*

(1974) to positvely affect the release of hydrolases from the cell membrane. This suggests the suppression observed is based upon reduced autolytic activity during division.

The best suppression of *ftsI23* occurred in the absence of PBP4 and PBP5. In this strain the inactivation of PBP5 is expected to increase the amount of pentapeptide and the deletion of *dacB* to reduce the hydrolysis of DD-peptide cross-bridges. This assumption is supported by the 2% increase in Ala-Dap cross-bridges observed in D456. It is possible this marginal increase is large enough to maintain saccular integrity even in the presence of high salt and increased hydrolase activity. However it is not certain why the deletion of *dacA* and *dacB* should produce the best suppression.

The T46 strain is the most sensitive to salt. The PBP6 protein has less DD-Cpase activity than PBP5 both in vitro and in vivo (Amanuma & Strominger, 1984; Van der Linden *et al.*, 1993). Its inactivation does not increase pentapeptides or have any other gross effect on the peptidoglycan synthesised. I have proposed PBP6's enzyme activity to be linked with PBP3. If this is correct a deletion of PBP6 would reduce the supply of substrate to PBP3. This would potentiate the thermal inactivation of PBP3, and only low levels of autolysin activity would enable suppression of *ftsI23*.

All these explanations assume a temperature sensitive PBP3 protein to have residual activity at the restrictive temperature. Like suppression by increasing DD-Cpase activity, a decreased level of murein hydrolase activity during division is proposed to enable thermally impaired PBP3 activity to complete a septum.

Several peptidases have been identified in *E.coli* . These include (in addition to PBP4) a penicillin-insensitive DD-endopeptidase MepA (Iida *et al.*, 1983; Keck & Schwarz, 1979.), a speculated LD-endopeptidase (Holtje & Tuomonen 1991) and recently the identification of a specialised DD-endopeptidase (T.Romeis, *pers.comm.*). Accompanied by the activities of muramidases and lytic transglycosylases these enzymes enable insertion, growth and recycling of peptidoglycan. Hydrolases have been implicated in division since the observation that penicillin treatment produced localised murein hydrolase activity at division sites (Schwarz *et al.*, 1969). More specifically DD-endopeptidase activity has been demonstrated to be enriched in minicell membranes (Goodell & Schwarz, 1977) and observed to peak just prior to division (Hackenbeck & Messer 1977). β-lactam tolerant mutants have been reported to exhibit reduced endopeptidase activity and lower levels of PBP4, implicating this enzyme in autolysis during division (Kitano *et al.*, 1980).

It is proposed that suppression of *ftsI*23 by deletion of the major DD-endopeptidase PBP4, is caused by reducing hydrolytic activity to enable residual PBP3 to complete synthesis of a septum. This sensitive suppression mechanism would be potentiated by deletion of PBP5 and the concomitant increase in DD-peptide bonds, or by a reduction in salt concentration and the inhibition of remaining hydrolase activity. The requirement of the T46 deletion for the lowest salt concentrations would be consistent with the PBP3-associated role proposed for PBP6 in chapter 2.

The most rapid lysis of T456 occured in the presence rather than the absence of salt. This would be predicted if salt positively regulated murein hydrolysis during division. The increased rate of autolysis for T56 and T456 is also consistent with the prediction that deletion of the two major DD-Cpases will potentiate PBP3 inactivation. This link between DD-Cpase and PBP3 was not demonstated with PBP3 specific antibiotics. It is assumed this is because these antibiotics also interact with other PBPs besides PBP3.

The results presented in this chapter suggest the normal construction of a septum involves coordination of DD-Cpase activity, and DD-endopeptidase activity with the synthetic activity of PBP3. Although not essential, the periplasmic DD-Cpases and PBP4 are required for the construction of a fully functional sacculus and the efficient construction of a septum.

CHAPTER 6

THE LD-CARBOXYPEPTIDASE.

The presence of DD-Cpase and LD-Cpase activities in the membranes of *E.coli* was first demonstrated by Izaki and Strominger (1968). Partial purification of a 43 kD protein which was proposed to form a dimer in vivo and exhibit LD-Cpase activity was achieved by Beck and Park (1977b). These workers used a toluenised *ftsA* temperature-sensitive strain to demonstrate LD-Cpase activity increased in dividing cells. The LD-Cpase of *G.homari* is essential and is sensitive to antibiotics with D-Alpha-aminoadipic acid or D-homoserine substituents (Hammes & Seidel, 1978). In *G.homari* the LD-Cpase can also catalyse an exchange reaction which will replace the terminal D-alanine of a tetrapeptide with other D-amino acids (Hammes, 1978). The D-Alpha-aminoadipic acid or D-homoserine groups interfere with this reaction. Studies on ether treated mutants in *E.coli* showed by contrast with *G.homari* that two LD-Cpases existed. Termed LD-Cpase 'I' and 'II' the former could catalyse release of D-alanine from uridine linked tetrapeptide pre-cursors, while the latter released it from muramyl-tetrapeptide (Metz *et al.*, 1986a). The LD-Cpase I appeared to be inessential and its substrate specificity suggested it was a cytoplasmic protein. Purification showed LD-Cpase I to have a molecular weight of 12,000 kD and to be inhibited by D-amino acids and Norcardicin-A (Metz *at al.*, 1986b). LD-Cpase II was also inhibited by Norcardicin-A but its sensitivity to an unusual β-lactam; Thienemycin, was distinctive. More recently Ursinus *et al.* (1992) purified a Norcardicin-A sensitive LD-Cpase from *E.coli* using Norcardacin-A affinity chromatography. This protein appeared to be a monomer with a molecular weight of 32 kD, and exhibited the expected high specificity for muramyl-tetrapeptide. The enzymes' pH range, and isoelectric point were consistent with earlier reports of Izaki & Strominger (1968), but it did not show the thienymycin sensitiviy reported by Metz *et al.* (1986b).

Suppression of *ftsI*23 by increasing levels of PBP5 and PBP6, or the supply of normally inhibitory levels of D-Cycloserine led to the proposal that tripeptides acted as preferred acceptors for a PBP3 mediated transpeptidation. Due to the relative abundance of DD-Cpase activity and the fact that approximately 90% of it could be deleted without any obvious phenotype, it was considered that the LD-Cpase was more likely to be the key regulatory enzyme. This was consistent with the cycling of the LD-Cpase reported by Beck & Park (1976) and the observation that chain forming *envC* mutants release 6 times the level of LD-Cpase when induced to divide (Karibian *et al.*, 1981). Therefore an attempt was made to locate and sub-clone the gene encoding the periplasmic LD-Cpase.

6.1. Isolation of Suppressors from a Plasmid Library.

The two major DD-Cpases of *E.coli* could be deleted without affecting septum formation. This suggested that in the periplasm tetrapeptides destined for degradation to tripeptides existed in excess. While it could not be discounted that timing or localisation of tetrapreptides plays an important role in septum formation, it was predicted that the presence of the LD-Cpase on a multi-copy plasmid would be able to suppress *ftsI*23.

Two plasmid libraries already constructed in pBR322 were available and construction of a third was begun. Initially the *HindIII* and *BamHI* libraries were transformed into TOE23 and transformants selected at 30°C. These were patched at 30°C and 42°C, colonies exhibiting good growth at the restrictive temperature were subsequently streaked out from the corresponding 30°C patch. They were re-checked for suppression and mini-prep DNA prepared from 30°C overnight cultures. The DNA was restricted to liberate the insert from backbone and then separated by gel electrophoresis. Any transformants that exhibited good reproducible suppression were then cured and checked for reversion. It was intended that a more detailed analysis of the inserts' restriction pattern would screen out complementation by the wild type allele, and any known suppressers.

6.1.1 Reversion.

During five separate experiments 1000 TOE23 transformants were screened for temperature sensitivity. Approximately 25% of these grew when patched at 42°C. When restreaked only 2% of transformants consistently suppressed temperature sensitivity. Extragenic multi-copy suppression of *ftsI*23 would be expected if any of these plasmids carried *dacA*, *dacC*, or *sufI*. *ftsI*23 would also be complemented by a plasmid with an insert that carried the *ftsI*$^+$ gene.

Mini-preparations of plasmid DNA from the suppressed strains showed that two plasmids were responsible for the majority of suppression. These plasmids had inserts of 12 kb and 15 kb respectively. TOE23 was transformed with mini-preparations of these plasmids and rescreened for suppression. These transformants also grew at 42°C but when they were cured of their plasmids they did not regain temperature-sen sitivity. The transformants had either reverted or obtained a suppressor mutation. The *ftsI*23 allele is normally stable under permissive conditions. The size of the plasmid inserts corresponded to that which would be expected if they contained a *BamHI* or *HindIII* fragment carrying the *ftsI*$^+$ gene. It is therefore

possible that the loss of temperature sensitivity from the cured strains resulted from recombination between the chromosomal and plasmid copies of *ftsI*.

Attempts to isolate suppressors from a multi-copy library failed. None of the expected multi-copy suppressors had been isolated. Therefore it was decided to adopt a different strategy for obtaining the LD-Cpase.

6.2. Attempts to obtain an *N*-terminal Sequence.

A 32 kD periplasmic protein purified by Norcardacin-A affinity chromatography and SDS-PAGE has been demonstrated to exhibit LD-Cpase activity by J.V-Holtje's group in Tubingen. In collaboration with this group a purified sample of the protein was supplied to me with the intention that I obtain the *N*-terminal amino-acid sequence. This sequence could then be used locate the gene by probing a *E.coli* library with degenerate oligonucleotides.

The first sample of purified protein wassuppliedlypholised and problems were encountered with resuspension. Eventually a proportion of the material was solubilised in 5% Formic acid. This was then subjected to amino acid analysis and *N*-terminal sequencing by the WELMET protein sequencing service at Edinburgh University. No *N*-terminal sequence was obtained due to blockage.

In essence, protein sequencing involves coupling of Phenyl isothiocyanate (PITC) to the α-amino group of the *N*-terminal residue of a protein. This is cleaved to liberate an ATZ-amino acid which is removed and converted from an ATZ-amino acid to a stable PTH-amino acid. The PTH-amino acid is then identified by its elution time from a HPLC column. *N*-terminal modfications like acylation, formylation and cyclisation are inhibitory to the initial coupling, and result in blockage of the *N*-terminus.

Two more samples of the LD-Cpase were purified by the same procedure in Gemany. In the final step of purification the sample was eluted from an SDS-PAGE gel system in tris-glycine buffer. The quantity and purity of the LD-Cpase was estimated from an aliquot run on SDS-PAGE. The sample contained approximately 440 μg of protein, and was contaminated by two proteins with molecular weights of approximately 34 kD and 39 kD. These contaminants were not present in sufficient quantities to intefere with sequencing of the LD-Cpase, so to avoid any loss of material no further purification was attempted.

The presence of glycine interferes with the accuracy of amino acid analysis and *N*-terminal sequencing. To change the buffer the sample was split into two fractions

and one was dialysed against Tris-HCl (25 mM pH.7.5). Despite careful and extensive dialysis approximately 90% of the protein was lost. It was presumed that either the dialysis tubing was damaged or the protein had become associated with the membrane. The remaining sample was loaded into a 10 kD Centricon column and centrifuged for 35 minutes at 8,000 rpm. The filter in the Centricon column allows the solution to pass through during centrifugation while excluding any molecules larger than 10 kD. By repeated resuspension of the sample in sterile ddH_2O and repeated centrifugation through the 10 kD Centricon column, the buffer was changed and the sample concentrated. Approximately 90% of the original protein was estimated to be recovered by this procedure. Any subsequent concentration of sample or buffer changes were carried out using Centricon columns.

The protein sample was taken to the SERC protein sequencing facility in Aberdeen. Initial amino acid analysis indicated the actual amount of protein to be half that estimated by SDS-PAGE. This amino acid analysis differed from the one obtained with the previous sample. It was attempted to purify the protein further by FPLC. However the protein appeared to elute with the solvent front and was unrecoverable. Approximately 60 µg remained after FPLC and amino acid analysis, 10 µg of this was loaded directly onto the protein sequencer. The *N*-terminus of the protein appeared to be blocked, but a trace sequence was obtained. This trace sequence was identified from very low levels of amino acids, and consequently is more likely to be inaccurate. A database search for the 10 consecutive residues identified did not produce any good matches. However a run of 6 consecutive residues did match 5 amino acids located within the MreC protein (Figure: 6.1).

Figure: 6.1. Comparison of *N*-terminal Sequence with *mreC*.

mreC; (52) V S N A P R E L (59)
N-terminal Sequence; (2) I D N A P R A L (9)

Amino acids represented by single letter code. Numbers correspond to location of amino acids within their full sequences.

6.3 MreC.

MreC is encoded by the *mreC* gene which forms part of a bacterial shape determining operon located at 71 minutes on the *E.coli* chromosome (Wachi *et al.*, 1989). This operon includes the *mreB* and *mreD* genes and determines cell shape and

mecillinam sensitivity. Mutations in any of the three genes results in cell rounding (Wachi *et al.*, 1989), overproduction of PBP1B and PBP3, and a reduction in the levels of the 16 kD Peptidoglycan Associated Protein (PAL) (Okada *et al.*, 1992). MreC has a putative membrane anchor at its N-terminus. MreB shares sequence similarity with heat shock and regulatory proteins, and MreD is highly hydrophobic.

Table: 6.1. Comparison of Protein Profiles of "LD-carboxypeptidase" samples and the amino acid composition of MreC.

Amino Acid	Edinburgh %	Aberdeen %	MreC %
ASX	3.2	8.2	8.6
GLX	11.6	12	11.6
SER	2.7	-	5.9
GLY	14.1	–	7.3
HIS	0.7	–	0.7
ARG	0.8	6.4	7.3
THR	5.7	16.1	5.9
ALA	14.3	14.4	10
PRO	5.16	8.9	8.9
TYR	1.1	5.9	2.1
VAL	7.6	4.5	8.4
MET	2.2	2.0	2.4
CYS	–	0	2
ILE	7.2	4.5	4.3
PHE	4.9	3.8	1.3
TRP	–	0	0.2
LYS	0.62	4	2.2
LEU	17.7	10.7	11.4

If the trace sequence did represent MreC it would have to originate from a cleavage product. The deduced molecular weight of MreC is 39.5 kD, but it has been reported to run at 37.5 kD in SDS-PAGE gel systems (Wachi *et al.*, 1989). These molecular weights are significantly different from the 32 kD reported for the LD-Cpase (Ursinus *et al.*, 1992). Comparison of the two amino acid profiles obtained for the two LD-Cpase samples and the amino acid composition of MreC revealed some similarities but also significant differences (Table: 6.1). If the sequence does

correspond to MreC it is more likely to have come from one of the minor contaminants visible in the original sample. The major contaminant had a molecular weight determined by SDS-PAGE of 36.5 kD. This is closer to the molecular weight of MreC. For MreC to co-purify with the LD-Cpase it would have to share a similar affinity for Norcardacin-A.

Norcardacin-A combines a β-lactam ring with a D-amino acid side chain which resembles the terminal D-alanine of a tetrapeptide side chain. Tetrapeptides can act as the substrate for the LD-Cpase, LD-endopeptidase and LD-transpeptidase. Mutations in the *mre* locus have also been reported to reduce PAL (Okada *et al.*, 1992). This suggests that they interfere with the LD-transpeptidation required for cross-linkage of peptidoglycan to PAL. The function of MreB; a regulatory protein (Okada *et al.*, 1992), and MreD; a lipid carrier molecule (Matsuhashi *pers comm.*), have been suggested. The function of MreC has not been elucidated. If the sequence obtained is the result of low levels of MreC co-purifying with the LD-Cpase on a Norcardacin-A affinity column, it would suggest that this protein is the LD-transpeptidase responsible for attachment of lipoprotein.

Preliminary work to sub-clone the *mreC* gene was begun with the amplification of the appropriate Kohara phage and isolation of its DNA. Four preparations of phage DNA were purified, two covering the desired DNA and two from the flanking regions. Only one of these phage contained chromosomal DNA that matched the expected region of the chromosome. This phage DNA did not include the *mre* locus and no further work was continued on the isolation of this gene because the sequence for the LD-Cpase became available.

6.4. Attempts to obtain a free *N*-Terminus.

An attempt was made to digest the remaining 50 µg of protein and release a peptide with a free *N*-terminus. Two specific proteases were chosen, Lys-C and Asp-N. Lys-C cleaves *C*-terminal to lysine residues while Asp-N cleaves after either N-aspartic or cysteine residues. Approximately 25 µg of protein was resuspended in the appropriate buffer, and the protease added.

The reaction was stopped by boiling and it was attempted to purify individual peptides by FPLC. The separation of proteolytic cleavage products by HPLC usually produces a trace of closely grouped peaks. The number and size of these peaks vary according to the protein and protease involved. However, the pattern obtained for the LD-Cpase digests was unusual. Only three clear broad peaks were obtained and

Amino acid analysis demonstrated that none of these contained peptides. It was thought possible that the protein was resistant to digestion and undigested protein had either eluted immediately with the solvent peak or failed to enter the column.

The final protein sample of 220 μg was obtained from the Max-Plank. This sample had been obtained by the same procedure used for the previous two samples, so it was assumed that the *N*-terminus would again be blocked. Therefore proteolytic cleavage and sequencing of peptides was thought to be the best strategy for obtaining an amino acid sequence. In the previous attempt to obtain sequence by this method problems had been encountered with both protein purification by FPLC, and protease digestion. To overcome problems with digestion it was decided to use a less specific protease. To avoid the problems encountered with FPLC peptides were purified by SDS-PAGE and then electroblotted onto PVDF membranes.

6.4.1. Protein Digestion and Electroblotting.

An efficient method for obtaining *N*-terminal sequence from 7-250 pmole amounts of protein separated by SDS-PAGE has been described (Matsudiara, 1987). The main limiting factor for obtaining *N*-terminal sequence by this technique remains *N*-terminal blockage, and the major cause of *N*-terminal blockage during SDS-PAGE is proposed to be the presence of polymerisation by-products (Moos *et al.*, 1987). To overcome this, several adaptations have been developed. These include pre-running the gel, adding glutathione or sodium thioglycolate to the running buffers and using PDA instead of acrylamide. Pre-running reduces the interaction between charged unpolymerised material and the protein sample, while glutathione and sodium thioglycolate scavenge charged impurities and uncharged reactive species in the gel system (Moos *et al.*, 1987). Acrylamide monomer is considered the major uncharged reactive species in a gel system. This can largely be eliminated by the use of PDA instead of acrylamide.

Along with these precautions against *N*-terminal blockage, amido-black protein staining was used in preference to coomassie-blue. This procedure is also reported to increase the yield of sequencable material (B.Dunbar *pers.comm.*).

The initial sample had proved refractive to digestion by two relatively specific proteases. Therefore it was decided to use two less-specific proteases, elastase and trypsin. Elastase cuts *N*-terminal to alanine and glycine residues, while trypsin cuts *N*-terminal to arginine and lysine residues.

Initial experiments to establish the optimum conditions for digestion used 21 μg of protein suspended in a final volume of 50 μl. Proteases were added to a final concentration of 5 ng per μg of protein. To avoid the particular protease adhering to the reaction vessel, elastase digestions were performed in plastic eppendorfs and trypsin digests in glass tubes. The protein was digested with Trypsin at 25°C and Elastase at 37°C.

In the initial experiments digestion was stopped at three time points of 5, 25 and 60 minutes. The reaction was stopped immediatly by adding SDS-PAGE loading buffer and boiling. Proteolytic cleavage products were then separated using a SDS-PAGE mini-gel system and visualised with coomassie blue staining.

The best digestion was judged to be the one that produced discrete bands. After 5 minutes incubation elastase produced a smear of cleavage products while Trypsin produced two discrete bands . After two more repetitions using shorter digestion times the optinum time for trypsin digestion was judged to be 5 minutes. During these experiments it was also established that the best separation of the two peptides was achieved with a 15% resolving gel.

The two discrete bands produced by trypsin digestion coresponded to approximate molecular weights of 25 and 28 kD. These bands began to appear after 30 seconds and were still faintly visible after 25 minutes, suggesting they represented major proteolytic cleavage products. Once the optinum time period for digestion was determined, the procedure was scaled up for 45 μg of protein. In this experiment digestion was carried out for 4 and 7 minutes in a volume of 50 μl Samples were then loaded onto a pre-run 14 cm x 16 cm SDS-PAGE gel system with a 7% and 15% PDA/Bis-acrylamide resolving gel. Rainbow markers, protein standards and a trypsin only control were run alongside the sample.

The gel was run until good separation of the rainbow marker bands in the 40 kD to 20 kD range was achieved. The gel was then carefully removed and prepared for electroblotting. The transfer of protein to PVDF membrane was carried out in a Trans-blot vertical transfer cell. A 10 mM CAPs transfer buffer (pH 11) was used and a current of 60 mA applied for 60 minutes. To determine the efficiency of transfer to the PVDF membrane it was stained with amido-black and the gel with coomassie blue.

Only 50% of the protein appeared to have transferred. This did not represent sufficient material for sequencing. This was the case for both the 4 and 6 minute digests. A second sample of 45 μg was digested for 5 minutes and blotted by the same procedure for 4 hours. A better transfer of material was achieved but the amount of peptide transfered to the PVDF membrane still appeared too low for sequencing.

The final sample of 45 µg represented all the material available for 12 months. Despite the problems encountered in achieving efficient protein transfer, none of the scaled up digests had produced the expected amount of peptides. No band for undigested material had been visible in any of the scaled up digests. Therefore it was considered possible that the lower than expected level of peptides was due to digestion proceeding too long. For the final sample the digestion time was reduced to 3 minutes and the transfer time extended from 4 to 12 hours.

This final preparation transferred almost completely to PVDF. Two distinct bands with the expected molecular weights were visible on the membrane. These were cut out and sent to the protein sequencing unit in Aberdeen for analysis.

The larger band of approximately 28 kD was blocked at its *N*-terminus. This may have been the result of this peptide including the original *N*-terminus. The second band of approximately 25 kD was run for 19 successive cycles and 13 amino acids were identified. These included a run of 8 successive residues which were suitable for the design of an oligonucleotide probe (Figure: 6.2).

Table: 6.2. Amino Acid Sequence of the 25 kD Peptide.

Residue Number	Amino Acid/ Acids	Amount Sequencing (pM)
1	?	–
2	Val	2.0
3	Gly	1.1
4	Pro	3.0
5	Asn	2.8
6	Thr	0.9
7	Gly	1.0
8	Val	1.3
9	Leu	1.0
	Gly	0.7
10	Ala	0.8
	Pheo	0.6
11	Val	1.1
12	?	–
13	Gly	1.1
14	Ser	0.4
15	?	–
16	?	–
17	Ala	0.6
18	?	–
19	Ser	0.6

Figure: 6.2. Nucleotide Sequence encoding residues 2 to 9.

```
5' GTA GGA CCA AAC ACT GGA GTA CTA  3'
     C   C   C   T   C   C   C T C
     G   G   G       G   G   G   G
     T   T   T       T   T   T   T
```

Figure: 6.3 *N*-Teminal Sequence of LD-carboxypeptidase.

		V	G	P	N	T	G	V	L	X	V	X	G		
FtsW	(360)	**V**	**G**	A	A	A	**G**	M	**L**	P	T	K	**G**		(371)
			G	P	N	T	G	V	L	X	V	X	G	S	
DD-Cpase *Enterococcus.*	(370)		S	**P**	K	**T**	**G**	E	**L**	L	**V**	L	A	**S**	(381)
		V	G	P	N	T	G	V	L	X	V	X	G		
FtsX	(224)	**V**	S	A	M	I	**G**	**V**	**L**	M	**V**	A	A		(236)
			G	P	N	T	G	V	L	X	V	X	G	S	
RlpA	(211)		**G**	**P**	G	G	D	I	**L**	P	**V**	S	N	**S**	(222)

Identities are shown in bold typeface and conserved residues appear underlined. The numbers in brackets refer to the location of the amino acids within the respective protein sequence.

The complete sequence was checked with trypsin and then analysed using the 'pir33' protein sequence database.The sequence did not corespond to trypsin and showed no good matches to any other known protein. This search did pick up two interesting proteins, namely FtsW and the DD-Cpase of *Enterococus hirae.* (Figure: 6.3). However these matches were not significant enough for any meaningful interpretation.

6.5. Probing with degenerate Oligonucleotides.

The run of 8 consecutive amino acids was used to design a degenerate oligonucleotide for probing the Kohara library. To avoid a high degree of degeneracy, inosine was selected at six positions and the degenerate position at the end of the final codon excluded. Inosine acts as a neutral base pairing in an equivalent manner to all four conventional bases (Martin *et al.*, 1985). Inosine has been successfully used at five positions in a 23mer with 50% GC content to locate the human gastrin gene (Ohtsuka *et al.*, 1985). The 23mer designed for the LD-Cpase had inosine at 6 positions and a GC content between 52% and 65% (Figure: 6.4.).

The alternative procedure for overcoming high degeneracy is to design guessmers using prefered codon usage. Prefered codon usage is usually associated with highly expressed proteins. The LD-Cpase was isolated as a minor component of the periplasm (Ursinus *et al.*, 1992) and would be predicted to be present at approximately the same level as the DD-Cpases. These enzymes are estimated to be present at between 600 and 1,800 copies per cell (Spratt, 1975). This suggests the LD-Cpase to be a poorly or moderately expressed protein, and therefore it was decided not to apply prefered codon usage to designing the probe.

The degenerate oligonucleotide designed had a calculate Tm of 69.7–74.6°C. The oligo was synthesised by OSWEL and its concentration determined by spectrophotometry. For probing, the oligo was end labelled with ATP. Labelling was confirmed by monitoring for the elution of a radiolabelled oligonucleotide from a NAP column. A Kohara gene maping membrane was prehybridised in a buffer consisting of 4xSSC, 0.5% SDS and 0.2 mg/ml Salmon testes DNA. Hybridisation was performed under high stringency at 65°C overnight. The membrane was then given four 15 minute washes. Two of these washes were in 2xSSC, 0.5%SDS at 37°C and two in 2xSSC at 30°C. The filter was then exposed to pre-flashed photographic film for 48 hours.

Figure: 6.4 Degenerate Oligonucleotide Probes.

Inosine Containing Oligonucleotide.

```
5' GTI GGI  CCI  AAC ACI GGI GTI CT 3'
                   T              T
```

23 mer containing 6 Inosines and with a degeneracy of 4. Calculated Tm of 69°C.

Oligonucleotide Designed by Preffered Codon Usage.

```
5' GTG GGT CCG AAC ACC ACC GGT GTG CT 3'
     T   C                   C   T
```

23 mer with a degeneracy of 16.

No signal was obtained from the initial hybridisation so it was decided to lower the temperature and consequently the stringency. Over successive experiments with two other genome maping membranes the probe was hybridised at 65°C, 60°C, and 55°C without any improvement. The absence of even non-specific binding suggested either the probe or filter were not functioning correctly. It was also possible that the stringency of hybridisation was too high to enable stable binding.

The elution profile from the NAP column suggested that the probe was labelling efficiently. The filters used were all obtained in one consignment from the Takeda corporation and had been stored according to manufacturers instructions for 12 months. The problems encountered after striping these filters meant a filter hybridised with a random-primed probe could not be recovered for successful probing with an end-labeled oligo. Due to the limited number of filters available to me none of these filters had been checked before use. When one of the filters used was probed with a random-primed *dacC* probe a pattern of non-specific binding was obtained. This did not correspond to the ordered array of plaques that should have been visible. Consequently new filters were ordered from Japan.

6.5.1 Preferred Codon Usage.

A new oligonucleotide was designed using preferred codon usage by a MacMolly tetra software package (Figure: 6.4.). It was checked for self-complementarity and the possibility it could form a secondary structure that would interfere with hybridisation. The program used for this was primarily designed for predicting RNA structure. It estimated the most favoured secondary structure to have a free energy of -1.9. For such a short oligonucleotide this level of free-energy indicated formation of a secondary structure to be unlikely (Zucker *et al.*, 1981).

It had also been considered possible that the high stringency used for the previous probings had interfered with hybridisation. Therefore it was decided to hybridise under low stringency and if necessary wash the the filter at successively higher temperatures. The first pre-hybridisation, hybridisation and initial washes were all performed at 37°C, and subsuquent washes at 30°C. After exposing the filter for 24 hours, the regular grid pattern of the Kohara filter was visible. Several individual plaques gave stronger signals. Each Kohara phage carries a chromosomal insert that partially overlaps the insert carried by the next phage. A positive signal would therefore be expected to include two successive phage. Only two sets of plaques corresponding to overlapping Kohara phage produced the diffuse signal characteristic

of an end-labelled oligonucleotide. These two overlapping sets represented Kohara phages 137 and 138, and 317 and 318.

In an attempt to reduce non-specific binding the filter was washed at 40°C. This had no affect on reducing the background signal, so the filter was washed at 45°C. This washing resulted in the loss of all the signal.

6.6. Isolation of Kohara Phage 138 and 318.

The four potential positives coresponded to two regions located at approximately 8 minutes and 37 minutes on the *E.coli* genome. The 8 minute region carried by phage 137 (9F1) and 138 (10AG) included the *lac* and *cyn* operon (Rudd *et al.*, 1990). The region overlapping 137 and 138 had largely been sequenced and contained *cynR*, *codA*, and *codB*. The *cod* operon genes encode cysteine deaminase, and cysteine transport proteins. *cynR* is a regulator of the *cyn* operon of cyanate hydrolases. The region contained by both 317 and 318 was largely unsequenced but did include the *sodB* gene, encoding superoxide dismutase.

The phage 138 and 318 were amplified up from Kohara lysates. These lysates were then used to infect a 250 ml culture for the large scale preparation of DNA. Once isolated and purified the phage DNA was restricted and separated by gel electrophoresis. Phage 138 was restricted with *EcoRI*, *EcoRV*, *BamHI*, and *PvuII*. The *BamHI*, and *EcoRI* digests gave the restriction pattern predicted from the most recent map of the *E.coli* genome (Rudd *et al.*, 1990). *EcoRV* digestion produced bands of approximately 11 kb, 5 kb, 3.9 kb, 3.4 kb, 2.8 kb and 2.6 kb. The expected pattern of 1.2 kb, 1.6 kb, 1.8 kb, 3.1 kb, 9.5 kb, and 10 kb was not produced. Similarly *PvuII* digestion did not produce several of the small bands expected.

To establish if the correct phage had been isolated, a sample of phage taken during the preparation of phage DNA was tested. The 138 phage carries the *lac* operon and should be capable of complementing a *lac*- strain. A lawn of TP8503 (*lac*-) was poured onto MacConkey lactose agar plates and left to dry. The putative 138 phage sample and Kohara phage 136 137 and 139 were all spotted onto the lawn and incubated overnight. Four distinct plaques were produced but only the λ138 produced the discolouration of the agar associated with the utilisation of lactose. This confirmed that the DNA prepared was from the correct phage.

Phage 318 was digested with *HindIII*, *EcoRV*, *EcoRI*, and *BamHI*, and the DNA fragments separated by gel electrophoresis. The *HindIII*,*EcoRI*, and *BamHI* digests all matched the expected restriction pattern. The *EcoRV* digest produced the

predicted number of bands, but three of these were 1 kb larger than predicted. This was not considered significant for a region that was largely unsequenced.

6.7. Confirmation of the approximate gene locus for the LD-Cpase.

Phages 138 and 318 were digested with the same enzymes used for their restriction analysis. The DNA was separated by gel electrophoresis and then transferred to a Hybond-N membrane. This membrane was then probed using the end labelled oligonucleotides designed by the MacMolly tetra program. The protocol previously used for the successful probing of the Kohara filter was used to probe the new filter.

After hybridising at 37°C and washing down to 30°C several clear bands were visible (Figure: 6.5.). These included multiple bands in some lanes, and even hybridisation to the molecular weight markers. To remove the non-specific binding the filter was washed at increasingly higher stringency. After each wash the filter was exposed and then the procedure repeated at a higher temperature. By this process the filter was washed at 45°C, 50°C, 55°C, and 60°C.

After the 60°C wash non-specific binding to the molecular weight markers and the 138 phage DNA had completely disappeared. Four single bands in each of the lanes containing λ318 DNA still retained the probe (Figure: 6.5). These bands coresponded to 14.7 kb *BamHI*, 20 kb *HindIII*, 8.7 kb *EcoRI*, and 5.2 kb *EcoRV* fragments. As was expected from the Kohara filter results, these fragments all overlapped for a region carried by both λ317 and λ318 (Figure: 6.6). This region includes part of the *sodB* gene. It was assumed the approximate location of the gene for the LD-Cpase had been found.

The original intention of this work had been to identify and sequence the gene for the LD-Cpase. Unfortunately time was not available to complete this. The λ318 phage DNA and the approximate location of the LD-Cpase were supplied to J.V-Holtje's group at the Max-Plank Institute. This region has subsuquently been sub-cloned onto a multi-copy plasmid. Cells transformed with this plasmid are super sensitive to NorcardacinA and filament in stationary phase (M.Templin *pers. comm.*).

Notes for Figure 6.5:

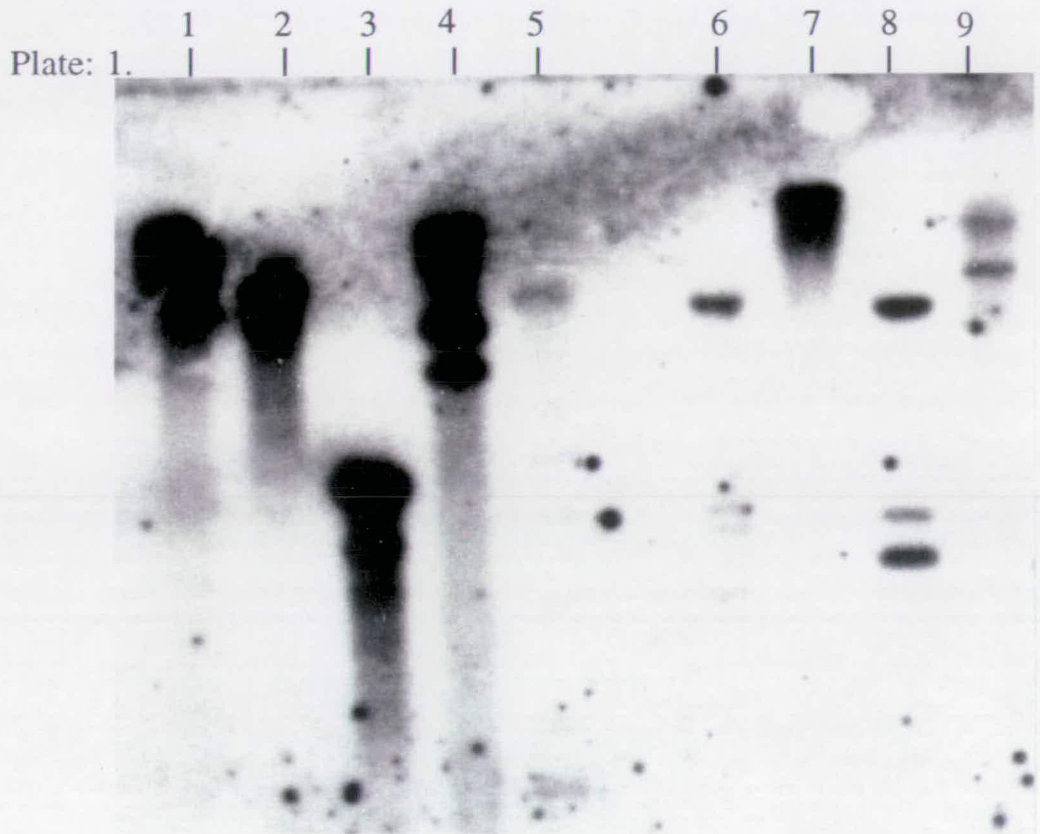
Plate 1: Filter after 37°C wash.

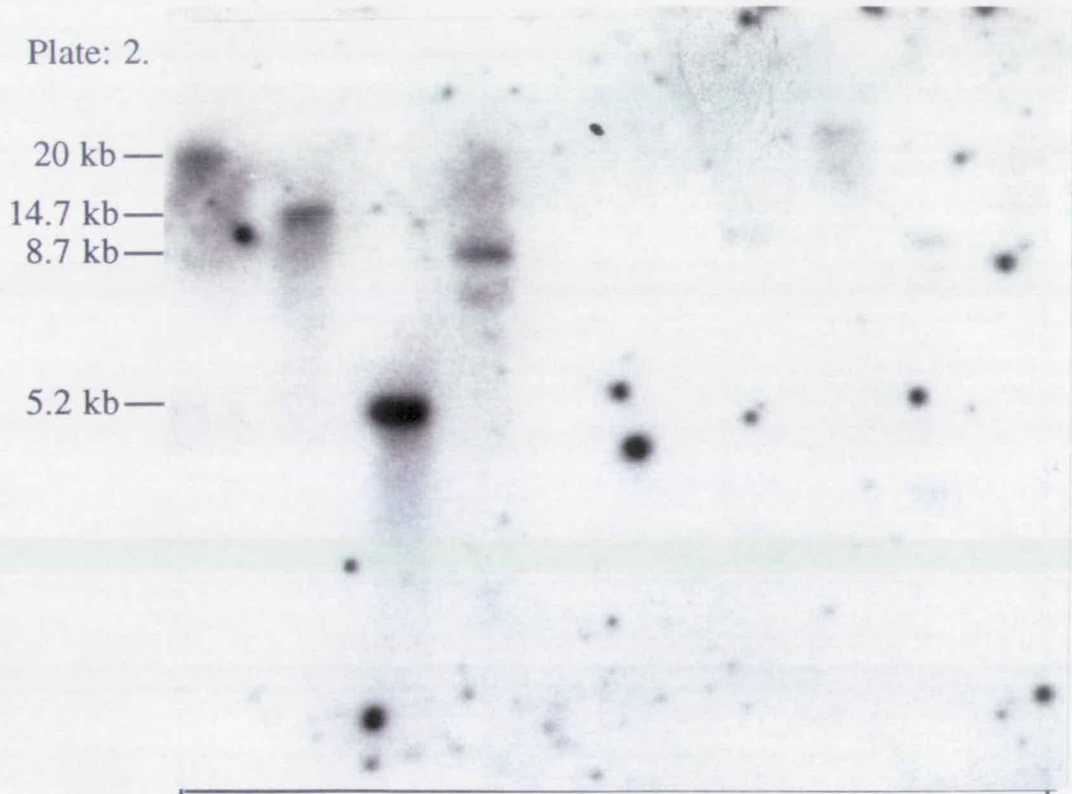
Lane 1; λ318 *HindIII* digest.
Lane 2; λ318 *BamHI* digest.
Lane 3; λ318 *EcoRV* digest.
Lane 4; λ318 *EcoRI* digest.
Lane 5; Markers (1 kb ladder)
Lane 6; λ138 *HindIII* digest.
Lane 7; λ138 *BamHI* digest.
Lane 8; λ138 *EcoRV* digest.
Lane 9; λ138 *EcoRI* digest.

Plate 2: Filter after 60°C wash.

Lane 1; λ318 *HindIII* digest.
Lane 2; λ318 *BamHI* digest.
Lane 3; λ318 *EcoRV* digest.
Lane 4; λ318 *EcoRI* digest.
Lane 5; Markers (1 kb ladder)
Lane 6; λ138 *HindIII* digest.
Lane 7; λ138 *BamHI* digest.
Lane 8; λ138 *EcoRV* digest.
Lane 9; λ138 *EcoRI* digest.

Figure: 6.5 Probing Kohara phage 138 & 318.





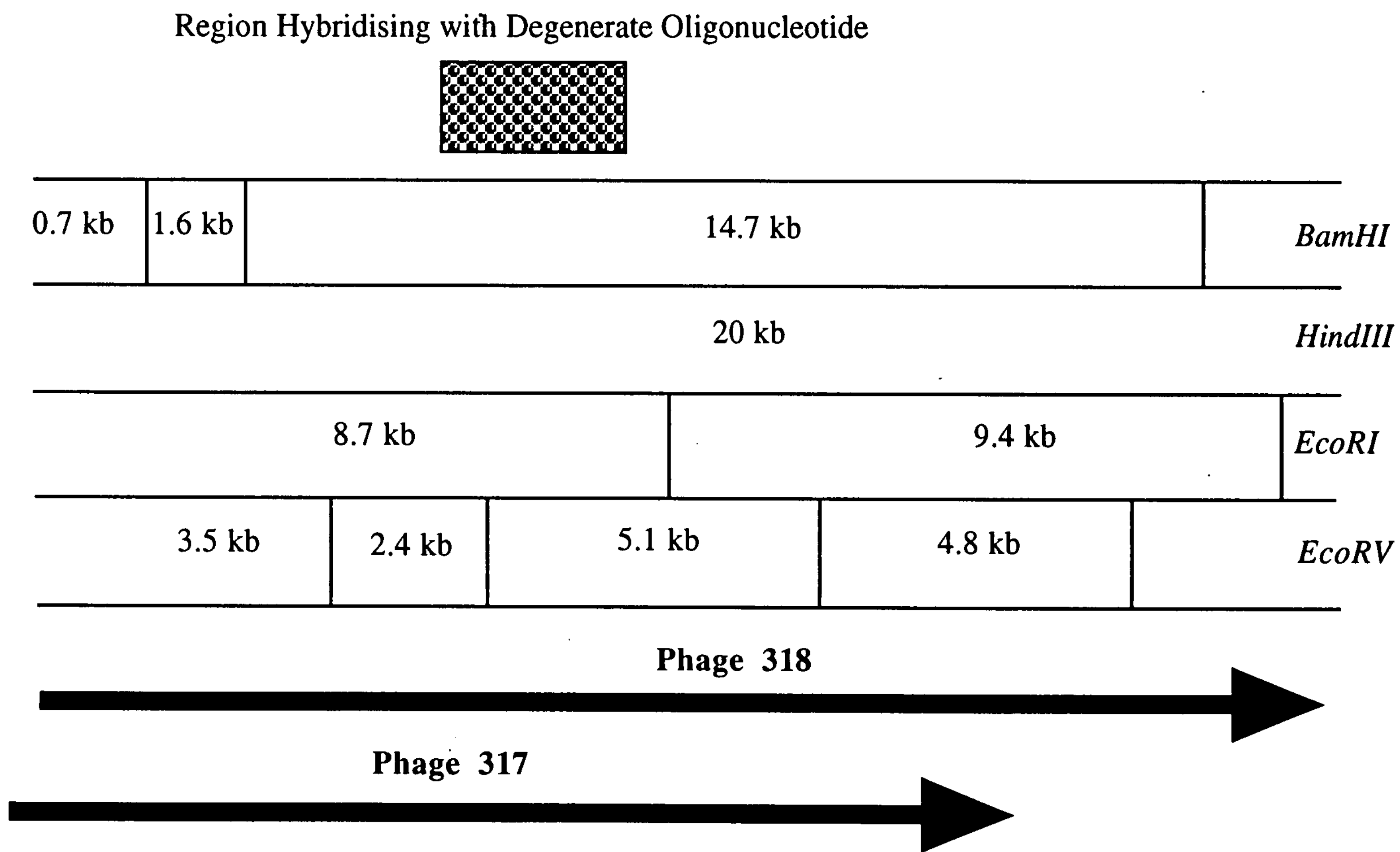


Figure: 6.6 Approximate Location of the LD-Cpase gene at 37 minutes.

CHAPTER 7

GENERAL DISCUSSION

The shape and integrity of Gram-negative bacteria is maintained by a rigid cell wall. In *E.coli* the cell wall is essentially a monolayer of peptidoglycan and is capable of protecting against osmotic pressures as high as 5 atmospheres (Mitchell & Moyle, 1956). The cell wall is not essential under all circumstances, a fact which has been shown by the isolation of L-forms that can grow and divide (Onoda, *et al.*, 1987). Division by these bacteria in the absence of a rigid cell wall shows that growth of peptidoglycan does not drive division, but follows it.

The earliest event in division is thought to be the assembly of a circumferential ring of FtsZ protein. It is now generally believed that contraction of this cytokinetic ring mediates the division process (Lutkenhaus, 1993). However contraction of the FtsZ ring alone is not sufficient for division of wild type cells. Under normal circumstances the construction of a lateral cell wall, or septum, is essential. Central to this process is the septum specific peptidoglycan synthetase PBP3 (Spratt, 1975; Ishino & Matsuhashi, 1981).

In this work the principal line of study has involved the investigation of septum assembly. To this end, suppression of a temperature sensitive *ftsI* allele, *ftsI*23, has been investigated. It has been shown that overproduction of DD-Cpases or deletion of the major DD-endopeptidase, PBP4, can enable a residual amount of mutant PBP3 to complete division. This has provided an insight into the process of septation and to understand it further, a strain deleted for *dacB* (PBP4), *dacA* (PBP5) and *dacC* (PBP6)was constructed. Studies with another suppresser of *ftsI*23; *sufI*, also showed DD-Cpase, and particularly PBP6, to be important in suppression.

When considering the synthesis and restructuring of murein there are several reasons why it is difficult to interpret results from deletion and suppression studies. First, the complete set of enzymes, lipid carrier molecules, and associated proteins involved with peptidoglycan is not known. The existence of important enzymes such as penicillin-insensitive peptidoglycan synthetases, LD-transpeptidases and LD-endopeptidases has been proposed (Nanninga, 1991; Holtje & Tuomanen 1991). For any clear impression of how assembly of a septum occurs, the identification and characterisation of these enzymes has to be achieved. Secondly, the work on separate *fts* genes and their products has suggested the existence of some form of division complex (Guzman *et al.*, 1992; Pla *et al.*, 1993). Therefore deletion of a gene or overproduction of a protein cannot be assessed in isolation, but primarily in the context of how that might affect other proteins. The third reason is the probability that the sacculus has evolved to become a highly resilient structure which exhibits several

attributes that maybe considered 'Safety mechanisms'. At their simplest, these safety mechanisms include redundancy of enzyme function. Examples of this exist for lytic transglycosylases (Romeis *et al.*, 1993), DD-Cpases (Annamura & Strominger 1984), DD-endopeptidases (Iwaya & Strominger, 1977; Keck & Schwarz, 1979) and DD-transpeptidases (Suzuki *et al.*, 1975). The value of redundancy is clearly demonstrated by TOE23. Although PBP3 is an essential protein, its absence in TOE23 does not lead to lysis through abortive division. Instead the cell uses other peptidoglycan synthetases to continue constructing a lateral cell wall, and the bacterium filaments. As soon as permissive conditions are restored, PBP3 can rapidly complete an aborted septa and the filament divides.

On a more complex level it appears that levels of peptidoglycan metabolising enzyme are affected by the levels of other peptidoglycan metabolising enzymes. This has been reported with, PBP1A and PBP1B (Tameki *et al.*, 1977), and PBP5 and PBP6 (this work). Mutations that affect peptidoglycan metabolising enzymes, such as *rodA*sui (Begg *et al.*, 1989) and *ftsI*23 (this work), also alter the relative proportions of PBPs. It is known that levels of PBP6 and PBP7 are growth-phase dependent (Buchanan & Sowell, 1982; Spratt, 1977; Tuomanen & Schwartz, 1987), and that PBP3 is degraded in stationary-phase (de la Rosa *et al.*, 1983). It is also known that mutations in *mreB* alter the levels of PBP1B and PBP3 (Wachi *et al.*, 1987) and that a *mreA* mutation increases the levels of PBP1A and PBP2 (Tameki *et al.*, 1977; Doi *et al.*, 1988). All these processes are evidence that the level of peptidoglycan metabolising enzymes is carefully regulated. This regulation in itself may also be considered a safety mechanism as it enables the cell to modify peptidoglycan metabolism in response to specific conditions. I have proposed in the case of PBP5 and PBP6, that one of these regulatory mechanisms may be related to the *amp* system of β-lacatamse induction.

Despite these problems genetic studies have enabled septation to be placed into discrete stages mediated by essential proteins. With the study of murein structure a model of events in vivo can be assembled. From the results presented in this thesis I would like to propose my interpretation of some of these events.

Division begins with the assembly of two FtsZ rings, one on either side of the future division site. I suggest two in order to account for the amount of FtsZ available*ie.* 10,000–20,000 molecules/cell (Bi & Lutkenhaus, 1991). Two rings contracting either side of the septum would also allow the symmetrical addition of peptidoglycan and avoid the problem of removing FtsZ from the centre of the

completed septum.

The contraction of the FtsZ rings would be responsible for the switch to localised incorporation of peptidoglycan at the division sites. This may be the result of an association between the FtsZ ring and specific peptidoglycan carrier molecules, or as Norris and Manners (1993) suggest, by altering the structure of the cytoplasmic membrane and stimulating translocase activity. Initially, the nascent peptidoglycan is incorporated by PIPS.

At the same time as peptidoglycan synthesis becomes localised (Wientjes *et al.*, 1989) contraction of the cytoplasmic membrane will also stimulate hydrolase activity. This can be achieved by placing the murein at the division site under stress. As a consequence of stress the energy of activation to hydrolyse cross-bridges will be reduced. Stress on the murein net may also activate allosteric hydrolases bound to the sacculus. A good example of this would be the Slt enzyme. Slt is an allosteric enzyme * that is bound to the peptidoglycan by its inhibitor binding site (Templin *et al.*, 1992). In this inactive form it is distributed throughout the sacculus (Walderich & Holtje 1991). Evidence exists to suggest that Slt does become active during division (Templin *et al.*, 1993). It is conceivable that stress transmitted through the glycan strands could alter the interaction of the Slt with its inhibitor, and enable it to become active. Such a mechanism is appealing as it allows safe and localised release of hydrolase activity.

The alterations in DD-Cpase and LD-Cpase activity predicted by this work may also be co-ordinated by contraction of the cytoplasmic membrane. It is equally possible that this may depend upon assembly of a complex including PBP3 (Broome-Smith *et al.*, 1985; Pla *et al.*, 1993). The increased levels of tripeptides produced by periplasmic carboxypeptidases and tripeptide lipid carrier molecules, will allow PBP3 to produce template defining cross-bridges in nascent murein (as suggested in Chapter 3). This enables the successive addition of triplets to the ingrowing septum, as has been proposed by Holtje (1993). Increased hydrolase activity will accompany the localised addition of murein and produce the increased release of peptidoglycan observed during septation (Park, 1993). Critical among these hydrolases are the DD-endopeptidases which cleave the PBP3 synthesised cross-bridges. A reduction in DD-endopeptidase activity can enable a residual amount of PBP3 to complete septation (as shown in Chapter 4) (Figure : 7.1).

The septum appears to pass through another critical stage defined by a new *fts* mutation *ftsK* (K.Begg *pers.comm.*). This stage appears to be associated with a reduction in DD-Cpase activity. Constriction continues until finally a small aperture exists. Sterical hindrance will prevent the addition of further peptidoglycan and the

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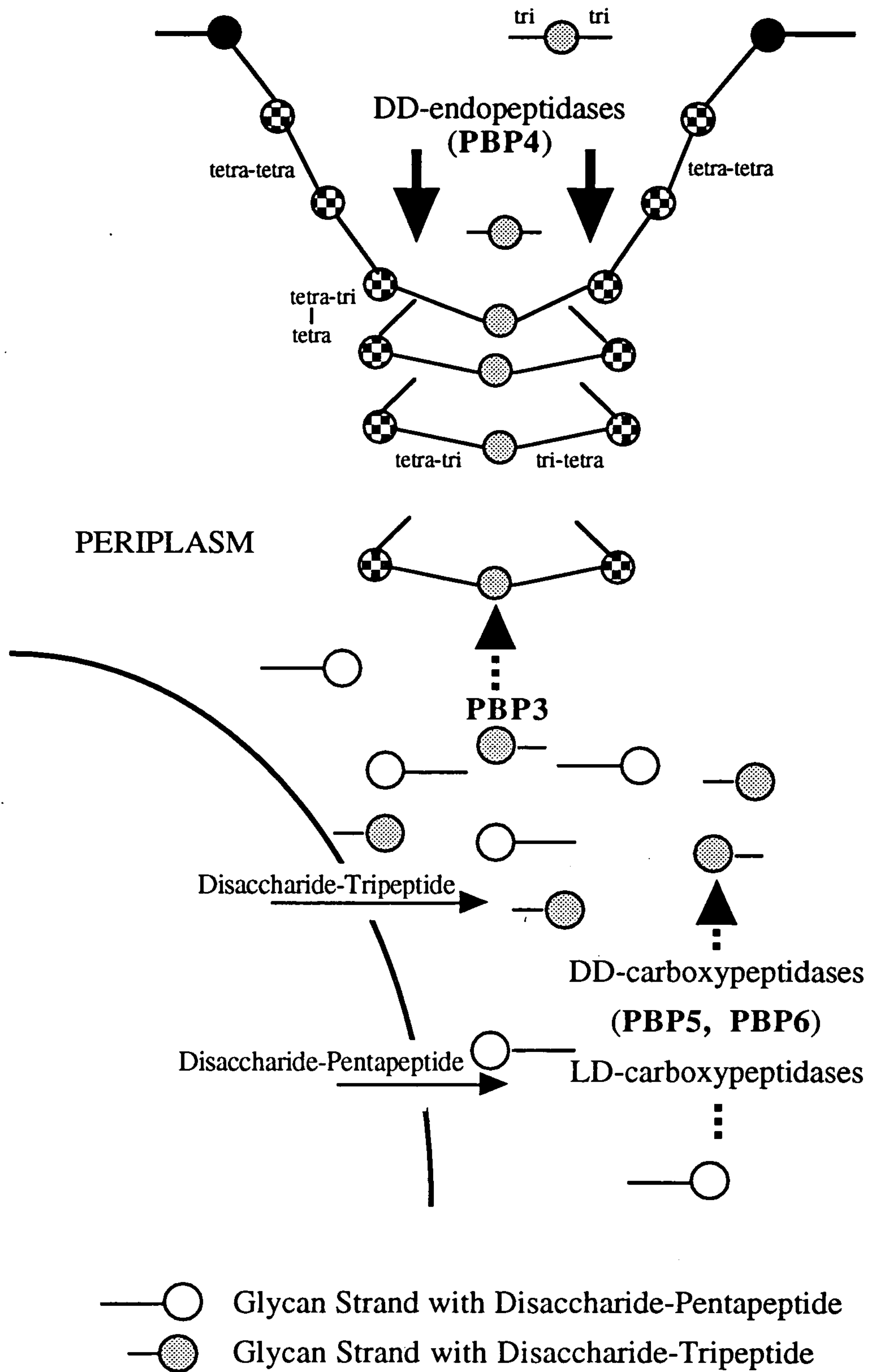
* The Slt is described in section 1.3.1.

two FtsZ rings will contract pulling the two cytoplasmic membranes apart. After this, the connections between the ingrowth of peptidoglycan can be hydrolysed and the cell wall form into two discrete septa. Finally in an EnvA mediated event the septa are split and the two daughter cells released.

Figure: 7.1 Construction of the Septum.



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APPENDIX I

Table; A.1. Murein Composition of Wild Type *E.coli* and Standard deviation for HPLC analysis. (Glauner, 1987)

Murein Components	*E.coli* KN126	*E.coli* W7	Standard Deviation (%)
Dipeptide	**2.72**	**2.61**	**9**
Tripeptide	**11.01**	**9.78**	**10**
Free	80.5	81.5	2
Acceptor (Ala)	18.0	16.6	11
Acceptor (*m*DAP)	1.5	1.9	15
Tri-Lys-Arg	**5.47**	**4.97**	**5**
Free	68.7	66.8	4
Acceptor (Ala)	26.9	27.3	7
Acceptor (*m*DAP)	4.3	5.9	15
Tetrapeptide	**54.25**	**54.59**	**4**
Free	69.1	67.7	3
Acceptor (Ala)	28.7	29.3	4
Acceptor (*m*DAP)	2.2	3.0	10
Pentapeptide	**0.15**	**0.16**	**18**
Free	40	50	15
Acceptor (Ala)	60	50	13
Tetra-Gly4	**2.50**	**3.33**	**8**
Free	70.0	72.7	2
Acceptor (Ala)	28.6	25.4	3
Acceptor (*m*DAP)	1.4	2.0	23
Penta-Gly5	**0.39**	**0.44**	**17**
Free	62	75	11
Acceptor (Ala)	38	25	20
1,6-Anhydro ends	**3.00**	**3.30**	**7**
Uncrosslinked	40.7	36.4	4
Crosslinked (Ala)	46.2	47.1	5
Crosslinked (*m*DAP)	13.1	16.6	7

Murein Components	*E.coli* KN126	*E.coli* W7	Standard Deviation (%)
Mean Chain length	**33.3**	**30.3**	**5**
Crosslinkage			
total	**23.06**	**23.73**	**3**
Ala	**21.40**	**21.54**	**3**
*m*DAP	**1.66**	**2.19**	**8**
Monomers	**55.30**	**54.07**	**2**
Dimers	**40.52**	**41.42**	**2**
Trimers	**4.06**	**4.36**	**9**
Tetramers	**0.13**	**0.15**	**1**

Table: A.2 Competition of β-lacatam Antibiotics for the PBPs of *E.coli.*

Antibiotics	I_{50} for PBP (μg/ml)							
	1A	1B	2	3	4	5	6	MIC
Penicillin G	0.5	3.0	0.8	0.9	1.0	24	19	16
Azlocillin	0.8	1.6	0.4	0.05	3.2	6.4	6.4	6
Aztreonam	0.5	70.0	>100	0.1	>100	>100	>100	0.1
Furazlocillin	3.0	6.0	0.5	0.05	6	6	12	0.5
Cefsulodin	0.47	3.7	>250	>250	>250	>250	>250	25
Mecillinam	>500	>500	0.04	>500	>500	>500	>500	0.05
Imipenem	0.2	0.6	<0.1	9.8	<0.1	0.3	0.6	0.1

Note: This table is adapted from "The Role of Penicillin-binding Proteins in the Antibacterial Activity of β-Lactam Antibiotics." by M.V.Hayes and J.B.Ward in Antiobiotics in Laboratory Medicine (Editor: Victor Lorian. Publisher: Williams and Wilkins. London.).

APPENDIX II

Publications.

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