Studies on Cell Division and Shape in *Escherichia coli.*

Yevgeny A. Nikolaichik

A thesis presented for the Degree of Ph.D.

Institute of Cell and Molecular Biology
The University of Edinburgh

1999

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Declaration

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

Acknowledgements

First of all, I would like to thank my wife, without whose support and understanding this thesis would have never been completed. I would also like to thank the Darwin Trust (and Ken Murray) for providing the studentship that made my study in Edinburgh possible. A lot of thanks to Willie Donachie, my supervisor, for always being helpful, supportive and ready to answer even the most bizarre questions.

I also wish to acknowledge all members of ICMB who have helped me, with special thanks to Millie Masters, Medhat Khattar, Dave Boyle, Steve Addinall and Sean MacAteer. A separate big thank you goes to Neil McLennan for help with protein work, to Ken Begg for his mutants and for always having the strains I needed and to Ken McClure for his books.

And thanks to my friends Sveta, Tanya and Taciana for helping me not to forget one very nice language.

Thank you.

Abstract

The original aim of this thesis was to utilise *Vibrio harveyi* luciferase as a reporter of the expression of cell division genes during the cell cycle. Several plasmids expressing *luxAB* genes from *ftsZ* promoters were constructed. To achieve maximal luciferase expression, the ribosome binding site in front of the *luxA* gene was improved, which led to increased expression of luciferase. The level of expression of the improved luciferase reporter from plasmids was sufficiently high to be detected in single cells, although not high enough to be used in lower copy number constructs. However, luciferase activity showed significant fluctuations that did not appear to be linked to cell cycle events. These fluctuations made the detection of any cell cycle related changes in luciferase expression impossible.

Another direction of this thesis is represented by the studies on the topology of the cell shape determining RodA protein. The ampicillin resistance levels were measured in 52 fusions with the topology probe BlaM made to different parts of the RodA protein. The combination of the BlaM fusion data and computer modelling argue for a topological structure of this protein consisting of ten transmembrane segments with both ends of the protein located in the cytoplasm. An important feature of the protein appears to be a large periplasmic loop - a highly conserved part of the protein possibly interacting with other members of the shape controlling system. The topology of the protein as well as the location of conserved regions suggests a possible function as a transporter of peptidoglycan precursors.

During the course of RodA characterisation a mutation was isolated that permits stable growth of a *rodA* null strain in rich media. The cause of this stabilisation appears to be a simultaneous increase in the amounts of FtsZ and FtsA proteins. In two other situations simultaneous overexpression of FtsA and FtsZ was necessary and sufficient to induce divisions both in polar and central division sites. On the other hand, overproduction of FtsZ alone could only induce divisions at cell poles. A hypothesis to explain this data is suggested to be sequestration of FtsA at cell poles after division, possibly as the result the action of Min proteins.

Abbreviations

Amp	Ampicillin
ATP	Adenosine 5'-triphosphate
bp	Base pair
BSA	Bovine serum albumin
dATP	Deoxy ATP
DNA	Deoxyribonucleic acid
dNTP	Nucleotide 5'-triphosphate
E. coli	*Escherichia coli*
EDTA	Diaminoethanetetraacetic acid
Kan	Kanamycin
kb	Kilobase
kDa	Kilodalton
OD_w	Optical density wavelength
ORF	Open reading frame
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
PIPES	Piperazine N,N'bis (2-ethane sulphonic acid)
RBS	Ribosome binding site
RNA	Ribonucleic acid
rpm	Revolutions per minute
SDS	Sodium dodecyl sulphate
Spc	Spectinomycin
Str	Streptomycin
TEMED	N,N,N',N',-tetramethylethylethylenediamine
Tet	Tetracycline
Tris	Tris(hydroxymethyl)aminomethane
ts	Temperature sensitive
X^R	Resistance to antibiotic X
X^S	Sensitive to antibiotic X

CHAPTER 1

INTRODUCTION

1.1. Introduction.

If one is watching a bacterium under a microscope, it is easy to make an observation that bacterial life is essentially a constant process of growth that at certain point leads to division. To divide successfully, a bacterium has to double its mass, replicate its chromosome and build a special structure, separating two daughter cells - a septum. Although the basic division process, that of cytokinesis, may occur similarly in all bacteria, details of division as a whole vary significantly depending on the shape of a bacterium and the composition of its cell wall. The object of this study, *Escherichia coli,* is a Gram-negative bacterium that has been extensively studied genetically and biochemically and is probably the organism which is better understood than any other living thing on Earth. The shape of this bacterium is determined by a rigid layer of peptidoglycan or murein located between outer and inner cell membranes. Peptidoglycan is a huge single molecule forming a bag-like structure that, combined with high internal osmotic pressure, acts as an exoskeleton determining the constant shape of the cell and its mechanical strength in a variety of environments with different osmolarity. Peptidoglycan is a unique polymer composed of glycan strands that are cross-linked by short peptide bridges. The presence of such a rigid structure puts its imprint on the whole physiology of this bacterium, and, more specifically, on the division process that is the subject of this thesis. The peptidoglycan sacculus has to be duplicated every cell cycle, and in such a way that ensures constant integrity of this structure, which is essential for the maintenance of the shape and viability of the cell. This is achieved through the action of a sophisticated assembly of proteins including peptidoglycan synthetases, lytic enzymes and a number of proteins specific to division, but not related directly to peptidoglycan metabolism. The

expression of these proteins is subjected to a complex regulatory network controlling the presence of the right amounts of the right proteins in the right places at the right time and co-ordinating cell division with chromosome replication and cell growth.

What follows below is an overview of genes and regulatory processes related to cell division in *E.coli*.

1.2. Peptidoglycan sacculus.

As peptidoglycan is a central component in the division of *E.coli*, the proteins responsible for its synthesis should probably be covered here, although alteration of expression of most of these usually doesn't have any striking morphological effect - cells die quietly without filamentation or any other cell shape change.

1.2.1. Murein structure.

The glycan strands of murein are made of a repeating subunit composed of two aminosugars, *N*-acetylglucosamine (GlcNAc) and *N*-acetylmuramic acid (MurNAc) linked by β-1,4 glycosidic bonds. The length of these strands is highly variable (Harz *et al.*, 1990) with an average of 29 disaccharide subunits (Glauner *et al.*, 1988). The glycan strands are cross linked with short peptide bridges. The pentapeptide L-ala-D-glu-DAP-D-ala-D-ala (DAP is *meso*-diaminopimelic acid) is attached to the lactyl group of MurNAc through an amide bond with the L-alanine residue. The presence of a dibasic acid (DAP in *E. coli*) is necessary for the formation of the cross-link between the neighbouring glycan strands. The cross linking is performed by a transpeptidase that cleaves the terminal D-alanine and forms the peptide bond between the remaining D-alanine and the free amino group of DAP from a pentapeptide connected to

another glycan strand. The formation of a peptide bridge still leaves an amino group free in one of the DAP residues which makes possible the linking of an additional glycan strand. Such linkage connecting three glycan strands occurs in about 5% of cases (Glauner & Höltje, 1990; Glauner *et al.*, 1988). In the mature peptidoglycan one or both remaining D-alanines are removed from the peptide bridges by carboxypeptidases. The peptide chains are arranged helically along glycan strands at angles of about 90° to each other (Labischinski *et al.*, 1985). If murein is single layered as in *E.coli* the peptide bridges could therefore connect every second MurNAc residues in the neighbouring glycan strands. The real degree of cross linking is close to this expectation at least in certain conditions (Glauner *et al.*, 1988). This composition results in a firm covalently linked net-like structure, effectively a single huge molecule, enclosing most of the bacterial cell. Such structure makes it possible for the murein sacculus to withstand the internal pressure of several atmospheres. And it is the combination of the high internal pressure with the rigid murein "bag" that determines the fixed shape of a bacterial cell. Clearly such a structure provides many advantages for a bacterium, but it also creates several problems, the most important of which is the growth of the rigid sacculus - it has to be grown and divided in such a way that the integrity of the whole structure opposing the internal osmotic pressure is never compromised. The bacterial solutions to this problem will be discussed later.

1.2.2. Synthesis of the cytoplasmic peptidoglycan precursors.

Murein synthesis starts in the cytoplasm with the activation of GlcNAc by linking it to UDP and the formation of UDP-GlcNAc-enolpyruvate which is catalysed by MurZ (Brown *et al.*, 1995;

Marquardt *et al.*, 1992). This intermediate is then converted to UDP-MurNAc by MurB (Pucci *et al.*, 1992). Then the pentapeptide chain is formed by sequential addition of five amino acids to the lactyl group of MurNAc. This step is performed by several enzymes. MurC adds L-alanine (Liger *et al.*, 1995), MurD - D-glutamic acid (Mengin-Lecreulx *et al.*, 1989), MurE - DAP (Maruyama *et al.*, 1988) and MurF adds a D-alanine:D-alanine dipeptide (Parquet *et al.*, 1989) previously linked by one of the D-ala-D-ala ligases, DdlA (Zawadzke *et al.*, 1991) or DdlB (Robinson *et al.*, 1986). Alanine racemases *alr* or *dadX* (Wijsman, 1972; Wild *et al.*, 1985) and glutamate racemase *murI* (Doublet *et al.*, 1993) are necessary for the formation of two peptidoglycan-specific D-amino acids. The UDP-MurNAc-pentapeptide is then connected through pyrophosphate to the C_{55} isoprenoid undecaprenol (bactoprenol), forming the lipid I precursor. This reaction is catalysed by MraY (Ikeda *et al.*, 1991). The addition of GlcNAc to lipid I by MurG (Mengin-Lecreulx *et al.*, 1991) yields the final cytoplasmic precursor, lipid II, the bactoprenol-linked disaccharide-pentapeptide. Lipid II is then somehow transported to the periplasm, but what protein(s) is (are) responsible for this transfer is not known. A possible candidate for the transporter was hinted by (Dai & Ishiguro, 1988) who described a mutant, *murH*, defective in transport of DAP to the periplasm. The mutation was mapped to 90.2 minutes, but nothing else is known about it as yet.

1.2.3. Assembly of the mature peptidoglycan in the periplasm: Murein synthases.

After the flipping of lipid II to the periplasmic side of the membrane the precursors are incorporated into the existing peptidoglycan molecule during the processes of cell elongation and division. This occurs in transglycosylation and transpeptidation reactions. Transglycosylases connect the disaccharide subunits by β-

1-4 bonds to form glycan strands. The reducing end of the glycan strand is blocked by the formation of 1,6-anhydromuramic acid (Höltje *et al.*, 1975). Transpeptidases cross link the glycan strands by peptide bridges. Several enzymes with these activities have been described. Most of these reactions are catalysed by the periplasmic (cell membrane anchored) high molecular weight penicillin binding proteins (PBPs) PBP1A, PBP1B, PBP2 and PBP3. The names of these proteins come from their ability to bind penicillin and the numbering is in the order of increasing mobility (decreasing molecular weights) on PAGE.

PBP1A and **PBP1B** are both bifunctional enzymes and are usually the ones performing most of the transglycosylation. They also carry out the transpeptidation reaction. Blocking the action of both PBP1A and PBP1B by a specific antibiotic results in the complete inhibition of peptidoglycan synthesis, whereas blocking of PBP2 leads to 60% and blocking of PBP3 - to 35% inhibition of peptidoglycan synthesis (Wientjes & Nanninga, 1991). PBP3 was mostly inhibited during constriction whereas inhibition of PBP2 was observed throughout the cell cycle and correlated with cell shape. This suggests that PBP1A and PBP1B have a general peptidoglycan synthesising activity on the basis of which other "modifying" PBPs perform their special tasks. Of these "modifying" enzymes PBP2 and PBP3 appear to be elongation and septation specific and are described in more detail later.

1.2.4. Murein hydrolases.

As the peptidoglycan sacculus is a bag completely closed by covalent links, splitting of the existing bonds is necessary for the insertion of the new material during growth and division of the cell. Therefore, not only murein synthases are required for the growth of the sacculus, but murein hydrolases as well. These enzymes should

also be essential for the existence of the cell, but this appears to be difficult to prove, as there is a great redundancy for each class of these enzymes. Practically every bond in the murein molecule can be cleaved by a specific hydrolase, and there is usually more than one enzyme catalysing the same reaction (Shockman & Höltje, 1994). Some of these enzymes act on the intact murein whereas others can only utilise soluble degradation products as substrates.

Soluble periplasmic (**Slt70**) or outer membrane bound (**MltA**, **MltB**, **MltC**) lytic transglycosylases cleave the glycosidic bond between GlcNAc and MurNAc (Dijkstra & Keck, 1996; Ehlert *et al.*, 1995; Engel *et al.*, 1991; Lommatzsch *et al.*, 1997). Interestingly, these enzymes block the reducing end of the glycan strands through the formation of 1,6-anhydro muramic acid. The formation of this compound may be needed to distinguish the murein turnover products from murein precursors.

N-acetylmuramyl-L-alanine amidases cleave the peptides off the disaccharide subunits. At least two periplasmic enzymes, **AmiA** and **AmiB**, are known (Tomioka *et al.*, 1983; Tsui *et al.*, 1994), as well as a cytoplasmic **AmpD** amidase (Jacobs *et al.*, 1995).

DD-endopeptidases necessary for splitting peptide bridges in the existing murein, are represented by two membrane associated enzymes, **PBP4** (product of *dacB*) and **PBP7** (product of *pbpG*) (Henderson *et al.*, 1995; Korat *et al.*, 1991), as well as periplasmic **MepA** (Keck *et al.*, 1990). PBP8, originally described as a separate protein, appears to be a product of PBP7 proteolysis (Romeis & Höltje, 1994).

DD-carboxypeptidases remove one D-alanine residue from pentapeptides. Three specific enzymes have been described - PBP5 (product of *dacA*), PBP6 (product of *dacC*), PBP6B (product of *dacD*) (Baquero *et al.*, 1996; Broome-Smith *et al.*, 1988). In addition, PBP4 also has DD-carboxypeptidase activity. The exact cellular role of these enzymes is not clear.

1.2.5. Models for the peptidoglycan sacculus elongation and division.

As it was discussed above, any addition of new material to the monolayered murein sacculus requires breaking the bonds between the existing peptidoglycan strands at one stage or another. The vast spectrum of lytic enzymes can definitely cope with this task, but there is an important problem these enzymes face - they have to work in a way that will not compromise the strength of the existing peptidoglycan layer. The murein sacculus has to constantly withstand the internal osmotic pressure during growth and division. Therefore the action of murein hydrolases has to be somehow correlated with the action of synthases. Two models have been proposed to explain the correlated action of these two classes of murein metabolising enzymes. The first model (Burman & Park, 1984; Park, 1996) relies heavily on the results of pulse-labelling experiments showing that single strands are inserted into the sacculus during elongation (de Jonge *et al.*, 1989; Park & Burman, 1985). It was suggested that a bifunctional (with both transglycosylase and transpeptidase activities) murein synthase in a tight co-operation with a transpeptidase moves unidirectionally along the glycan strands and inserts a single new strand between the preexisting ones. This model explains both the original formation of the cross links between the "old" and "new" strands and the appearance of the linking between the newly synthesised strands some time later - the appearance of the cross links between the newly synthesised strands would mark the beginning of the next strand insertion. For this model to work, a really tight integration between murein synthase and endopeptidase is needed. This hypothesis, however, cannot explain the function of the trimeric muropeptides and the role of the active murein turnover which is observed.

Another possible way of inserting new material into the sacculus would be the "make-before-break" strategy utilised by gram-positive bacteria (Koch & Doyle, 1985), where new murein layers are added in a relaxed state underneath the outside layers bearing the mechanical stress. These new strands are brought into play slowly as the outside layers are degraded by hydrolases. A similar form of sacculus growth is proposed for *E. coli* in another model (Höltje, 1996; Höltje, 1993). This model postulates that new material has to be attached to the existing murein layer before any covalent bonds can be cleaved. The hypothesis explains the role of the trimeric cross bridges and in fact relies on their existence. The trimeric bridges are thought to be the attachment sites for the new material being added. The smallest attachment structure is a single glycan strand, but the minimal piece of new murein added to this attachment structure has, for structural reasons, to consist of three strands. To explain the experimentally observed insertion of single glycan strands, Höltje postulates the existence of a "primer" strand synthesised by a monofunctional transglycosylase. No evidence for the existence of such "primer" strands is available, but an enzyme capable of producing such strands, a monofunctional glycosyltransferase, is known (Di Berardino *et al.*, 1996). The triplet of glycan strands is assumed to be formed by the synthesis of two new strands and their simultaneous cross-linkage to the pre-existing primer strand. This triplet is then attached to the free amino groups of DAP from the peptide bridges on both sides of the docking strand in the existing murein. The docking strand is then removed and three new strands are pushed into the murein plane by the internal pressure.

Both models for sacculus growth described above require tight co-ordination of the action of several enzymes. The "three-for-one" hypothesis requires more enzymes to coordinate their action in space and time, and it was logical to suggest that murein synthesis is performed by a multienzyme complex, a holoenzyme of murein

synthesis (Höltje, 1996). Support for the existence of this complex is provided by affinity chromatography studies that demonstrated direct protein-protein interactions between several murein hydrolases and synthases. Immobilised lytic transglycosylases were able to bind and retain bifunctional murein synthases PBP1A and PBP1B, transpeptidases PBP2 and PBP3, and endopeptidases PBP4 and PBP7 (Romeis & Höltje, 1994; von Rechenberg *et al.*, 1996). The same interactions were seen when one of the PBPs was immobilised on a column. As this group of enzymes provides all the enzymatic activities needed for sacculus growth according to the "three for one" model, Höltje (1996) suggested that the "murein replicase" complex is composed of a dimer of bifunctional murein synthase (PBP1A or PBP1B) that adds to the primer strand to form a murein triplet, a dimer of transpeptidase (PBP2) attaching the triplet to the sacculus, a dimer of an endopeptidase (PBP4 or PBP7) splitting peptide bonds connecting the old strand, and a lytic transglycosylase (Slt70, MltA or MltB) cutting the glycan strand.

A minor modification to the multienzyme complex composition may be sufficient to carry out murein synthesis during cell division - PBP3 would be used instead of PBP2 and possibly a monofunctional transglycosylase would be added - the triplets may be synthesised in one step as, contrary to what happens during elongation, several new murein strands are inserted side by side during division (de Jonge *et al.*, 1989; Nanninga *et al.*, 1990). A slight delay in the removal of the docking strand would lead to murein invagination as the result of the addition of a new murein triplet before the removal of the docking strand for the previous one.

1.2.6. The sacculus as a dynamic structure.

The model for the sacculus growth described above suggests that half of the peptidoglycan existing in the newly divided cell has to be

removed by the time of the next division as the result of the "three-for-one" process of insertion of new strands. It would be logical for the cell to reuse this material. Whether or not the "three for one" hypothesis is true, the removal of about 50% of cell wall peptidoglycan per generation was shown, and about 90% of the released material is reinserted into the sacculus (Goodell, 1985; Park, 1993).

The products released from the peptidoglycan by lytic transglycosylases and D,D-endopeptidases can be reutilised through two pathways. The first turnover products are usually monomeric 1,6-anhydromuropeptides. These can be first split in the periplasm into the disaccharide and the peptide by the AmiA amidase (van Heijenoort *et al.*, 1975). The peptide usually lacks both D-alanines by that time due to the action of carboxypeptidases. The tripeptides are transported into the cell by a periplasmic peptide permease, either murein specific MppA (Park *et al.*, 1998) or general peptide permease OppA (Goodell & Higgins, 1987), in conjunction with OppBCDF proteins. It is not clear what happens to the released disaccharide. Another way of taking up the turnover products into the cell is through the AmpG permease transporting intact muropeptides (Lindquis *et al.*, 1993). Tripeptides are then released by the cytoplasmic amidase AmpD (Höltje *et al.*, 1994). The tripeptide can be included in the normal pathway of the murein precursor synthesis by specific tripeptide ligase Mpl producing UDP-MurNAc tripeptide (Mengin-Lecreulx *et al.*, 1996).

1.3. Cell division genes.

The definition of a specific cell division gene may be somewhat difficult. Cell division is, of course, not possible without many of the genes described above and mutations in many of them are lethal, but

the term "cell division gene" is usually used when a mutation directly leads to a visible defect in division without other immediate effects on cell growth, cell wall formation, or DNA replication or partition. Normally, blocking division does not cause the immediate cessation of growth and cells continue to elongate for a while. This results in the formation of long filaments, hence most of the essential cell division genes are called *fts* (filamentous temperature sensitive). If one talks about a gene that is absolutely essential for the division of every bacterium, then the only gene of those known at present that fits this definition is *ftsZ* (even this gene is probably missing in at least one bacterium: recently completed *Chlamydia trachomatis* genome has no recognisable *ftsZ* homologue). But *ftsZ* by itself appears to be sufficient for division only of the simplest bacteria, for example *M. genitalium*, that live in an ideal nutrient rich environment at constant temperature and constant osmotic pressure and hence don't need (and don't have) peptidoglycan cell wall. Other bacteria need to have additional structures to be able to survive in nature. One of these additional structures is the peptidoglycan cell wall, typical for the vast majority of *Eubacteria*. The presence of cell wall immediately creates serious problems during division. Being rigid and under the constant osmotic pressure from the inside, peptidoglycan cell wall requires several proteins to split it in two during division without breaking the whole structure, and these proteins have to act coordinately with other proteins needed for division as such.

ftsZ is the cell division gene that has received most attention in recent years, following the discovery that its product forms a ring on the inner face of the cytoplasmic membrane at the cell midpoint (Bi & Lutkenhaus, 1991). The *ftsZ* gene, located in the *mra* cluster at 2.5 minutes of the chromosomal map, codes for the most abundant cell division protein, which is present in 5,000 to 20,000 copies per cell (Bi and Lutkenhaus, 1991). FtsZ protein shares some sequence

similarity with eukaryotic tubulins, which is in agreement with its weak GTPase activity (de Boer *et al.*, 1992). The recent solution of FtsZ and tubulin crystal structures (Lowe & Amos, 1998; Nogales *et al.*, 1998) confirmed that the 3D structures of these proteins are remarkably similar despite the low level of primary sequence similarity. The N-terminal GTP binding domains of these proteins are virtually identical, and the C-terminal domains also are very similar although primary sequence similarities in the C termini could not be detected. The functional similarity of FtsZ with tubulins is supported by the fact that it can assemble into protofilament sheets and mini rings that closely resemble the structures formed by tubulin . Given the diameter of these protofilaments and diameter of the cell, the amount of FtsZ in a cell is sufficient to form a protofilament encircling the cell 20 times (Erickson *et al.*, 1996). The ability of FtsZ to polymerise (dimerise) has been recently demonstrated *in vivo* (Di Lallo *et al.*, 1999).

It is currently assumed that constriction of the ring formed by FtsZ molecules causes progression of the septation machinery at the division site. Formation of this ring seems to be the earliest sign of division and can be detected very early in the cell cycle (Addinall *et al.*, 1997). It was also shown that FtsZ rings can form in *ftsA, ftsQ* and *ftsI* mutants (Addinall *et al.*, 1996), as well as in *ftsW* (Boyle *et al.*, 1997) and *ftsK* (Wang & Lutkenhaus, 1998) strains, thus indicating that products of these genes are not necessary for the assembly of the ring structure and rather act later in the division process. FtsZ can also form structures other than rings - arcs in a *rodA*^{sui} mutant and spirals in an *ftsZ26* strain. These structures can contract and thus determine the shape of an aberrant septum (Addinall & Lutkenhaus, 1996). This also indirectly suggests that assembly of the FtsZ ring starts at a nucleation site on the inner membrane (possibly another cell division protein), from which its bi-directional polymerisation starts. A different approach, based on

observation of FtsZ-GFP fusions *in vivo*, also confirms the presence of Z rings in live cells; moreover, when the fusion protein is overproduced, spiral structures are also observed (Ma *et al.*, 1996). Also, FtsZ-GFP sometimes forms double rings in the cell centre. Deletion of the C-terminal 67 non-conserved residues of FtsZ in the fusion protein prevents formation of the Z ring, but doesn't stop polymerisation, in fact, polymers formed with this deletion variant (spirals and thick sheets), appear to be much more stable and have brighter fluorescence (Ma *et al.*, 1996).

FtsZ homologues have been found in wide variety of *Eubacteria* and *Archaea*, including all those for which complete genome sequences are available (except *C. trachomatis*); it was even found in a higher plant, *Arabidopsis thaliana*, where it is encoded by a nuclear gene but apparently required for division of the chloroplasts (Osteryoung & Vierling, 1995).

Properties of the Z ring, mainly its formation early in the cell cycle, location in the middle of the cell at the future division site, and its visible constriction during the division process, provide an attractive basis for the speculations that FtsZ ring can be i) an assembly site for other proteins involved in division and ii) a motor force or at least part of it, driving septation. For both i) and ii) to work, FtsZ must interact with at least some of the suggested participants of this division complex. Some evidence for such an interaction has appeared recently and is discussed later in this chapter.

The ***ftsA*** gene located upstream of *ftsZ* codes for a 46 kDa protein present at about 150 molecules per cell. The correct ratio of FtsA to FtsZ (1:100) is thought to be essential for successful division (Dai & Lutkenhaus, 1992; Dewar *et al.*, 1992). The cytoplasmic form of FtsA can be phosphorylated and in the phosphorylated state it is capable of binding ATP, whereas in the non-phosphorylated form it

appears to be associated with the cell membrane (Sánchez *et al.*, 1994). The protein is a member of a large family of ATP binding proteins that include actins and heat shock proteins (HSP70) and was proposed to be folded in a way typical for this family (Bork *et al.*, 1992; Sánchez *et al.*, 1994). However, mutating residues that are supposed to be essential for ATPase function, as well as phosphorylatable residues, does not change the ability of the protein to complement known temperature sensitive *ftsA* mutations (Sánchez *et al.*, 1994). It has been recently shown by immunofluorescence microscopy (Addinall & Lutkenhaus, 1996) and by using GFP fusions (Ma *et al.*, 1996) that FtsA closely follows FtsZ at the time of division - a ring can be seen at the middle of a cell, a bit later than FtsZ ring itself, and these structures behave in exactly the same way in different mutants that have FtsZ structures other than rings (spirals or arcs) (Addinall and Lutkenhaus, 1996). FtsA is probably recruited to the forming septal structure immediately after the FtsZ ring is formed. This statement is based on the fact that FtsZ rings can form in *ftsA* mutants, but not *vice versa* (Addinall *et al.*, 1996; Addinall and Lutkenhaus, 1996). Another piece of evidence to support this is that FtsA-GFP fusion protein, normally localised in cell centres, appears to follow spiral patterns (seen for FtsZ-GFP fusion) when the concentration of FtsZ is increased (Ma *et al.*, 1996). Also, part of the FtsA-GFP fusion protein appeared to be sequestered in the membrane, something that correlates with the evidence for two sub populations of FtsA, localised to the cytoplasm or the cell membrane according to their phosphorylation state.

ftsQ lies upstream of the *ftsA* gene. Its product is a 31 kDa cytoplasmic-membrane protein with a single N-terminal transmembrane α-helix and most of the protein located in the periplasm (Robinson *et al.*, 1984; Yi *et al.*, 1985). The FtsQ protein was estimated to be present in 50 to 100 copies per cell (Carson *et*

al., 1991). Mutants of this gene produce filaments without signs of constrictions, but constrictions are visible when a second mutation in *rodA* gene is introduced, which suggested FtsQ acts after FtsZ (Begg & Donachie, 1985). The cytoplasmic and transmembrane domains of FtsQ appear to be non-specific for its function and can be replaced by a membrane anchor from another protein (Buddelmeijer *et al.*, 1998; Dai *et al.*, 1996). The periplasmic domain was the only part of the protein required for septal localisation and was sufficient to complement the *ftsQ1 ts* mutant (Buddelmeijer *et al.*, 1998). Correct localisation of FtsQ was dependent on cytoplasmic FtsZ and FtsA, but not on periplasmic FtsL or FtsI (Chen *et al.*, 1999). This suggests that FtsQ acts at an intermediate stage of septation and probably interacts with a periplasmic protein, other than FtsL and FtsI, to achieve correct localisation at the time of division.

ftsW is situated between *murD* and *murG* in the *mra* gene cluster. *ftsW* mutants are filamentous (Ishino *et al.*, 1989) and show no constrictions even when combined with spherical mutations, which suggests an early role in cell division (Khattar *et al.*, 1994). High hydrophobicity and homology with RodA suggest inner membrane location of the protein. FtsW protein has been proposed to interact with PBP3 (Matsuhashi *et al.*, 1990), but there is no direct evidence for this interaction. The early block of division in *ftsW* mutants might be due to stabilisation of the FtsZ ring, as the ring is absent in 50% of filaments in an *ftsW* null strain and the number of rings is reduced in another 50% (Boyle *et al.*, 1997).

The ***ftsN*** gene located at 88.5 minute codes for a 36 kDa protein with a transmembrane segment near its N terminus, and short N terminal cytoplasmic and large periplasmic domains. The protein is estimated to be present in about 50 molecules per cell (Dai *et al.*, 1993). The cytoplasmic domain of the protein appears to be

completely dispensable and the transmembrane segment can be replaced by the membrane-spanning domain of MalG or even by the cleavable MalE signal sequence (Dai *et al.*, 1996), suggesting that only the periplasmic part of the protein determines its specific function. On the other hand, translocation of the protein to the periplasm is absolutely required for its function. The gene was originally isolated as a multicopy suppressor of the *ftsA12* mutation and was later shown to suppress *ftsI23* and *ftsQ1* mutations (Dai *et al.*, 1993), two *ftsW* mutations (M. Khattar, personal communication) and *ftsK44* (Draper *et al.*, 1998). As FtsN depletion leads to filamentation (long aseptate filaments), it is assumed to be an essential cell division protein. The mutant phenotype, protein abundance and location led to a suggestion of FtsN functioning together with *ftsQ*, *ftsI* and *ftsL* in a 'stoichometric complex' at the site of septum formation (Lutkenhaus, 1993).

ftsL *(mraR)* lies close to the very beginning of the *mra* gene cluster (Ishino *et al.*, 1989). Its product is a 13.6 kDa cytoplasmic-membrane protein with one membrane-spanning domain and most of the protein located in the periplasm. The protein is present in 30 to 40 copies per cell, and mutation leads to production of aseptate filaments (Guzman *et al.*, 1992; Ueki *et al.*, 1992). The protein contains a leucine zipper which may be essential for its function, possibly controlling its dimerisation. FtsL is a membrane protein with small cytoplasmic and transmembrane domains and a large periplasmic domain. Unlike some other division proteins, both cytoplasmic and membrane spanning domains appeared to be specific and essential for the correct functioning of this protein (Guzman *et al.*, 1997). Recent work (Ghigo *et al.*, 1999) has demonstrated that, like several other cell division proteins, FtsL forms a ring structure at the cell midpoint rather late in the division process. This localisation is dependent upon the function of FtsZ,

FtsA and FtsQ, but not FtsI. On the other hand, localisation of FtsQ, FtsA and FtsZ does not require FtsL (Ghigo *et al.*, 1999).

The ***zipA*** gene was discovered recently during a search, using an affinity blotting procedure, for proteins specifically interacting with *ftsZ* (Hale & de Boer, 1997). The gene appears to be essential, with insertional inactivation in the presence of a ts complementing plasmid producing long aseptate filaments at nonpermissive temperature. Interestingly, overexpression of ZipA also appeared to block cell division and this latter block can be reversed by increasing amounts of FtsZ, further supporting the possibility of interaction between these two proteins. Moreover, ZipA::GFP fusion proteins appeared to be localised in a ring structure in the middle of cells in 91% of cases, sometimes being visible even in very young cells. This closely resembles the behaviour of FtsZ rings described by (Addinall and Lutkenhaus, 1996). ZipA is a protein with a predicted molecular weight of 36 kDa that migrates at 50 kDa on SDS-PAGE gels. The very N-terminal part of the protein is presumably localised to the inner membrane with the rest of it located in the cytoplasm. Immediately after the membrane-spanning domain there is a 103 residue proline and glutamine rich region that may form a rigid linker between the membrane anchor and the C-terminal domain of the protein. This structure as well as the location of the protein makes it a good candidate for a "connector" between the membrane and FtsZ ring. The possibility of direct interaction between ZipA and FtsZ is supported by the presence of the sequence motifs in ZipA, similar to microtubule-binding signatures of several eukaryotic proteins and *in vitro* demonstration of stabilisation of FtsZ protofilament assembly by ZipA (RayChaudhuri, 1999). Hale and de Boer originally suggested the possible role of ZipA as a nucleation centre for the assembly of the FtsZ ring (consistent with its abundance at 1-10 % of FtsZ). This, however, may not be the case, as

later work has shown that Z rings still form in the absence of ZipA, although the number of rings formed decreases (Hale & de Boer, 1999). Midcell localisation of ZipA itself appeared to be FtsZ, but not FtsA or FtsI dependent (Hale and de Boer, 1999; Liu *et al.*, 1999). And, in turn, the septal localisation of FtsA did not depend on ZipA.

ftsI lies in the same cluster as *ftsQAZ* and codes for a septum specific murein synthetase, PBP3 (Ishino & Matsuhashi, 1981). The protein is estimated to be present in 50-100 copies per cell (Spratt, 1977; Weiss *et al.*, 1997). PBP3 is a transmembrane protein with its catalytic domain located in the periplasm (Bowler & Spratt, 1989). Mutants are filamentous with constrictions visible only after introduction of a spherical mutation (Begg and Donachie, 1985). PBP3 is a division-specific transpeptidase. It was suggested that PBP3 requires an accessory protein to show transglycosylase activity in a way similar to PBP2 and RodA proteins (Ikeda *et al.*, 1989; Matsuhashi *et al.*, 1990). FtsW was proposed for this function on the basis of sequence similarity between FtsW and RodA; evidence for this interaction is, however, missing.

The protein becomes located at the septum at late stages of division; some of the protein is also observed at the cell poles (Weiss, et al., 1997). The correct septal localisation requires its own transmembrane part, FtsZ, FtsA, FtsQ, and FtsL (Weiss *et al.*, 1999)

The ***ftsK*** gene was originally characterised in this laboratory (Begg *et al.*, 1995). The gene is located in the 20 minute region of the chromosome downstream of an SOS-inducible promoter (*dinH*: Lewis *et al.*, 1992) and codes for a cytoplasmic membrane protein with a predicted size of about 147 kDa. The protein is highly homologous to several SpoIIIE proteins from Gram positive bacteria. In *B. subtilis* this protein has been shown to be responsible for correct partitioning of one of the sister chromosomes into the spore compartment, it may

also be involved in septum closure (Wu & Errington, 1994). In addition, the C-terminal part of the protein has significant homology to a number of Tra proteins of *Streptomyces* plasmids and the conjugative transposon Tn916. FtsK, like SpoIIIE, has a hydrophobic N-terminal region with several potential transmembrane segments and a large cytoplasmic domain containing nucleotide binding motif. The protein is localised to the cell midpoint at the time of cell division, and by fusion of the N-terminal membrane domain of FtsK to GFP protein it was shown that only the N-terminal 15% of the protein is required for correct localisation (Yu *et al.*, 1998). The same N-terminal part is sufficient to complement the *ftsK44* mutation. The original *ftsK44 ts* mutation resides in this part of the protein in one of the α-helical transmembrane segments (Begg *et al.*, 1995), and it actually prevents correct localisation (Yu *et al.*, 1998). The phenotype of the double *ftsK rodA* mutant suggested that the protein acts late in division (Begg *et al.*, 1995). This is supported by the timing of FtsK localisation - the FtsK-GFP fusion is localised at the division site only when there is a visible constriction (Yu *et al.*, 1998). On the other hand, immunofluorescence data shows that FtsK localises to the septum early which is supported by the smooth appearance of filaments produced by a null mutant (Wang and Lutkenhaus, 1998).

Recent data (Liu *et al.*, 1998) suggests that the C terminal part of the protein that has the highest homology with SpoIIIE and other proteins with "DNA-mobilising" function is indeed required for normal DNA partitioning into the sister cells during division. To summarise, the N-terminal part of FtsK probably has some role in the completion of the septum, while the C terminal domain is needed to move the DNA that might be trapped by the closing septum to the correct compartment.

1.4. The division process.

The division of a bacterial cell is thought to start from a rather small location in the cell envelope, a division site, where the assembly of the FtsZ ring begins (Lutkenhaus, 1993). Indirect support for this hypothesis is provided by the appearance of FtsZ structures in certain division mutants. In *ftsZ26* mutant a large proportion of cells has spiral FtsZ structures and FtsZ forms arcs in a *rodA* mutant. In both cases, the formation of these structures seem to start at a point attached to the cell membrane and proceed bi-directionally around the circumference of the cell (Addinall and Lutkenhaus, 1996). This strongly suggests that there is some sort of a membrane located nucleation site where the assembly of the Z ring begins. This site has to be generated at the onset of each division at midcell position. The nature of this site still remains a mystery. One possible candidate for the participation in this structure, an Era GTPase, was described recently (Britton *et al.*, 1997; Britton *et al.*, 1998). Mutants with decreased Era levels complete chromosomal replication but become arrested in the cell cycle before division. Little is also known about how the nucleation site is positioned and how the correct timing of this event is determined. The timing is somehow correlated with the completion of chromosome replication and the positioning of the septum seems to coincide with the nucleoid-free space in the cell centre. This suggests the involvement of partition of the replicated chromosomes in the determination of the division site.

1.4.1. Nucleoid partition.

Obviously, a cell has to replicate its chromosome during each cell cycle and ensure that the resulting copies end up in different daughter cells. Therefore, division must be somehow coordinated with chromosome replication and partition of nucleoids. As the speed

of DNA replication at a given temperature is constant (Cooper & Helmstetter, 1968), cells achieve the necessary number of complete chromosomes by the time of division through the control of replication initiation - intervals between initiation events are equal to intervals between divisions. This results in fast growing cells having multiple chromosomes.

Separation of the sister nucleoids seems to be a necessary prerequisite for division, and it looks like nucleoid-free space can determine the positioning of a septum (and, in turn, septum formation is inhibited in the areas where a nucleoid is present). There is no consensus at the moment on the mechanisms driving the separation of nucleoids, but it was recently demonstrated that *E. coli* (as well as other bacteria) has an active mitotic-like partition apparatus (Gordon *et al.*, 1997). Unfortunately, it is not known what proteins are involved in this rapid partition of chromosomal origins. Two possible candidates for this role are the *muk* gene products and FtsK.

mukB mutant cells appear to have a slightly filamentous phenotype and a significant number of anucleate cells. The irregular separation of the nucleoids can be easily visualised (Niki *et al.*, 1992; Niki *et al.*, 1991). MukB appears to be the largest prokaryotic protein characterised so far. It shares some similarity with eukaryotic myosins and has a domain structure reminiscent of the eukaryotic motor proteins kinesin and myosin, which is consistent with its suggested function of motor force protein moving nucleoids around. Supporting this possibility is recent demonstration of specific binding of MukB to microtubules (Lockhart & Kendrick-Jones, 1998). In the same operon with *mukB* two other genes, ***mukE*** and ***mukF*** are located. Inactivation of both also leads to the defect in partitioning and the production of anucleate cells (Yamanaka *et al.*, 1996).

The purified MukB protein has low ATPase and GTPase activities. The N-terminal domain of MukB binds with high affinity to

FtsZ. FtsZ can stimulate nucleotide turnover by MukB. These properties are consistent with MukB functioning as a motor protein using FtsZ as a track or anchor for generating force within *E. coli* (Lockhart & Kendrick-Jones, 1998). As the FtsZ ring is assembled early, MukB could use it as a "marker" to move nucleoids away from the site where the future septum will form. Consistent with this, Hiraga *et al.* (1998) demonstrated bi-directional migration of a replication initiation regulator SeqA. This migration occurred after the formation of Z ring and required MukB.

The ***ftsK*** gene product appears to be another candidate for participation in chromosome partitioning. FtsK has strong similarity in its primary structure to the SpoIIIE protein of *B. subtilis* and a number of Tra proteins from conjugative plasmids in *Streptomyces*, for which involvement in the DNA translocation have been demonstrated. The original *ftsK44* mutation didn't have any alterations in chromosome partition (Begg *et al.*, 1995), but, when the expression of the C-terminal domain was reduced, the partition defect was indeed detected (Liu, et al., 1998).

1.4.2. Distinguishing the division sites: the *min* system.

The function of the genes in the ***minB*** locus is to prevent formation of the septum in the wrong place. The locus consists of three genes, *minC, minD* and *minE*. The MinC and MinD proteins act together to form a non-specific inhibitor of septation blocking cell division at all available sites. MinE protein prevents action of this inhibitor at internal sites while still permitting it to act at cell poles (de Boer *et al.*, 1989). Although MinC normally needs activation by MinD to function properly, it can also act together with another division inhibitor, DicB (de Boer *et al.*, 1990; Labie *et al.*, 1990). In *minB* mutants (lacking MinC or MinD) a septum can form either

internally or at the cell poles, but the total number of septa is the same as in normal cells (Donachie & Begg, 1996; Teather *et al.*, 1974). Every polar division consumes a "quantum" of the "division potential" and one central division is lost, thus making a cell bigger. In addition, the time till next division is inversely proportional to the cell length, but even in the longest cells only one septum can form at a time (Donachie and Begg, 1996).

Location of MinD to the inner membrane was determined by immunoelectron microscopy. MinD protein was also shown to have ATPase activity *in vitro* (de Boer *et al.*, 1991).

Although small (88-AA), MinE protein was shown to have three distinct domains with different functions. The small N-terminal domain appears to be required to counteract the activity of the MinCD division inhibitor, the central region may be involved in dimerisation or oligomerisation (Pichoff *et al.*, 1995), and the larger C-terminal part determines the topological specificity of MinE function, only allowing it to act at internal division sites (Zhao *et al.*, 1995). MinE was recently demonstrated to form a ring structure near the middle of young cells (Raskin & de Boer, 1997). Formation of this ring required MinD but was independent of MinC and FtsZ. Thus, MinE looks currently to be the protein appearing at the forming division site first, which is presumably necessary for the correct localisation of the FtsZ ring, although not for the Z ring formation.

How exactly the Min proteins work is not yet known. An attempt to show direct interaction of those three proteins with their most likely target, FtsZ, failed (Huang *et al.*, 1996); although this approach, utilising yeast two-hybrid system, shows interaction between members of the min system, and that MinE reduces interaction between MinC and MinD. The nature of the topological signal recognised by the MinE protein is not known either.

1.4.3. The assembly and order of action of cell division proteins at the division site.

Bacterial cell division, although simple at first glance, is in fact quite a complex process in which many proteins take part. It is obvious that the action of these proteins must be coordinated somehow, and the easiest way such a co-ordination could be achieved is through the formation of a macromolecular complex at the division site. This, still largely hypothetical, structure called divisome (Nanninga, 1991) or septator (Ayala *et al.*, 1994) is thought to be assembled at the division site at the very beginning of division. The process probably starts at a nucleation site where the assembly of the Z ring begins. Other division proteins are thought to be recruited to the divisome after the formation of the Z ring.

Although genetic evidence suggesting the possibility of interaction between cell division genes has existed for some time, only the direct demonstration of protein-protein interaction could prove that this interaction really takes place. These data started to accumulate recently. The *zipA* gene was in fact discovered through direct binding of its product to FtsZ (Hale and de Boer, 1997). Specific binding of the purified N-terminal domain of MukB to FtsZ was recently demonstrated (Lockhart and Kendrick-Jones, 1998). Indirect evidence for FtsA-FtsZ interaction is provided by studies of the localisation of heterologous FtsA in *E. coli*. FtsA protein from *Rhizobium meliloti* or *Agrobacterium tumefaciens* was able to localise correctly in *E. coli* cells only if FtsZ protein from the same organism was also provided (Ma *et al.*, 1997). The interaction between FtsZ and FtsA was also observed using yeast two-hybrid system (Wang *et al.*, 1997). Also, some evidence exists for the possible interaction of FtsW with PBP3 (Matsuhashi, 1994) and of PBP3 and PBP1B (Höltje, 1996). The interaction between high molecular weight PBPs and lytic enzymes has already been discussed.

Recent localisation studies using immunofluorescence and GFP fusions provided information on mid-cell localisation of some proteins, which is the prerequisite for their participation in the divisome. Localisation at the division site was first shown for FtsZ using immunogold staining (Bi and Lutkenhaus, 1991) and then confirmed with immunofluorescence microscopy (Addinall and Lutkenhaus, 1996) and *in vivo* using GFP fusions (Ma *et al.*, 1996). Several other division proteins have also been localised to the septum - FtsA (Addinall and Lutkenhaus, 1996; Ma *et al.*, 1996), FtsQ (Buddelmeijer *et al.*, 1998), ZipA (Hale and de Boer, 1997; Hale and de Boer, 1999), FtsW (Wang *et al.*, 1998).

Recent experiments with division protein localisation in several *fts* mutants provide a key to the order of their recruitment to the divisome and, possibly, to the order of their action during division. Mid-cell location of FtsA depends on the prior formation of the Z ring (Addinall and Lutkenhaus, 1996). Localisation of FtsQ to the septum requires FtsZ and FtsA, but not FtsL and FtsI (Chen *et al.*, 1999). Septal localisation of FtsI appears to be dependent on the presence of both FtsZ and FtsA (Wang *et al.*, 1998), as well as FtsL and FtsQ (Weiss *et al.*, 1999). At the same time, the assembly of FtsZ rings is somewhat impaired if PBP3 is inactivated, and constriction of the ring does not occur (Pogliano *et al.*, 1997). FtsN also forms central rings, but requires not only FtsZ, but also FtsA, FtsQ and FtsI for correct localisation (Addinall *et al.*, 1997). Although the phenotype of the original FtsK mutant and the results obtained with GFP fusions (Yu *et al.*, 1998) suggested a late time of action for FtsK, immunofluorescence labelling showed it to be localised to the septum rather early; only FtsZ and FtsA, but not FtsQ or FtsI are required for correct localisation (Wang and Lutkenhaus, 1998).

To summarise, the available data suggests the following order of assembly into the "divisisome" of several division proteins: FtsZ-FtsA-FtsQ-FtsL-FtsI. FtsN may be recruited to the septum after FtsI

(Addinall *et al.*, 1997), but the multicopy suppression of mutations in the genes coding for FtsA, FtsQ, FtsI and FtsK (Dai *et al.*, 1993; Draper *et al.*, 1998) may indicate an earlier role for the protein. FtsK is recruited to the septum after FtsA, but it is not known if later proteins depend on its localisation. ZipA and FtsA both localise to the septum after FtsZ and do not require each other for this localisation (Hale and de Boer, 1999). FtsW may act before FtsZ, at least it is required for the stabilisation of the Z ring (Boyle *et al.*, 1997).

FtsI, an enzyme absolutely required for septal peptidoglycan synthesis, is one of the last proteins recruited to the divisome. On the other hand, the constriction of the Z ring requires FtsI activity (Pogliano *et al.*, 1997). This indicates that the whole divisome structure may need to be assembled before the synthesis of the septum and the constriction begin. FtsI may also provide a link between the periplasmic peptidoglycan synthesising complex (discussed above) and cell division proteins. Although direct interaction (binding) of FtsI with any other cell division protein has not been demonstrated, the indirect evidence for such interactions strongly suggests that FtsI is a part of the divisome (Ikeda *et al.*, 1989; Matsuhashi *et al.*, 1990; Tormo *et al.*, 1986). As the direct binding of FtsI to other peptidoglycan synthases and lytic enzymes has been detected (Höltje, 1996; Romeis and Höltje, 1994), these proteins must be parts of the divisome as well.

An important problem arises from the fact that many of the cell division proteins are present in amounts that are much lower than that of FtsZ. For example, the FtsA to FtsZ ratio is normally 1:100. FtsQ, FtsL (?) and FtsN (?) are probably present in smaller amounts than FtsZ. It has been suggested that "minor" proteins form complexes with each other, "subassemblies", that are attached to the Z ring but are located far apart from one another at the beginning of constriction, moving closer together only as constriction progresses

(Nanninga, 1998). It is not known what force is responsible for the constriction of the ring and the progress of division.

The final stage of division must be different from constriction because the divisome must be disassembled and the dividing cells must separate which may or may not require additional proteins for splitting peptide bonds that probably hold two cells together. Alternatively, this step could be performed by the "normal" lytic enzymes.

1.5. Regulation of cell division.

The most important question about the regulation of cell division, that of how the correct timing and precise placing of the septum are determined, still remains largely unanswered. There is, however, some information on other aspects of division regulation - regulation of transcription of several division genes, involvement of protein regulators, either on the transcription level or direct protein-protein interactions and a special case of division regulation during the SOS response.

1.5.1. Transcriptional regulation.

Most of cell division genes appear to be located in the large *dcw* (*d*ivision and *c*ell *w*all) or *mra* cluster at 2 minutes of *E. coli* chromosome. Several *mur* genes involved in the synthesis of peptidoglycan are also located in this cluster. No transcriptional terminators have been found within this large cluster of 16 genes, which has led to the suggestion that the whole cluster constitutes a single operon. Genes in the cluster are usually separated by only a few base pairs, and in some cases even overlap, which suggests the possibility of translational coupling. As operon structure usually

implies some sort of co-ordinate gene regulation, studies have been conducted on the regulation of transcription of the genes in this region. Most of the genes in the cluster have several promoters, contributing differently to their transcription and sometimes responding to different environmental stimuli.

A strong promoter is located in the very beginning of the *dcw* cluster. This *mra* promoter appears to be necessary for the full expression of the first nine genes, up to *ftsW* . Inactivation of this promoter is not lethal to the cells if the first nine genes of the cluster are provided *in trans*, which indicates the presence of additional promoters capable of transcribing the downstream genes (Hara *et al.*, 1997). It appears, however, that transcription of all downstream genes including *ftsZ* is reduced if this promoter is inactivated (Mengin-Lecreulx *et al.*, 1998).

Although the *mra* promoter is likely to be important for normal transcription of all the genes in the *dcw* cluster, additional promoters within the cluster are also important and were actually characterised first. Due to its importance in cell division, regulation of *ftsZ* transcription has been studied most extensively. The gene is preceded by a set of promoters of different strengths and types which probably reflects the necessity to adjust the expression of the gene in different conditions. Initially, most of transcription of *ftsZ* was attributed to the two proximal *ftsZ* promoters. It was, however, later shown that the seemingly stronger of these "promoters" is in fact an RNAse E cleavage site (Cam *et al.*, 1996). When this was taken into account, it appeared that almost half of *ftsZ* transcripts originated from two promoters within *ddlB* (Flärdh *et al.*, 1997). It was, however, shown later that even stronger promoters are located further upstream, with two thirds of transcripts coming from upstream of *ddlB* (Flärdh *et al.*, 1998). This correlates with the earlier observation that sequences further than 6 kb upstream of *ftsZ* are required for its normal expression (Dai & Lutkenhaus, 1991).

Regulation of *ftsZ* expression from its promoters appears to be important for the proper functioning of the division machinery. Insertion of a regulatable *tac* promoter just in front of *ftsZ* led to noticeable alterations to the normal division cycle, although the cells remained viable (Palacios *et al.*, 1996). This may indicate that normal *ftsZ* promoters somehow regulate transcription of the gene during the cell cycle (the result of this regulation may be maximising the number of viable cells per mass). This possibility is supported by the observation of oscillating *ftsZ* transcript levels in synchronised cells (Garrido *et al.*, 1993). (Zhou & Helmstetter, 1994) also observed variations in *ftsZ* transcript levels, but attributed them to transient inhibition of transcription after replication of this chromosomal region. Transcription originating upstream of *ftsA* exhibited the periodicity and accounted for a significant proportion of the transcripts entering ftsZ while proximal *ftsZ* promoters were transcribed constitutively through the cell cycle.

Although these reports of oscillating transcript levels during the cell cycle provide a possible connection between regulation of gene expression and initiation of cell division, it is not known how the transcript levels are controlled, *i.e.* what proteins and promoters are responsible for these oscillations. On the other hand, regulation of transcription from two promoters located within *ddlB* has been studied in more detail.

The ***sdiA*** gene (*s*uppressor of *d*ivision *i*nhibition) was isolated by its ability to suppress the action of several division inhibitors. Its product induced minicell formation when overexpressed in wild-type cells and allowed the *ftsZ84* mutant to divide at the non-permissive temperature (Wang *et al.*, 1991). These effects appeared to be due to the SdiA protein activating transcription from one of the promoters in *ddlB*, upstream of *ftsQAZ* and *envA* genes (Wang *et al.*, 1991). The significance of this activation is not clear as the gene is dispensable.

However, the gene may play a role in stationary phase, because the protein has extensive homology to the large family of *luxR*-like quorum-sensing transcriptional activators. All known activators of this family work in pair with another gene (*luxI*-like), whose function is to produce a small freely diffusible molecule, N-acyl-homoserine lactone, with an acyl chain of a length that varies amongst different species. As this molecule accumulates with increasing population cell density, the *luxR*-like gene activates transcription of the corresponding regulon. Interestingly, *E.coli* clearly doesn't have a homologue of *luxI*, which raises a question: what acts as the activator of the *sdiA* product? Two conflicting reports have addressed this problem. (Garcia-Lara *et al.*, 1996) didn't find any evidence for an effect of the putative autoinducer on the regulation of the *sdiA*-dependent promoter (*ftsQp2*). In fact, they observed a slight (30-35%) decrease of transcription from this promoter during growth in "conditioned" medium that had previously supported growth of *E.coli*. This effect was less than the decrease in transcription from the same promoter (40-60%), resulting from the insertional inactivation of the *sdiA* gene. Contrary to this report, Sitnikov et al. (Sitnikov *et al.*, 1996) not only demonstrated an increase in transcription from the *ftsQp2* promoter in stationary phase, but also demonstrated activation of this promoter by *V. fischeri* and *V. harveyi* autoinducers, as well as by conditioned medium. Therefore, *sdiA* seems indeed to act like other *luxR*-like regulators, although the native autoinducer molecule in *E.coli* remains unknown.

rpoS, a gene coding for a stationary phase sigma factor, is also involved in the regulation of transcription of the *ftsQAZ* genes. In particular, *ftsQp1*, one of the two promoters shown to be responsible for the transcription of the *ftsQA* genes (and contributing to the transcription of *ftsZ*), is RpoS dependent (Ballesteros *et al.*, 1998;

Sitnikov *et al.*, 1996), but no other promoter in the *ddlB-ftsZ* region is (Smith *et al.*, 1993).

stfZ is a small gene suggested to be transcribed from the antisense strand in the *ftsZ* 5' region. The presence of both promoter activity and a functional terminator in this region has been shown (Dewar & Donachie, 1993), and inhibition of cell division at elevated temperatures when this DNA piece is present in multiple copies led to the suggestion that it acts as antisense RNA blocking *ftsZ* translation in a manner similar to a phage relic gene, *dicF* (Dewar and Donachie, 1993; Tétart & Bouché, 1992).

1.5.2. Post-transcriptional regulation.

Although many, if not all, genes in the *mra* cluster are translated from polycistronic messengers, the relative efficiency of translation of different ORFs varies significantly. This is not surprising, as the products of different cell division genes are present in the cell in quite different amounts, with FtsZ being probably the most abundant (up to 20,000 molecules per cell) and FtsQ one of the least abundant (Carson *et al.*, 1991). Several of the ORFs in the region do not have good ribosome binding sites and, in addition, several genes have start codons other than ATG. This implied that translation of different ORFs in this region should demonstrate significant differences in efficiency, and this, indeed, is the case (Boyle, 1997; Mukherjee & Donachie, 1990)

Another important aspect of post-transcriptional control of cell division is division inhibition during the SOS response. DNA damage in *E.coli* induces the expression of several genes that perform two functions important for cell survival. Some of the induced proteins are involved in repairing DNA, but others act to delay cell division

until the DNA is repaired. The SOS induced division block appears to be the result of SulA protein action.

The ***sulA*** (*sfiA*) gene product blocks division by preventing Z ring assembly (Bi & Lutkenhaus, 1993). The use of a yeast two-hybrid system revealed the direct interaction of SulA with FtsZ (Huang *et al.*, 1996). It has also been shown that SulA prevents the polymerisation of FtsZ *in vitro* (Trusca *et al.*, 1998). As FtsZ ring assembly is a key to the whole division process, SulA induction completely blocks division. Once the SOS induction is over, SulA is rapidly degraded by Lon protease and division resumes.

FtsK also seems to be involved in the SOS response. One of the suggested functions of this protein is to ensure that chromosomes are not trapped by the closing septum. Inactivation of only the cytoplasmic part of FtsK allows continued cell division but induces the SOS response, presumably due to consequent DNA damage (Liu *et al.*, 1998).

1.6. Cell shape control.

The maintenance of the normal rod shape is important for *E. coli* cells. Cell division genes are, of course, involved in cell shape control by ensuring that cells divide and do not get too long. But there exists a set of genes determining the rod shape as such. Reducing expression of these genes results in cells becoming spherical. The most important control over the shape is exerted by the *rodA* and *pbpA* genes located in the *mrd* cluster at 14.5 minutes on the *E. coli* chromosome (Tamaki *et al.*, 1980). These genes are discussed in more detail in Chapter 4.

Another class of round mutants was mapped at 71 minutes in the five gene ***mre*** operon (Wachi *et al.*, 1989; Wachi *et al.*, 1987;

Wachi *et al.*, 1991). The spherical phenotype can be caused by mutation of ***mreB,*** as well as by deletion of *mreBCD* (which is not complemented by the *mreB*$^+$ gene alone). Overproduction of MreB causes cells to filament, which was shown to be correlated with a decrease in the amount of PBP3; correspondingly, the amount of PBP3 is increased in the *mreB* mutant (Wachi & Matsuhashi, 1989). MreB was found to be homologous to FtsA (Doi *et al.*, 1988), which led to the suggestion of a possible role for MreB in opposing the function of FtsA and thus favouring cell elongation over septation (Wachi and Matsuhashi, 1989).

The ***dacA*** and ***dacC*** genes coding for D-alanyl:D-alanine carboxypeptidases, when overproduced, make cells grow as spheres (Markiewicz *et al.*, 1982); their overproduction also suppresses the division defect in an *ftsI* mutant (Begg *et al.*, 1990). Both effects were attributed to increased amounts of preferred substrate for PBP3 action, which may suggest a possible role for DacA (PBP5) and DacC (PBP6) in the process of septum formation. Nevertheless, both genes, together with *dacB* coding for a protein (PBP4) with similar enzymatic activity, could be deleted simultaneously without any decrease in cell viability or alteration in morphology (Edwards & Donachie, 1993).

The ***bolA*** gene controls changes in cell shape occurring as the cells enter stationary phase and its overexpression makes cells spherical (Aldea *et al.*, 1988). Transcription of the gene requires the product of *rpoS*: the stationary phase sigma factor σ^s (Aldea *et al.*, 1989; Lange & Hengge-Aronis, 1991). A possible evolutionary reason for this mechanism is maximisation of the number of viable cells per biomass when nutrients have been exhausted (Donachie, 1992).

CHAPTER 2

MATERIALS AND METHODS

2.1. Bacterial strains, phages and plasmids

Bacterial strains used in this study are listed in Table 2.1. Bacteria were either maintained on L-broth plates stored at room temperature, or, for longer-term storage, in tightly closed L-broth stabs sealed with Parafilm.

The only bacteriophage used in this study was P1. Phage lysates were stored at 4°C as broth suspensions to which a few drops of chloroform had been added to prevent microbial growth.

Plasmids used and constructed in the course of this study are listed in Table 2.2.

Table 2.1. Bacterial strains

Strain	Genotype	Source/Reference
AB2497	*thr leu proA his argE lac gal ara xyl mtl thi thy rpsL*	Laboratory stock
TOE13	AB2497 *ftsA13*	K.J. Begg
TC22	AB2497 *ftsA22*	K.J. Begg
JM101	F' *traD36 lacI^q Δ(lacZ)*M15 *proA$^+$B$^+$/supE thi (lac-proAB)*]	Laboratory stock
JM101 *rodA*	As JM101 *rodA^{ts}*. Tet^R	This work
JM109	*e14$^-$Δ(lac-proAB) thi gyrA96 recA1 endA1 hsdR17 relA1 supE44/* F' *traD36 lacI^q Δ(lacZ)M15 proA$^+$B$^+$*	Laboratory stock
KEN90	*ara lac galK galE trp ilv his thy ftsZ84^{ts}*	K.J. Begg
KH201	*ftsW^{ts} leu*::Tn*10 ara^{am} lac125^{am} galK42^{am} galE trp^{am} ilv his thy suIII A81^{ts}*. Tet^R.	Khattar *et al.*, 1994
KJB24	As W3110 *rodA^{am}*. Kan^R	K.J. Begg

Strain	Genotype	Source/Reference
MM38*Δpcn*	*argG6 hisG1 leuB6 metB1 pyrE gal-6 lacY1 xyl-7 bgl*$^{+}$ *fhuA2 T1*^{R} *φ80*^{R} *gyrA rpsl104 tsx-1 TGR uhp ΔpcnB*::Kan^{R}	S. MacAteer
P678	*thr leu thi*	Laboratory stock
P678-54	P678 *minB*	Laboratory stock
SP5211	*his pro purB thi mtl xyl galK lacY rpsL rodA52*^{ts}	B. Spratt
TOE1	AB2497 *ftsQ1*	K.J. Begg
W3110	F- *sup*^{0} λ^{s} λ^{-}	Laboratory stock
W3110 *ddl::Tn5*	*ddl::Tn5* transduced into W3110 from KJB24.	K.J. Begg
XL1-Blue	F' *lacI*^{q} *Δ(lacZ)M15 proA*$^{+}$*B*$^{+}$ *Tn10/Δ(lac-proAB) thi gyrA96 recA1 endA1 hsdR17 relA1 supE44*	Laboratory stock

Table 2.2. Plasmids

Plasmid	Description	Source/reference
pADD5	*Sma*I chromosomal fragment carrying *pbpA*, *rodA* and *rlpA* cloned into the same site of pACYC177. Amp^{R}.	S. Addinall
pADD11	*Kpn*I-*Hin*dIII fragment from pADD5 with *rodA* and *rlpA* cloned into the same sites of pUC19. Amp^{R}.	S. Addinall
pBAD18	ColE1 based replicon. P_{BAD} promoter upstream of polylinker. Amp^{R} transcribed from P_{BAD}. 4.4 kb. Amp^{R}.	Guzman *et al.*, 1995.
pBR322	Amp^{R}, Tet^{R}, pMB1 replicon	Bolivar 1978
PET-3c	pBR322 based replicon. The N-terminal portion of T7 gene 10 is transcribed from the φ10 promoter. 4.4 kb. Amp^{R}.	Rosenberg *et al.*, 1987

Plasmid	Description	Source/reference
pHP45Ω	Contains the Ω cassette on *Sma*I, *Eco*RI, *Bam*HI and *Hin*dIII fragments in a pBR322 background. 4.4 kb. Amp^R Spc^R Str^R.	Prentki and Kirsch 1984
pJBS633	pBR322 derivative with '*blaM* topology probe. Kan^R	Broome-Smith and Spratt, 1986
pJF118HEW	Full length *ftsW* with improved RBS cloned into the expression vector pJF118HE under P_{tac} promoter	M Khattar
pUC18	ColE1 derived replicon (pMB1). P_{lac} promoter region and polylinker. 2.7 kb. Amp^R.	Laboratory stock
pUC19	As pUC18 but with the polylinker reversed.	Laboratory stock
pZ*AQ	pZAQ with 2 base pair insertion at *Bst*BI site within *ftsZ*	This work
pZA*Q	pZAQ with 4 base pair insertion at *Bgl*II site within *ftsA*	This work
pZAQ	Chromosomal fragment carrying *ftsQAZ* cloned into *amp* gene of pBR322. Tet^R	Ward and Lutkenhaus, 1985
pZAQ*	pZAQ with 4 base pair insertion at *Mlu*I site within *ftsQ*	This work
pZH1	*luxAB* genes from *Vibrio harveyi* PCR amplified with mutagenic primers to introduce *Nde*I-containing RBS cloned into *Nde*I-*Ava*I sites of pUC19	This work
pZH2	pUC18 containing *Eco*RI-*Sma*I fragment with *luxAB* genes from *Vibrio harveyi* under control of P_{lac}	This work
pZH3	*Nde*I-*Bam*HI *luxAB* fragment from pZH1 cloned into the same sites of pET-3c to create fusion of gene-10 RBS to the *luxA* gene.	This work

Plasmid	Description	Source/reference
pZH5	P_{ftsZ}::*luxAB* fusion plasmid. *Sph*I-*Eco*RI Ω-P_{ftsZ} fragment from pZlux ligated into *Sph*I-*Xba*I cut pZH3 (*Eco*RI and *Xba*I ends filled in with Klenow enzyme).	This work
pZH6	*Ava*I *ftsZ*'-luxAB fragment subcloned from pZH5 into the same site of pUC18	This work
pZH7	Same as pZH6, but *ftsZ*'-luxAB in the opposite orientation	This work
pZH24	*Nhe*I-*Eco*O109I *rodA* fragment from pADD5 cloned into the *Nhe*I-PvuII sites of pJBS633. *Eco*O109I ends filled in with Klenow enzyme. BlaM fused to 75th amino acid of RodA	This work
pZH25	*Nsp*I-*Ssp*I *rodA* fragment from pADD11 cloned into the *Sph*I-*Pvu*II sites of pJBS633. BlaM fused to 321st amino acid of RodA	This work
pZH28	The complete *rodA* gene with flanking sequences cloned in pJBS633 as an *Nhe*I-*Sal*I fragment	This work
pZH31	pUC18 with *EcoR*I-*Hind*III fragment containing PCR amplified *ftsW* without the first 90 bp of *ftsW* ORF	This work
pZlux	2.3 kb EcoRI fragment containing the promoter region for *ftsZ* fused to *luxAB* genes from *Vibrio harveyi*	S. Dewar

2.2. Growth media and buffers

L-broth and L-agar were used routinely for most bacterial manipulations. For phage P1 2.5 mM $CaCl_2$ was added to the

medium. In certain cases VB minimal agar, supplemented with appropriate carbon sources, vitamins and amino acids, was used.

2.2.1. Growth Media

L-broth	Difco bacto tryptone	10 g
	Difco bacto yeast extract	5 g
	NaCl	5 g
	pH to 7.2 with NaOH	
	Distilled water to 1 litre	
L- agar	L-broth + 15 g Difco agar per litre	
LB top agar	L-broth + 6.5 g Difco agar per litre	
Nutrient broth (NB)	Oxoid No.2 nutrient broth	25 g
	Distilled water to 1 litre	
NB agar	Nutrient Broth + 12.5 g Davis NZ agar	
VB minimal media	20x VB salts	25 ml
	20% carbon source	5 ml
	Supplements as required	
	Distilled water to 1 litre	
VB minimal agar	As VB minimal media + 15 g Difco agar per litre	
20x VB salts	$MgSO_4.7H_2O$	4 g
	Citric acid	40 g
	KH_2PO_4	400 g
	$NaNH_4HPO_4.4H_2O$	70 g
	Distilled water to 1 litre	

SOC broth	Bacto tryptone	4 g.
	Bacto yeast extract	1 g.
	5M NaCl	0.4 ml
	1M $MgCl_2$	2 ml
	1M KCl	0.5 ml
	1M $MgSO_4$	2 ml
	Glucose	0.72 g.
	Distilled water	to 200 ml

2.2.2. Commonly used buffers

TAE buffer

Working solution:

40 mM Tris–acetate

2 mM EDTA

50x Conc. stock solution:

Tris base	242 g
Glacial acetic acid	57.1 ml
0.5 M EDTA (pH 8.0)	100 ml
Distilled water	to 1 litre

TBE buffer

Working solution:

89 mM Tris–borate

89mM boric acid

5x Conc. stock solution:

Tris base	54 g
Boric acid	27.5 g
0.05 M EDTA (pH 8.0)	20 ml
Distilled water	to 1 litre

TE buffer

10 mM Tris–HCl (pH 8.0)

1 mM EDTA (pH 8.0)

Phage buffer	Na_2HPO_4	7 g
	KH_2PO_4	3 g
	NaCl	5 g
	$MgSO_4$ (0.1 M)	10 ml
	$CaCl_2$ (0.1 M)	10 ml
	1% gelatine solution	1 ml
	Distilled water to 1 litre	
Bacterial buffer	KH_2PO_4	3 g
	Na_2HPO_4	7 g
	NaCl	4 g
	$MgSO_4.7H_2O$	2 g
	Distilled water	to 1 litre

2.2.3. Minimal medium supplements

The only minimal medium supplement used in this work was thiamine hydrochloride, which was stored as a 1 mg ml^{-1} water solution and added to minimal medium to final concentration of 2 µg ml^{-1}. In cases when rich minimal medium was required, casamino acids (CAA) were added to the final concentration of 1-5 µg ml^{-1} from 100 µg ml^{-1} stock solution.

2.2.4. Growth of Bacteria

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

2.2.5. Selection of Antibiotic Resistance

The concentrations for the antibiotics used in this work are shown in Table 2.3. All antibiotics were used in both complex and minimal media at the same concentrations.

Table 2.3. Antibiotic Solutions

Antibiotic	Abbreviation	Solvent	Concentration	
			Stock	Final
			(mg ml^{-1})	(µg ml^{-1})
Ampicillin	Amp	H_2O	100	20–100
Kanamycin sulphate	Kan	H_2O	25	25
Naladixic acid	Nal	0.1 M NaOH	20	20
Spectinomycin dihydrochloride	Spc	H_2O	50	25
Streptomycin sulphate	Str	H_2O	100	20
Tetracycline hydrochloride	Tet	50% ethanol	10	5
Rifampicin	Rif	Ethanol	50	50

2.3. Bacterial and phage techniques

2.3.1. Preparation of competent cells and transformation with plasmid DNA

To prepare competent *E. coli* cells the method of Chung *et al.* (1989) was employed. Five ml of L-broth was inoculated with a single colony and incubated overnight with shaking at a suitable temperature. This culture was diluted 1 in 50 into fresh L-broth and grown with good aeration to an OD_{600} of between 0.3 and 0.4. The culture was chilled on ice, transferred to a universal bottle and

centrifuged at 5000 rpm for 10–15 min. The supernatant was removed and the bacterial pellet resuspended in 0.1x the original volume of ice-cold TSS buffer. At this point the cells could be frozen at –70°C, or could be used immediately for transformation. Freshly prepared cells always gave the highest transformation efficiency. The plasmid DNA (typically 1–100 ng in <10 µl) was added to 0.1 ml aliquots of the competent cells, mixed gently and stored on ice for 15–30 min. After this time between 1 ml of L-broth + 20 mM glucose was added and the cells incubated with aeration at an appropriate temperature for 1 h to allow expression of plasmid antibiotic-resistance genes. Two hundred microlitres of this mixture was then spread onto antibiotic-containing plates and incubated until bacterial colonies appeared.

TSS buffer:

Difco Bacto Tryptone	10 g
Difco Yeast Extract	5 g
NaCl	10 g
PEG 3350	100 g
$MgSO_4$	20 mM
DMSO	50 ml
PIPES Buffer pH 6.5	10 mM
distilled water	to 1 litre

2.3.2. Preparation of cells for electro-transformation.

1 litre of L broth was inoculated with 1 ml of a fresh o/n culture and incubated with good aeration until the OD_{600} reached 0.4. The culture was chilled on ice for 15-30 minutes. The cells were pelleted in 250 ml GSA centrifuge bottles at 10,000 rpm for 5 minutes. The supernatant was discarded and the pellets resuspended in 250 ml of chilled sterile water and repelleted as before. This process was repeated using decreasing volumes of chilled sterile water (125 and

50 millilitres). Then the pellets were resuspending in 20 millilitres of chilled 50% glycerol in distilled H_2O, (v/v) and spun at 10,000 rpm for 5 minutes in a Sorvall SS34 rotor chilled to 4°C. The supernatant is discarded and the pellet resuspended in 1 ml of chilled 50% glycerol. The final volume of the suspension was adjusted to give an OD_{600} of 250-300 (1:100 dilution of this suspension was used for OD measurement). These cells were used immediately or aliquots of 50 microlitres in Eppendorf tubes were snap frozen in a dry ice/ethanol bath and stored at -70°C for later use.

2.3.3. Electro-transformation.

The DNA to be used for electro-transformation needed to be salt free to allow for application of high electric field strength. Therefore, ligation reactions were ethanol precipitated, the pellet washed twice with 500 µl of 70% ethanol and resuspended in 10 µl of distilled water. 1-3 µl of this DNA water solution was mixed with 20 µl of electro competent cells (thawed on ice) in chilled Eppendorf tubes and left on ice for 5 min. The mixture was then transferred to chilled 1 mm electroporation cuvettes and subjected to electric pulse in GenePulser apparatus BioRad set to 25 mF, 1.7 kV and shunt resistor 200 Ohms to deliver a 17 kV/cm pulse with time constant of about 4 milliseconds. Immediately after the pulse, 1 ml of SOC broth was added to the cuvette, the cells transferred to a flask and incubated for 1 hour with gentle aeration at a suitable temperature to allow for the expression of antibiotic markers. After this aliquots of the suspension were spread on selective plates and incubated 20-40 hours.

2.3.4. Preparation of phage P1 lysates

Cells were grown in L-broth + 2.5 mM $CaCl_2$ until late log phase. 1 ml of the culture was then mixed with 10^6 phage, incubated

at 37°C for 30 min, and 3 ml of L-top agar containing 20 mM $MgSO_4$. This was poured onto a L-agar plate, left to set and incubated at 37°C overnight or until visible lysis occurred. The layer of top agar was scraped off into a sterile universal bottle. Equal volume of L-broth + 2.5 mM $CaCl_2$ and 100 µl of chloroform were added to the bottle. The bottle was vortexed vigorously for 1 min, centrifuged 5 min at 5,000 rpm. The supernatant was transferred to a fresh bottle and a few drops of chloroform were added. The lysate was stored at 4°C.

2.3.5. Phage P1-mediated transduction

The recipient strain was grown up to late-log phase in L-broth. The cells were harvested by centrifugation and the bacterial pellet resuspended in 0.1x the original volume of L-broth + 2.5 mM $CaCl_2$. 100 µl aliquots of the concentrated culture were mixed with either 100 or 10 µl of phage P1 lysate. These were incubated at 37°C for 30 min. 1 ml of phage buffer was added, cells concentrated tenfold and plated onto selective plates. When expression of a selective marker (e.g. antibiotic resistance) was necessary, 1 ml of L-broth + 10 mM sodium citrate was used instead of sodium citrate and cells were incubated for 1 hour prior to plating. The plates were incubated for 1-2 days at an appropriate temperature.

2.3.6. Determining the minimal inhibitory concentration of ampicillin

Ampicillin resistance of *E. coli* cells harbouring hybrid plasmids expressing β-lactamase was determined as follows. Overnight cultures were diluted in bacterial buffer to 10-20 cells per µl concentration. 2 µl drops of these dilutions were placed on L-agar plates with doubling concentrations of ampicillin starting with 1 µg

ml^{-1} of antibiotic. The minimal antibiotic concentration sufficient to inhibit the formation of colonies after 24-30 hours of growth at 37°C was taken as the measure of ampicillin resistance.

2.4. Luciferase imaging set-up

The Hamamatsu Argus 50 system was used for all measurements of bacterial luciferase activity and observation of luciferase expression in single cells. The system consisted of highly sensitive CCD camera (Hamamatsu C2400-77 type 01) connected to an image analyser and a computer. The camera was mounted on top of a black box where all samples were placed.

Luciferase reaction requires oxygen and reduced FMN in addition to substrate, an aliphatic aldehyde; therefore log-phase cultures grown with good aeration were always used for luciferase assays.

To measure luciferase activity in bacterial populations, 100 µl of bacterial culture was placed in a 400 µl well of a black microtiter plate. 3 µl of luciferase substrate (nonyl aldehyde) was added as a 1% ethanol solution. The plate was placed 10-30 cm from the camera lens and light emission was immediately measured for several seconds. In these conditions light emission was fairly stable for about 30 seconds after addition of the substrate.

Observation of luciferase expression in single cells was performed by plating a small number of exponentially growing cells onto a 2 mm thick agar slide (with appropriate medium) so that about 20-30 cells appeared in the field covered by the camera lens. The slide was placed into a petri dish sealed with Parafilm, with a drop of nonyl aldehyde. In such conditions, the cells are expected to have saturating amounts of both the luciferase substrate, which is

very volatile, and oxygen. The plate was then placed under the camera in the black box and incubated further at room temperature. The camera was equipped with an objective and suitable extension rings to bring the lens as close to the sample as possible (about 7 mm). The image analyser was set up in the photon counting mode and images were sequentially taken, with photons continuously accumulating for some time.

For quantitation of luciferase output, the image analysis programme included with the Argus system was used.

2.5. DNA Techniques

Most DNA manipulations were performed according to (Sambrook *et al.*, 1989) with minor modifications or according to instructions of manufacturers of certain reagents/kits.

2.5.1. Plasmid preparation

All preparations of plasmid DNA were performed using a modification of the alkaline lysis method of Birnboim and Doly (1979). When possible, DNA was prepared from an *endA* strain, typically XL1-Blue, to avoid DNA degradation by the EndA endonuclease.

For **large scale plasmid preparations** 250 to 500 millilitres of L-broth (plus suitable antibiotic) were inoculated with a single colony of the plasmid-bearing strain, and incubated overnight with continuous shaking at the appropriate temperature (typically 37°C). The culture was chilled on ice for 15 minutes and cells were collected by centrifugation at 6,000 rpm in a GSA or GS-3 rotor for 10 minutes at 4°C. The supernatant was discarded, and the bacterial pellet resuspended in 20 ml of 25mM Tris-HCl/50mM EDTA pH 8.0. To this cell suspension 40 ml of 0.2M NaOH/1% SDS were added, mixed

by gentle swirling of the tube and incubated on ice for 5 min. 30 ml of 5M K acetate (pH 4.8) was then added, the solution mixed gently by swirling and left on ice for a further 5 min. After the formation of a heavy flocculent precipitate the mixture was centrifuged in a GS3 rotor for 15 minutes at 10,000 rpm to pellet the precipitated chromosomal DNA and insoluble cellular debris. The supernatant was transferred to clean GS3 bottles and 0.6 volumes of isopropanol were added. The mixture was incubated on ice for at least 10 minutes and then centrifuged at 10,000 rpm for 15 minutes. The pellet was resuspended in 5 ml of TE buffer in 30 ml glass tubes, 2.4 ml of 8M ammonium acetate were added. After 20 minutes on ice the mixture was centrifuged for 15 min at 10,000 rpm in an SS-34 rotor, the supernatant was transferred to clean 30 ml glass tubes and 15 ml of ice cold ethanol was added. The mixture was left on ice for at least 10 minutes and centrifuged for 15 min at 10,000 rpm. The pellet was dissolved in 750 µl TE and transferred to an Eppendorf tube. 5 µl of DNAse free RNAse A solution (10 mg ml^{-1}) were added. The mixture was incubated at 37°C for 30 min. Then phenol/chloroform extraction was performed as follows. An equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) was added, the suspension was mixed vigorously by inverting the tube or by vortexing until homogeneous, and centrifuged in a microcentrifuge at 10,000 rpm for 3 minutes, after which the aqueous phase was carefully transferred to a clean Eppendorf tube. The procedure was repeated until the interface between the phenol and aqueous phases appeared clean. Then 1/10 volume of 3M sodium acetate and two volumes of ethanol were added, DNA was precipitated by centrifugation and washed with 70% ethanol. The final DNA pellet was resuspended in 500 ml of TE. The concentration of DNA was measured by absorbance at 260 nm.

This procedure usually resulted in clean DNA suitable for all applications including sequencing.

For small-scale preparations the above procedure was scaled down so that all stages could fit in Eppendorf tubes and all incubation times were reduced. Typically, 3 ml of an overnight culture of a plasmid bearing strain were spun down and resuspended in 200 µl of 25mM Tris-HCl/50mM EDTA pH 8.0. 400 µl of 0.2M NaOH/1% SDS were added and mixed by inverting the Eppendorf tube 3-4 times. 300 µl of ice cold 5M potassium acetate were added immediately or as soon as cell lysis was obvious. The preparation was mixed again by inverting the tube 3-4 times and centrifuged at maximal speed in a microcentrifuge at 4°C for 10 min. 600 µl of ice-cold isopropanol were added to the supernatant in a clean tube, mixed, and the tube centrifuged in a microcentrifuge at 4°C for 10 min. The pellet was resuspended in 100 µl of TE, 50 µl of 8M ammonium acetate was added, mixed and the mixture left for 5-10 minutes on ice before the next centrifugation. 300 µl of ethanol were added to the supernatant, mixed and centrifugation repeated. The resulting pellet was washed with 70% ethanol and resuspended in 50 µl of TE. The resulting DNA preparation was clean enough for restriction by most endonucleases. Sometimes, larger volumes of culture were taken at the first stage (10 to 25 ml). RNAse treatment and phenol extraction were usually necessary in that case.

2.5.2. Phenol extraction and ethanol precipitation of DNA

Phenol extraction followed by ethanol precipitation was the standard method of purification of crude DNA preparations and inactivation of enzymes after reactions.

An equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) was added to DNA solution, the suspension was mixed vigorously by

inverting the tube or by vortexing until homogeneity, and centrifuged in a microcentrifuge at 10,000 rpm for 1 minute. The aqueous phase was carefully transferred to a clean Eppendorf tube. If a white precipitate was present at the aqueous/organic interface, the procedure was repeated (several times if necessary).

DNA was routinely precipitated from aqueous solution by adding 1/10 volume of 3M sodium acetate (pH5.5) and 3 volumes of absolute ethanol, mixing thoroughly and leaving on ice for a minimum of 10 min (usually longer). This was then centrifuged in a microcentrifuge at maximal speed for at least 10 min. The supernatant was discarded, the pellet washed in an equal volume of 70% ethanol, recentrifuged as above for 5 min and the supernatant again discarded. Remaining drops of water were removed by aspiration and the pellet was left to dry on a bench for a couple of minutes. The dried DNA pellet could then be resuspended in a suitable volume of TE buffer (for storage and further manipulations) or water (for electroporation). Sometimes, to keep the total volume small, 0.6 volume of isopropanol was used instead of ethanol. After isopropanol precipitation and centrifugation, the pellet was washed with 70% ethanol as described above.

2.5.3. Determination of DNA concentrations

DNA concentrations were determined by measuring the absorption of appropriately diluted (usually 1:500) solutions at 260 nm, as an OD_{260} value of 1.0 corresponds to double-stranded DNA concentration of 50 mg ml^{-1}. DNA purity was determined by measuring the ratio of absorption at 260 and 280 nm. Pure DNA should have a 260/280 ratio of at least 1.8.

2.5.4. Restriction of DNA

Endonuclease reactions were typically performed in volumes of 20 µl for cutting up to 1-2 µg of DNA or in volumes of up to 200 µl for

larger amounts of DNA, especially of the chromosomal DNA because of its usually low concentration and purity. The reactions contained the appropriate Boehringer Mannheim or New England Biolabs restriction buffer at 1x concentration. BSA was added (to 1 mg ml^{-1}) if recommended by the manufacturer of an enzyme. The restriction enzyme was usually present in a two- to fivefold excess. The digests were made up to their final volume using distilled water. The complete restriction mixtures were incubated at the temperature recommended for a particular enzyme for 1–3 h. The products of the reaction were either directly analysed by agarose gel electrophoresis, or phenol extracted, ethanol precipitated and dissolved in a suitable volume of TE buffer for further manipulations.

2.5.5. Ligation of DNA

DNA ligation was usually performed in a final volume of 10-20 µl. These contained between 0.5–1 µg of total DNA with insert DNA in a 2- to 20-fold molar excess over the vector DNA, 1x Boehringer Mannheim ligation buffer and T4 DNA ligase. 0.2 unit of ligase was used for the ligation of cohesive DNA termini, and 1 unit of the enzyme for the ligation of blunt-ended molecules. The reactions were incubated for at least 30 min at room temperature for cohesive termini or at least 8 h at 16°C for blunt end ligation. Between 2 and 5 µl of the reaction mixture was directly used to transform competent cells of an appropriate strain of *E. coli*. For electroporation, ligated DNA was ethanol precipitated, washed twice with 70% ethanol and dissolved in water to achieve a salt-free solution.

2.5.6. 'Filling in' of recessed 3' termini

The Klenow fragment of DNA polymerase I (Klenow enzyme) was used to give blunt-ended DNA molecules by filling in the recessed 3' termini generated by various restriction enzymes. Reactions were

performed in a final volume of 20 µl containing up to 1 µg DNA, 1x Klenow buffer, all four dNTPs each at a concentration of 20 mM and 2 units of Klenow enzyme. Alternatively, dNTPs and Klenow enzyme were sometimes added directly to the restriction reaction mix after heat inactivation of endonucleases, as Klenow enzyme works well in most restriction buffers. The reactions were incubated at room temperature for 30 min. The reactions were stopped and the unincorporated nucleotides removed by increasing the reaction volume to 100 µl with TE, phenol extracting and ethanol precipitating the DNA.

2.5.7. Generation of deletions with *Bal*31 nuclease.

Nested deletion series in plasmids were created by treatment with *Bal*31 nuclease. This nuclease rapidly degrades double stranded linear DNA from both 5' and 3' ends, hence the plasmid to be digested was linearised with an appropriate enzyme prior to *Bal*31 digestion. Reactions were performed in the *Bal*31 buffer at 30°C for 1 to 30 minutes. First, a minimal concentration of the nuclease resulting in visible degradation of the plasmid was determined, starting with 5 U of the enzyme per 1 µg of DNA. Then half of that concentration was used to perform a time course with samples removed from the reaction every minute and the reaction stopped by the addition of 0.1 volume of 0.5 M EGTA (a specific chelator of Ca^{2+}). Products of the reaction were analysed by agarose gel electrophoresis, and samples with deletions of the required sizes were used further. As *Bal*31 gives a mixture of blunt and staggered ends, DNA was phenol extracted, ethanol precipitated and treated with Klenow enzyme to repair the DNA ends prior to recircularisation with T4 DNA ligase.

*Bal*31 *buffer:*

50 mM Tris-HCl, pH 7.5

10 mM $CaCl_2$

10 mM $MgCl_2$

600 mM NaCl

50 μg ml^{-1} BSA

Distilled water to 50 μl.

2.5.8. Agarose gel electrophoresis

Agarose gels for electrophoretic analysis of DNA were prepared with TAE buffer. Agarose concentration varied according to the sizes of expected DNA fragments. 0.8% agarose was used for separation of all fragments larger than 700 bp, 1.5% agarose was used for 300-700 bp fragments and 3% agarose for fragments smaller than 300 bp. Several types of electrophoretic equipment from Hybaid, Bio-Rad and BRL were used. Most of these were of "minigel" type with gel sizes 5 x 7.5 to 7 x 10 cm, allowing for rapid visualisation of results and simultaneous checking of 8 to 36 samples, depending on the comb size. Samples containing 1x loading dye (6x stock is 0.25% bromophenol blue, 0.25% xylene cyanol and 40% (w/v) sucrose in H_20) were always loaded after immersion of the gels in TAE buffer with 0.5 μg ml^{-1} of ethidium bromide. For rapid fragment separation the gels were run at 80-100 mA at room temperature or at up to 150 mA at 4°C. These conditions usually led to good fragment separation in less than an hour. For better separation, especially with larger fragments and higher agarose concentration, the gels were run at 20-40 mA for several hours and sometimes overnight at 10-15 mA. After the run the DNA fragments were visualised by UV illumination.

2.5.9. Isolation of DNA from agarose gel slices

DNA fragments within the size range 500bp-15kb were isolated from agarose gels using Geneclean II kit from BIO 101. The procedure is based on a specific DNA binding to silica matrix (Glassmilk) at high salt concentration and its further release in low salt solution. The appropriate DNA bands were excised from ethidium bromide stained agarose gels under UV light in the minimal gel volume possible. The gel piece was placed in an Eppendorf tube, weighted and three volumes of 6 M NaI solution added. The tube was incubated at 50°C with periodic mixing until the agarose was dissolved (usually 2-5 minutes). 5 µl of Glassmilk was added, mixed and the tube stored on ice for at least 5 minutes with periodic mixing to keep the Glassmilk suspended. Silica matrix with bound DNA was pelleted by brief (5 seconds) centrifugation. The supernatant was discarded and the pellet washed three times with 400 µl of New Wash (an ethanol based buffer supplied with the kit), centrifuging and resuspending the pellet each time. The washed pellet was resuspended in 5 µl of TE buffer and incubated at 50°C for 2-3 minutes. The mixture was centrifuged for 30 seconds and DNA containing supernatant collected. The elution step was sometimes repeated to extract the DNA still bound to the matrix after the first elution.

2.5.10. Small scale preparation of chromosomal DNA

1.5 ml of an overnight culture of an appropriate strain (incubated with vigorous shaking) was spun down in a microcentrifuge. The pellet was resuspended in 567 µl of TE buffer. 30 µl of 10% SDS and 3 µl of proteinase K solution (20 mg ml^{-1}) were added, mixed thoroughly and the cells were left to lyse for 1 hour at 37°C. 100 µl of 5M NaCl were added, mixed thoroughly. Then 80 µl of

hexadecyltrimethyl ammonium bromide (CTAB)/NaCl solution was added mixed thoroughly and the tube was incubated at 65°C for 10 minutes. An equal volume of chloroform/isoamyl alcohol (24:1) was added, mixed thoroughly and spun in a microcentrifuge for 5 minutes. The aqueous phase was transferred to a clean tube and phenol extracted once. DNA was then precipitated by the addition of 0.6 volume of isopropanol, washed with 70% ethanol and redissolved in 100 µl of TE buffer. 10-20 µl of this preparation was usually sufficient for a restriction digest.

CTAB/NaCl solution:

4.1 g NaCl was dissolved in 80 ml of H_2O and 10 grams of CTAB slowly added while stirring. If necessary, the mixture was heated to 65°C to dissolve. The final volume was adjusted to 100 millilitres.

2.5.11. DNA sequencing techniques

DNA sequencing was performed using the Pharmacia T7 Sequencing Kit. The kit is based on the chain-terminating dideoxynucleotide sequencing method developed by Sanger *et al.* (1977) and utilises T7 DNA polymerase that yields longer chain termination fragments than the Klenow enzyme used in the original procedure.

Double stranded templates were used in all reactions.

Annealing of primer to double stranded template

Concentration of the template to be sequenced was adjusted so that 32 µl contain 2 µg (for a plasmid of pUC19 size, for larger plasmids the amount of DNA was increased). 8 µl of 2 M NaOH were added to 32 µl of the template solution and mixed thoroughly. The tube was incubated at room temperature for 10 minutes. 7 µl of 3 M sodium acetate (pH 4.8) and 4 µl of water were added followed by 120

µl of 100% ethanol. The mixture was placed on dry ice for 15 minutes and centrifuged for 10 minutes. The pellet was washed with ice-cold 70% ethanol, dried by aspiration and redissolved in 10 µl of distilled water. 5-10 pmol of an appropriate primer was added in a volume of 2 µl followed by 2 µl of annealing buffer. The mixture was incubated at 65°C for 5 minutes, transferred to 37°C, incubated for 10 minutes and then left at room temperature for at least 5 minutes.

Sequencing reactions

Because of the high reaction rates the primer extension reactions are performed in two stages, a labelling reaction and a termination reaction. Dideoxynucleotides are excluded from the first stage of the reaction, then added for the second.

Four wells of a microtiter plate were labelled 'A', 'C', 'G' and 'T' and 2.5 µl of the corresponding dideoxynucleotide mix added to each well. 3 µl of the Labelling mix-dATP, 1 µl of [α-^{35}S]dATP, and 2 µl of diluted (1:5) T7 DNA polymerase were added to the tube containing the annealed template and primer. The tube was incubated at room temperature for 5 minutes. During this incubation, the four sequencing mixes were warmed in a water bath at 37°C. 4.5 µl of the labelling reaction was added to each of the four sequencing mixes and these were incubated at 37°C for 5 minutes. The reactions were stopped by adding 5 µl of stop solution to each well.

Prior to electrophoresis the samples were heated at 80°C for 2 min to denature the DNA and 2.5-3 µl of each sample was immediately loaded onto the gel.

DNA sequencing gel electrophoresis

A 30 x 40 cm BRL sequencing apparatus was used for DNA sequencing. The glass sequencing gel plates were thoroughly cleaned with detergent and ethanol, assembled using 0.2 mm spacers and taped together to minimise the possibility of leakage.

The gel was prepared by adding together the following:

40 % (w/v) acrylamide solution*	15 ml
urea	43 g
water	35 ml
10x TBE	10 ml
ammonium persulphate 10% w/v	1 ml
TEMED	35 µl

*19:1 mix of acrylamide and N,N`-methylenebisacrylamide

This solution was poured between the sequencing plates. The flat edge of a 60-well shark-tooth comb was pushed between the plates to layer the top of the gel. Clingfilm was wrapped round the exposed areas of the gel and each edge of the gel was clamped with bulldog clips. After polymerisation the tape and comb were removed and unpolymerised acrylamide from the top of the gel was washed with distilled water. The comb was then replaced with the points downwards just touching the surface of the gel. The gel was then clamped into the sequencing apparatus and 1x TBE solution poured into the top and bottom reservoirs. The gel was then pre-run at about 50 W (~1500 V) for 1 h and then loaded with the sequencing reactions. The gel was then electrophoresed at 50 W until the blue dye-front ran off the end of the gel. Once electrophoresis was complete the top plate was removed and a sheet of wet blotting paper was placed on top of the gel followed by a dry blotting paper sheet. Even pressure was applied, dry paper removed and wet paper carefully peeled off the glass plate taking the gel with it. The gel was covered with SaranWrap and dried in a vacuum gel-drier for 1 h at 80°C. The gel was then placed in an autoradiography cassette and allowed to develop at room temperature for 24 h.

2.5.12. Amplification of DNA by polymerase chain reaction

Polymerase chain reaction (PCR) was used for amplifying fragments of known DNA. Plasmid or chromosomal DNA was used as template. Oligonucleotide primers were obtained from various commercial sources. Vent DNA polymerase from New England Biolabs was used when higher fidelity of reaction was required, otherwise *Taq* polymerase from Promega was used. Buffer supplied by the manufacturer was used with each polymerase. Magnesium was usually present in Taq buffer. When using Vent polymerase, magnesium chloride was added to concentration of 2 mM. BSA was only added when amplifying chromosomal templates from crude samples. The usual reaction mix was as follows:

Polymerase buffer (10x)	10 µl
BSA (10 mg/ml)	10 µl
dNTP mix (5 mM each)	4 µl
Oligonucleotide primers	20 pmol each
Thermostable DNA polymerase	2 units
distilled water	to 100 µl

Reactions were performed in a Hybaid™ thermal cycler in a volume of 20 to 50 µl with 20 µl of mineral oil on top of the reaction mix if a heated lid was not used. Typical cycling parameters were denaturing at 94°C (95°C for Vent polymerase) for 45 sec., annealing for 45 sec. at the appropriate temperature for the primer-template pairs (usually 50-55°C) and polymerisation at 72°C (75°C for Vent polymerase) for 1 minute (plus 1 minute for every kb of template above 1,000 bases).

PCR was sometimes used to screen potential recombinants after cloning for the presence of the correct insert. This was done when the primers flanking the site of insertion were available. *Taq* polymerase and buffer with BSA were used in this case. A small part of a single

bacterial colony was added to the complete reaction mixture with a 200 µl pipette tip. The heating of the reaction mix appeared sufficient to lyse enough cells to consistently provide sufficient amounts of template for a successful reaction.

2.6. Protein Techniques

2.6.1. Polyacrylamide gel electrophoresis of proteins

E. coli proteins were routinely separated using SDS–polyacrylamide gel electrophoresis with a discontinuous buffer system (Laemmli 1970). In virtually all cases a 10% resolving gel and 4% stacking gel were employed. A mini–gel apparatus (SE 250) manufactured by Hoeffer Scientific Instruments was used. This used 10 x 5 cm gels, which could be electrophoresed in about 45 min. The composition of typical gel solutions was as follows:

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 (freshly prepared)	0.25 ml
distilled water	12 ml
TEMED	15 µl
Total	25 ml

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 (freshly prepared)	0.1 ml
distilled water	6.3 ml
TEMED	10 µl
Total	10 ml

These solutions were made up immediately prior to use, with the ammonium persulphate solution and the TEMED being added last. The resolving gel solution was pipetted between the glass plates separated by 0.75 mm spacers. The depth of the stacking gel between the bottom of the comb and the resolving gel was about 1 cm. Once the resolving gel had been poured it was layered with isobutanol saturated with 1x resolving-gel buffer and allowed to polymerise for 1 h. The isobutanol was then discarded and the top of the gel was washed with distilled water. The stacking-gel solution was poured on top of the resolving gel, the comb inserted and polymerisation allowed to occur. The comb was then removed and the wells washed out with 1x reservoir buffer, which was also used to fill up the buffer chambers of the apparatus. The sample could be loaded onto the gel at this stage.

Samples (typically whole-cell extracts, grown to late log-phase in L-broth, centrifuged and resuspended in a sixth of the original volume of distilled water) were mixed 1:1 with 2x PAGE-loading buffer, boiled for 3 min (or kept at 37°C for 1 hour if hydrophobic membrane proteins were to be analysed), and centrifuged in a microcentrifuge for 5 min prior to loading. About 2–5 µl of sample per lane could be loaded onto the mini–gel. Samples were loaded using a 10 µl Hamilton syringe. Gels were typically electrophoresed at a constant current of 40 mA until the bromophenol blue dye-front had run off the bottom of the gel. Once electrophoresis was complete the glass plates were removed from the apparatus, separated carefully using a plastic wedge and the gel placed in staining solution for 45–60 min with constant gentle agitation. Gels were the transferred into destaining solution and left for anything between 2–24 h. For preservation, the stained/destained gel was soaked in destaining

solution plus 5% glycerol for 30 min and dried down on blotting paper using a vacuum gel drier at 80°C for about 1 h.

Solutions used in SDS–PAGE.

4x stacking-gel buffer (0.5 M Tris)

15.25 g of Tris base, dissolved in 200 ml distilled water, adjusted to pH 6.8 with concentrated HCl, made up to 250 ml, filtered and autoclaved.

4x resolving-gel buffer (1.5 M Tris)

45.5 g of Tris base, dissolved in 200 ml distilled water, adjusted to pH 8.8 with concentrated HCl, made up to 250 ml, filtered and autoclaved.

10x reservoir buffer

30.2 g of Tris base, 144 g of glycine dissolved in 600 ml distilled water, made up to a final volume of 1 litre and filtered. SDS was added to 0.1% in the final 1x buffer.

2x PAGE loading buffer

4x stacking gel buffer		0.125 ml
10% SDS		0.300 ml
50% glycerol		0.200 ml
2–mercaptoethanol		0.050 ml
0.1% bromophenol blue		0.200 ml
Distilled water		0.125 ml
	Total	1.0 ml

Stock acrylamide

37 g acrylamide, 1 g NN' methylene bis-acrylamide, made up to 100 ml with distilled water, filtered and stored at 4°C.

Staining solution

9% (v/v) acetic acid, 45% (v/v) methanol, and 0.1% (w/v) Coomassie brilliant blue.

Destaining solution

7% (v/v) acetic acid, and 5% (v/v) methanol.

2.6.2. Western blotting procedures

Western blotting was performed using a BioRad electrophoretic transfer cell. The previously run gel was trimmed down by removing the stacking gel and placed on top of several sheets of blotting paper saturated in protein-transfer buffer (which is 1x protein gel reservoir buffer but without any SDS). A sheet of nitrocellulose saturated in the same buffer was placed on top of the gel excluding any bubbles followed by several more sheets of saturated blotting paper. The whole sandwich was secured in the transfer apparatus and transfer was allowed to continue at 1 amp for 1 h.

After this, non-specific binding sites were blocked by immersing the membrane in 5% blocking reagent (skimmed milk) in PBS-T buffer for one hour. The membrane was briefly rinsed twice with fresh changes of PBS-T, then washed three times - once for 15 minutes and twice for 5 minutes with fresh changes of washing buffer. All washing steps, as well as incubations with antibodies were performed at room temperature with gentle agitation. At least 200 ml of the buffer was used for each washing step.

The membrane was incubated in the appropriately diluted primary antibody for 1 hour. Dilutions ranged from 1:500 to 1:5000.

The incubation was performed in square plastic dishes with 5 ml of antibody dilution. The washing step described above was repeated and the membrane was incubated in the secondary antibody (horseradish peroxidase conjugated anti-rabbit or anti-mouse Ig) diluted 1:1000 for 1 hour. The filter was then removed and washed in PBS-T as before.

The blot was removed and treated with 1:1 mixture of Amersham "ECL" detection reagents, with just enough liquid to cover the blot, for 1 min. Then the blot, excess liquid removed, was wrapped in SaranWrap and quickly exposed to a film. A series of exposures was performed, usually 15, 30, 60 and 120 seconds.

When more than two proteins were to be detected on the same blot, two approaches were used. If the proteins were giving good signals with low background, the blot was treated with antibodies to both proteins simultaneously (this could also involve mixing two secondary antibodies at the next incubation stage). This was the case for FtsZ and GroEL antibodies and resulted in good specific detection of both proteins with practically no background. FtsA, being a much less abundant protein, was detected separately, then the blot was washed thoroughly in PBS-T and reprobed with antibodies to FtsZ and GroEL.

Antibodies used in this work were polyclonal anti-BlaM antibody (5 prime->3 prime), polyclonal anti-GroEL (StressGen product no. SPA-804), F168-12 monoclonal anti-FtsZ from N. Nanninga and polyclonal anti-FtsA from M. Vicente.

Quantitative results were obtained by comparing sample band intensities on the scanned blot images with twofold dilutions of a standard (the sample with the highest concentration of protein) by using the NIH image program (http://rsb.info.nih.gov/nih-image). Correction for loading errors was made by normalising results to the amounts of GroEL

Phosphate buffered saline - Tween (PBS-T) pH 7.5

di-sodium hydrogen orthophosphate anhydrous	11.5 g
sodium dihydrogen orthophosphate	2.96 g
sodium chloride	5.84 g
Tween 20	0.1% (v/v)
distilled water	to 1 litre

2.7. Sequence analysis.

Most sequences were obtained from the EMBL database. Sequences from the ongoing genome projects were downloaded from the corresponding WWW sites. Sequence similarity searches were performed with BLAST2 programmes (Altschul *et al.*, 1997) using network server at NCBI, BLAST servers of the University of Oklahoma and Sanger Centre or FASTA programmes (Pearson, 1990) for some downloaded incomplete genome sequences. The ClustalX programme (an improved version of ClustalW, (Thompson *et al.*, 1994)) was used for multiple sequence alignment and MacBoxshade for shading the alignments. Hydrophobicity profiles were built with the TopPred II programme (Claros & von Heijne, 1994) utilising Kyte-Doolittle (Kyte & Doolittle, 1982) and GES (Engelman *et al.*, 1986) hydrophobicity scales. Topology predictions were made utilising PredictProtein (Rost *et al.*, 1995; Rost *et al.*, 1994) and TMAP (Persson & Argos, 1994) programmes. Sequence similarity plots were made with the PlotSimilarity programme from the GCG package.

CHAPTER 3

USE OF BACTERIAL LUCIFERASE FOR *IN VIVO* MONITORING *FTSZ* PROMOTER ACTIVITY IN SINGLE CELLS

3.1. Introduction

In a few reported cases when the periodicity of transcription of cell cycle genes was addressed, synchronised cell populations of one or another kind were used. Unfortunately, the very procedure of synchronising the cells inevitably disturbs their metabolism, and this by itself can cause certain cell processes to oscillate with periodicity typical of cell cycle events (Donachie & Masters, 1966). Development of *in vivo* reporter systems taking advantage of using bacterial luciferase with extremely sensitive low light measuring equipment, created for the first time in bacterial genetics the possibility of following *in vivo* gene expression in a single cell. It should be mentioned, however, that this possibility, although declared some time ago, was never reported to be used in a real, measured experiment.

As the ability to measure gene expression from a single cell could be very helpful in studying the cell cycle, the original aim of this project was to apply the bacterial luciferase reporter system to studying cell division gene expression during the cell cycle. The *ftsZ* gene was chosen for initial testing for the following reasons.

- It is one of the most important and best studied cell division genes.

- This is probably the cell division gene with the highest efficiency of transcription, so reporter expression might be expected to be high.

- It was reported that transcription of *ftsZ* oscillates during the cell cycle (Garrido *et al.*, 1993).

3.2. Pilot experiments with the luciferase reporter

Preliminary results obtained in this laboratory (Sue Dewar) have shown that single *E.coli* cells are capable of producing an amount of light sufficient to be detected by a Hamamatsu photon counting camera, if the luciferase reporter is transcribed from a multicopy plasmid by phage T7 RNA polymerase. Therefore initial constructs involving part of the *ftsZ* promoter region driving the expression of *Vibrio harveyi luxAB* genes were also placed onto a multicopy plasmid (a pBR322 derivative).

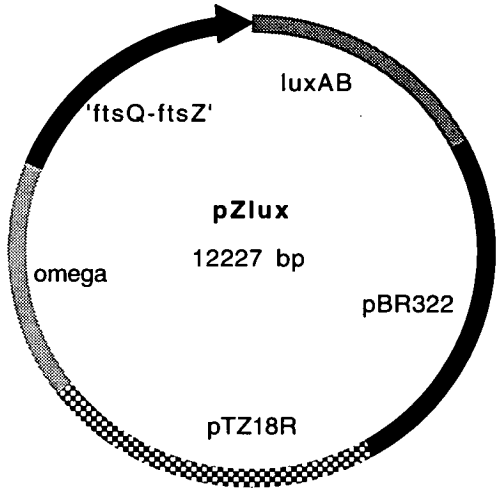


Figure 3.1. *The pZlux plasmid.*

The *luxAB* reporter plasmid used in the first experiments, pZlux, constructed by Sue Dewar in this lab, has a 2.3 kb *Eco*RI fragment containing the promoter region for *ftsZ* fused to *luxAB* genes from *Vibrio harveyi* (Figure 3.1.). The omega interposon introducing strong transcriptional terminators is present just in front of the promoter region. The plasmid is a derivative of pBR322 with a relatively high copy number. The promoter region contains the two proximal *ftsZ* promoters inside the *ftsA* gene as well as the one within *ftsQ*. This

region was thought at that time to be responsible for about 90% of *ftsZ* transcription (Garrido *et al.*, 1993). The plasmid produced enough luciferase for the colonies to be visible in a dark place after the addition of luciferase substrate (nonyl aldehyde).

As a control *luxAB* genes were cloned into pUC18 plasmid as an *Eco*RI-*Sma*I fragment, giving pZH2. This placed the luciferase genes under the cell division independent *lac* promoter.

Several important questions needed to be addressed to decide if the bacterial luciferase is a suitable reporter for the planned experiments. First, it was essential to know how stable the luciferase is. Cultures of JM101 cells with pZH2 were grown to log phase at room temperature with IPTG induction, then split in halves and rifampicin was added to one half to stop all protein translation and accumulation of the reporter protein. Then light emission was measured from small aliquots (50 µl) of the cultures placed into black microtiter plates at 10 minute intervals. Light emission by the rifampicin treated culture decreased by 50% only after about 100 minutes, which was longer than the length of the cell cycle in these conditions (about 60 minutes). This suggests that LuxAB luciferase is fairly stable, which is a bit unfortunate, as in the worst case the amount of cell division protein is only expected to double during one cell cycle. Hence, in this situation even if there is an abrupt increase in expression of a given cell division gene at a specific time in the cell cycle, light production from the reporter fused to such a gene may only be expected to increase two fold (or slightly more than that). An unstable reporter would have made this difference more pronounced thus simplifying it's detection. On the other hand, such instability would also result in lower signal levels.

The relative stability of the luciferase reporter means that, in case of periodic transcription, it may be necessary to detect twofold difference between light production levels. Therefore the sensitivity of the test system to the differences of that scale was tested. A

simulation was performed using cultures of JM101 strain with pZH2 induced with different levels of IPTG (final concentrations 50 and 25 $\mu g\ ml^{-1}$). 5 µl drops of each culture, diluted so that each drop would have about 40 cells, were placed beside each other on the surface of the same agar slide. Rifampicin was added to the medium to prevent further transcription. An image of the slide was then taken for 7 minutes and number of photons captured from each cell was then calculated (details of imaging setup described in 3.3.). It appeared that cells with light production levels differing by less than 50% could be clearly distinguished (Figure 3.2.)

The original attempts to detect luciferase expression driven by the *ftsZ* promoter region were unsuccessful, although the control plasmid pZH2 produced enough luciferase for single cells to be easily detected by the Argus 50 system with an integration time of 5 minutes and even less. Therefore, media conditions for the growth of the cultures were carefully optimised. It appeared that minimal media with addition of casamino acids (CAA) were better suited for this sort of experiment. Although rich media, such as LB, resulted in slightly higher levels of luciferase expression, the doubling time for the cultures was almost half of that on minimal medium, so minimal medium allowed the accumulation of more photons per cell cycle. Also, glycerol appeared to be a better carbon source than glucose. The switch from LB to CAA-glycerol resulted in about two fold increase of light output.

To optimise the detection of light production, single cells were placed on a white membrane filter in the hope that it would act as a reflector. Surprisingly, use of membrane filters resulted in about 3 fold increase in the photon counts rather than expected 2 fold. The reason for the larger increase remains unknown.

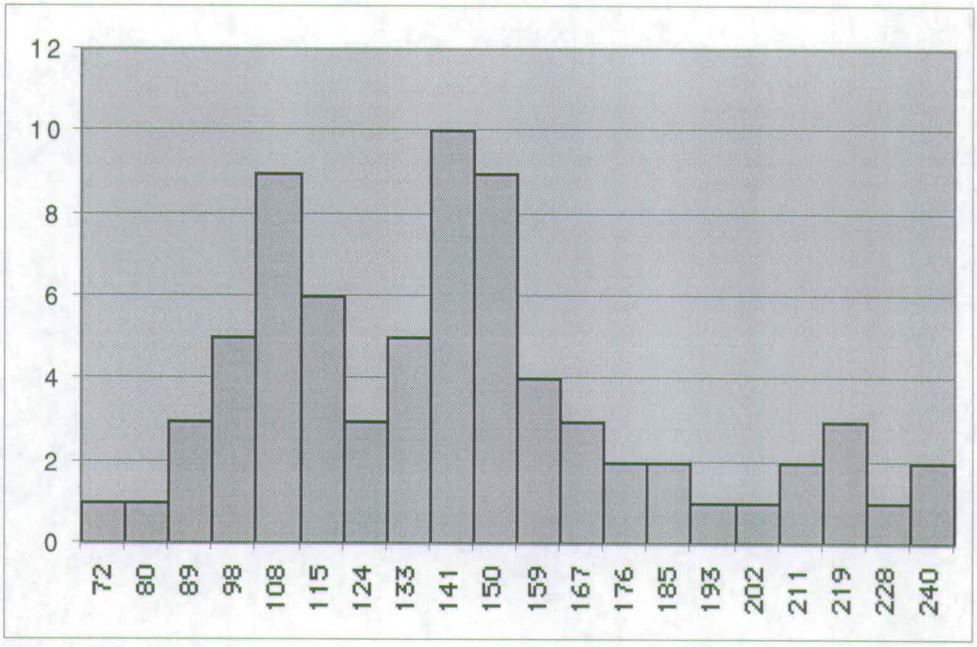


Figure 3.2. *The distribution of cells with different brightness in a mixed population.*
JM101 cells with pZH2 were induced with different concentrations of IPTG (25 and 50 μg ml^{-1}) and grown to OD_{540}=0.4. Photons from cells from each population were counted separately (30 and 43 cells respectively). Mean photon counts from the stronger induced subpopulation were higher by 40%.
Horizontal axis - photons per minute (mean values for each interval). Vertical axis – number of cells in each interval.

3.3. Luciferase expression in single cells.

Measuring expression of the P_{ftsZ}:*luxAB* fusion in single cells was performed by plating a small number of exponentially growing cells onto an agar slide so that about 30 cells appeared in the field covered by the camera lens. The slide was placed into a petri dish sealed with Parafilm, with a drop of luciferase substrate (nonyl aldehyde). In such conditions the cells are expected to have saturating amounts of both the luciferase substrate, which is very volatile, and oxygen, also required for the luciferase reaction. The plate was then placed (in a black box) under the Hamamatsu CCD camera connected to an Argus 50 image analyser and incubated

further at room temperature. The camera was equipped with an objective and suitable extension rings to bring the lens as close to the sample as possible (about 7 mm). The image analyser was set up in the photon counting mode and images were sequentially taken, with photons continuously accumulating for some time. The usual setting was 10 minutes, which allowed to have at least 10 points within one doubling time (usually 110 minutes in CAA-glycerol at room temperature).

With this setup, light emission was usually directly seen as bright spots on the image analyser screen if the system was set up in the "slice" mode, when one photon arriving at the photo multiplier of the camera resulted in a variable number of pixels displayed on the screen. This setup was optimal for rapid visualisation of light producing cells. However, for the quantitative measurements the "gravity" mode of the analyser was used. In this mode, the center of gravity of each spot resulting from the multiplication of a single photon, was calculated and displayed as a single pixel. The usual picture seen on the screen (in slice mode) was continuous appearance of spots that started to overlap with time in certain places and, eventually, some bright (in pseudo-colour) spots appeared. These spots always correlated with the pattern of microcolonies grown on the slide the next day. It was therefore safe to assume that each bright spot corresponded to a single cell originally placed on the slide and use the gravity mode further on for quantitative measurements of luciferase production. Although in the gravity mode it was very difficult to determine the positioning of cells in the first several images taken in an experiment, cell locations became clear by the end of experiments, so it was always possible to track back cell placement and photons emitted by them in the very first images.

For quantification of the image data, the area analysis function of Argus 50 system was used. Usually, circles of the minimum diameter to include all pixels corresponding to a single cell were

drawn around bright spots on the last image, and total photon count was calculated inside these areas. These calculations were repeated for the circles of the same diameter and coordinates for all images of an experiment.

The result of these experiments was a bit unexpected. It was not possible to tell from this data if photon emission by single cells is continuous or periodic. As the luciferase reporter appeared to be fairly stable, theoretical expectations were of a rather sharp, probably two fold, increase in the reporter output at a certain position in the cell cycle if *ftsZ* transcription in this plasmid construct is indeed periodic or a smooth increase if *ftsZ* transcription is continuous. The observed situation appeared to be neither of these two options. Some of the cells behaved as if the transcription was periodic, with rather sharp approximately twofold increases repeating at the right times for the expected cell cycle duration while other cells showed more or less continuous increase in photon counts (Figure 3.3). The signs of periodicity could be seen in 30-50% of the cells (37% - average of 95 cells tested in three experiments). 13% of tested cells were abnormal, showing huge fluctuations in light production without any sign of exponential increase throughout the experiment.

In the control experiments with pZH2 (Figure 3.4.) periodic increases of light production could be observed sometimes, but in less than 10% of the cells. It should be mentioned, however, that *lac* cells can sometimes show a sort of wave-like fluctuations, but these do not correspond to the length of the cell cycle and (or) to doubling in light intensity level.

The observed fluctuations of light production were more pronounced in the beginning of these experiments, when very few photons could be accumulated during each 10 minute interval. Even after all optimisations light production at the initial points of the time lapse experiments was at the limit of detection - about one to two photons per minute. These levels are too low and hence the input of

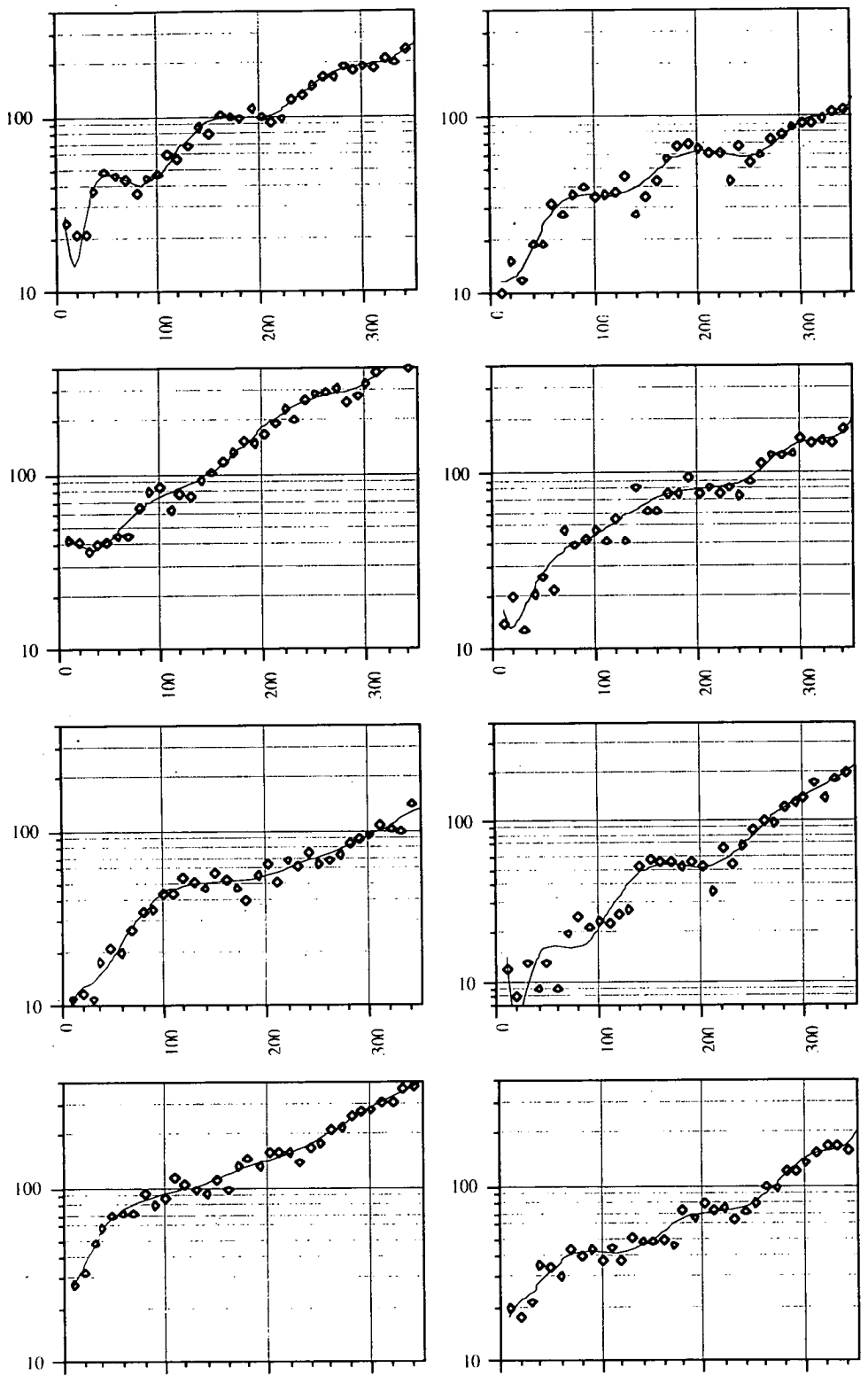


Figure 3.3. *Light production by single cells carrying P_{ftsZ}::luxAB fusion on pZlux plasmid.*
Vertical axis - photons per 10 min. Horizontal axis - time in minutes.

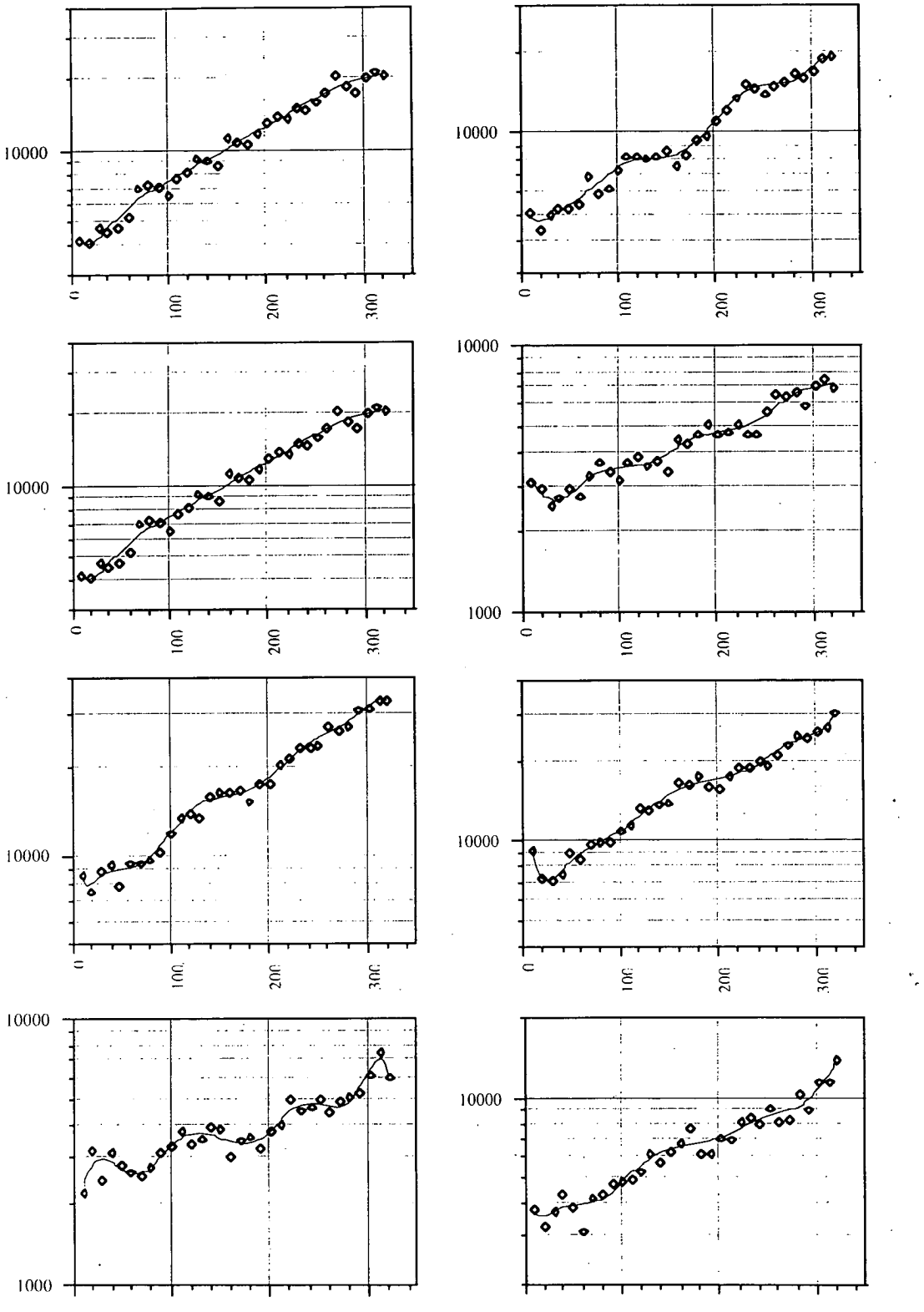


Figure 3.3. *Luciferase activity in single cells carrying pZH2.*
Axes as on figure 3.3.

random fluctuations of the observed photon counts is expected to be high in the initial phases of these experiments. Therefore, an attempt was made to optimise the reporter expression to increase the light output.

3.4. Constructing the efficient *fts::luxAB* reporter fusion.

Examination of the luciferase genes' sequence revealed that the sequence immediately upstream of the *luxA* gene did not match well against what is believed to be the optimal ribosome-binding site (RBS) (Figure 3.4a.). Therefore an obvious thing was to substitute a good ribosome binding sequence for the presumably inefficient original.

```
a)          CAAATAAGGAAATGTTATGAAATTT
                                M  K  F
b)    RBS         NdeI                                    BamHI
TAAGAAGGAGATATACATATGGCTAGCATGACTGGTGGACAGCAAATGGGTCGGATC
                    M  A  S  M  T  G  G  Q  Q  M  G  R  I
```

Figure 3.4. *Translation initiation regions of Vibrio harveyi luxA (a) gene and phage T7 gene 10 from plasmid pET-3c (b).*

The pET-3c protein expression vector (Rosenberg *et al.*, 1987) was chosen as the donor of a ribosome binding site as this plasmid proved to be very efficient in expressing various proteins placed under the ribosome binding site of gene 10 of phage T7. After T7 infection gene 10 product is produced more rapidly than any host protein which indicates the efficiency of translation.

To fuse the *luxAB* coding region to the RBS of gene 10, an *Nde*I site was introduced overlapping the start codon of *luxA*. This was done through PCR with mutagenic primers

5' GTCATATGAAATTTGGAAACTTCCT 3'

5' GTCCCGGGTTTACCAGTGGTATTTGACG 3'.

The product of PCR amplification of the pZlux template with this pair of primers was digested with *Nde*I and *Ava*I and ligated with pUC19 restricted with the same enzymes. The *luxAB* fragment was cut from the resulting plasmid, pZH1, with *Nde*I and *Bam*HI and cloned into the same sites of pET-3c. The resulting plasmid, pZH3, had the desired fusion of gene-10 RBS to the *luxA* gene. Then the *ftsZ* promoter region together with most of the omega sequence was cut from pZlux with *Sph*I and *Eco*RI (this removes one end of the omega but leaves both the streptomycin resistance gene and the required terminator untouched). This fragment was ligated into *Sph*I-*Xba*I cut pZH3 (both *Eco*RI and *Xba*I sticky ends were filled in with Klenow fragment of DNA polymerase I). The resulting plasmid, pZH5, was analogous to pZlux with the exception of the improved ribosome binding site in front of *luxA* (Figure 3.5.). To possibly further increase the luciferase expression, the resulting 'omega-'*ftsQ-ftsZ'-luxAB* construct was subcloned as an *Ava*I fragment from pZH5 into the same site of pUC18. This gave the pZH6 and pZH7 plasmids differing only in the orientation of the cloned fragment .

The measurements of luciferase production in liquid cultures had shown that the introduction of an efficient ribosome binding sequence resulted in a substantial increase in light production by cells with pZH5 compared to pZlux. As bacterial luciferase consists of two subunits, increasing the amounts of LuxA only should not lead to the increased amounts of active luciferase. The observed results must therefore indicate that the amount of LuxB is also increased, and this is possible to explain if the translation of the genes coding for the two luciferase subunits is coupled.

Luciferase expression from pZH7 and pZH6 was indistinguishable and slightly higher than from pZH5 (increase of about 50-100% when measured in liquid cultures).

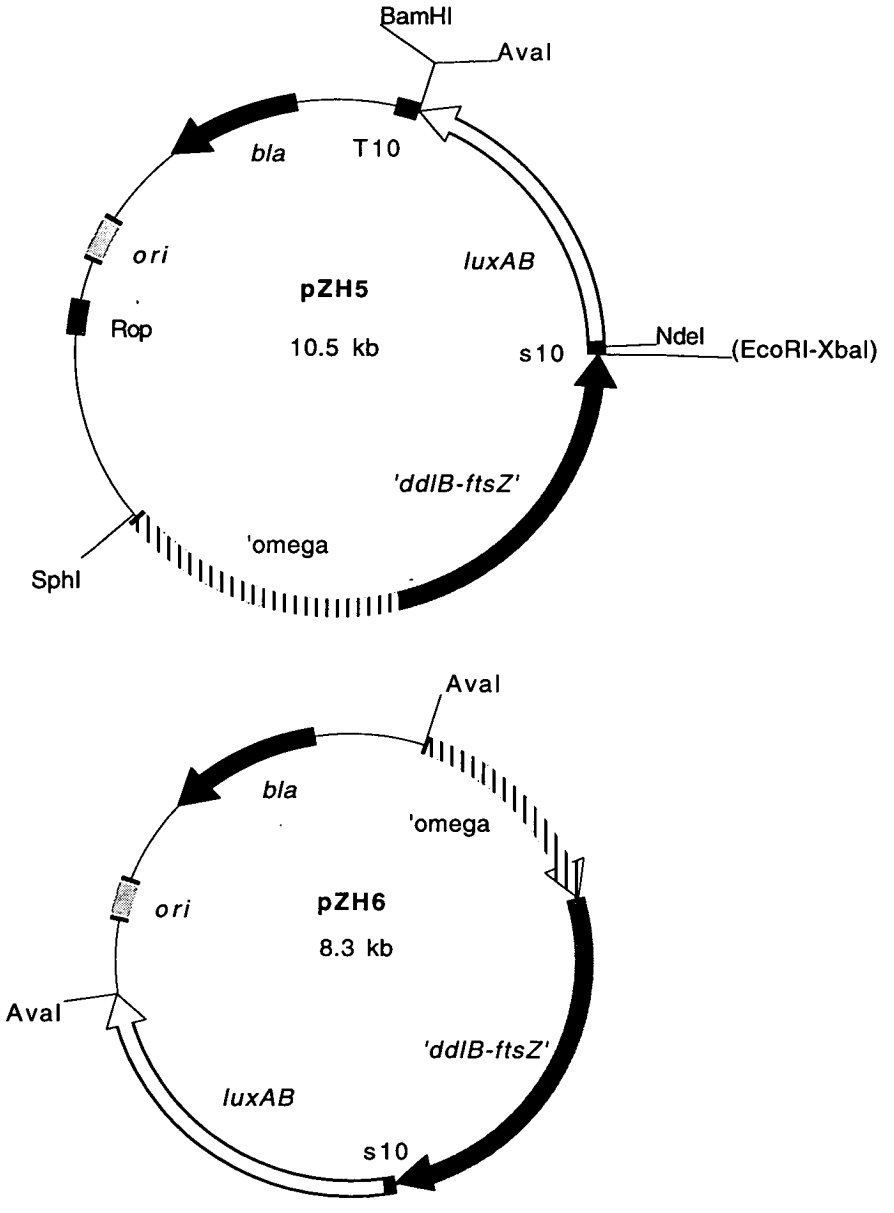


Figure 3.5. *Plasmid vectors with improved luciferase reporter.*

3.5. Expression of the improved luciferase in single cells.

As the increase in light output due to the improved luciferase reporter was significant, single cell experiments were repeated with

new plasmids. pZH6 was used first and experiments were done exactly as for pZlux. The observed average single cell levels of light output were approximately 20 times higher than these for pZlux in the beginning of the experiment; the difference was much more pronounced in the end (Figure 3.6.).

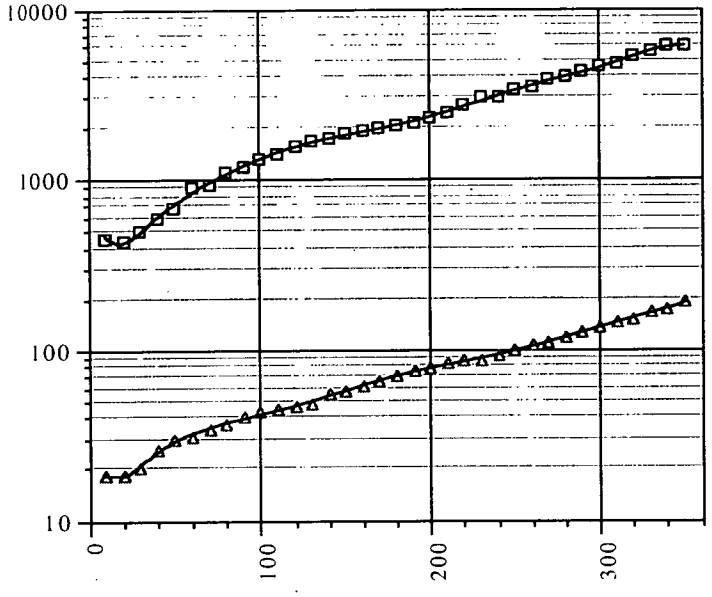


Figure 3.6. *Levels of light emission by JM101 cells with pZlux (triangles) and pZH6 (squares).*
Axes as on figure 3.3.

As with pZlux, stepwise increase in light production with the periodicity roughly corresponding to the cell cycle duration was observed for some cells (Figure 3.7.), but the fraction of these cells was smaller than with pZlux. Thorough examination of the data from about 200 single cells obtained in 8 independent experiments showed that "periodic" cells with the correct timing of the periodicity constitute not more than 10% in the analysed group (compared to about 30% for pZlux). Majority of the cells showed periodic changes in light production with no correspondence to the expected cell cycle parameters or showed relatively smooth increase of light output. As with pZlux, few cells showed erratic "ups" and "downs" in light production.

The patterns of light output by the cells carrying pZH6 resembled those of the control experiment with pZH2 (Figure 3.3.) more than the data with pZlux. Therefore, another control experiment was performed. In this case, induced cultures of JM101 with pZH2 were placed on an agar slide without IPTG and imaged as usual. With this setup, luciferase production was expected to halt soon after the placement on the agar. Therefore, any changes in light production due to fluctuations in transcription of the reporter were expected to be eliminated. The same fluctuations of light output were, however, still observed with the only difference being the expected decrease of light production with time (Figure 3.8.).

Fluctuations of this sort could not be attributed to changing amounts of luciferase. One possible explanation could be that light production fluctuations reflect changes in metabolic properties of the host cells, leading to a varying amount of reduced FMN which, together with aliphatic aldehyde and oxygen, are necessary components for the luciferase reaction. As both aldehyde and oxygen are almost certainly present in vast excess, only the one uncontrollable component of reaction, FMNH, could be responsible for unstable output. It may be worth noting, that due to the dependence on FMNH, the concentration of which in turn correlates with the metabolic activity of the cell, light emission by the bacterial luciferase is used in a number of applications as an indicator of changes in the metabolic state of bacterial cells in response to, for example, starvation (Meikle *et al.*, 1992) or biocide treatment (Walker *et al.*, 1992).

As the level of expression of the luciferase reporter from the created plasmids was sufficient for the detection with the Hamamatsu CCD camera in a reasonably short periods of time, an

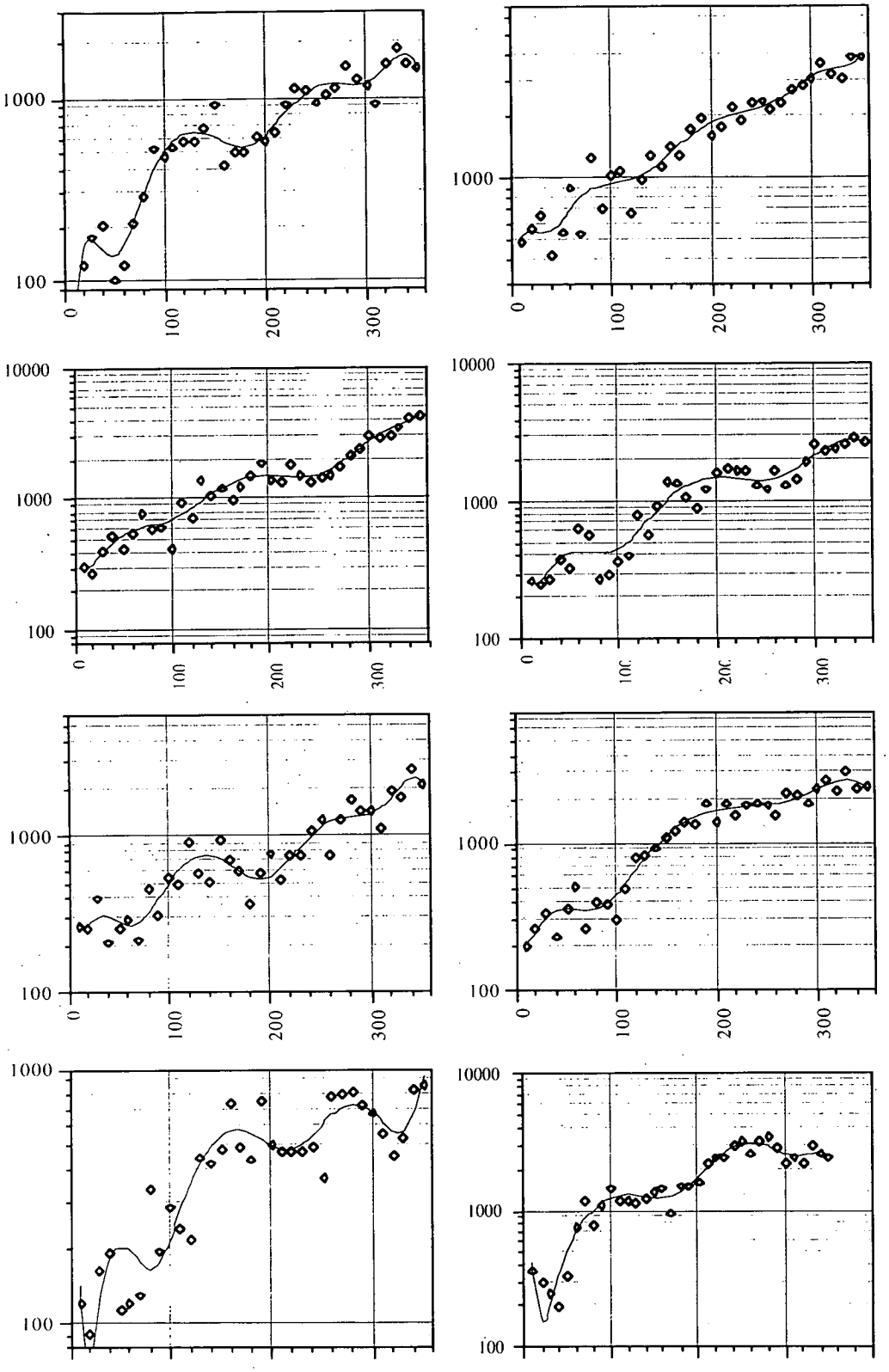


Figure 3.7. Light production by single cells of JM101/pZH6.
Axes as on figure 3.3.

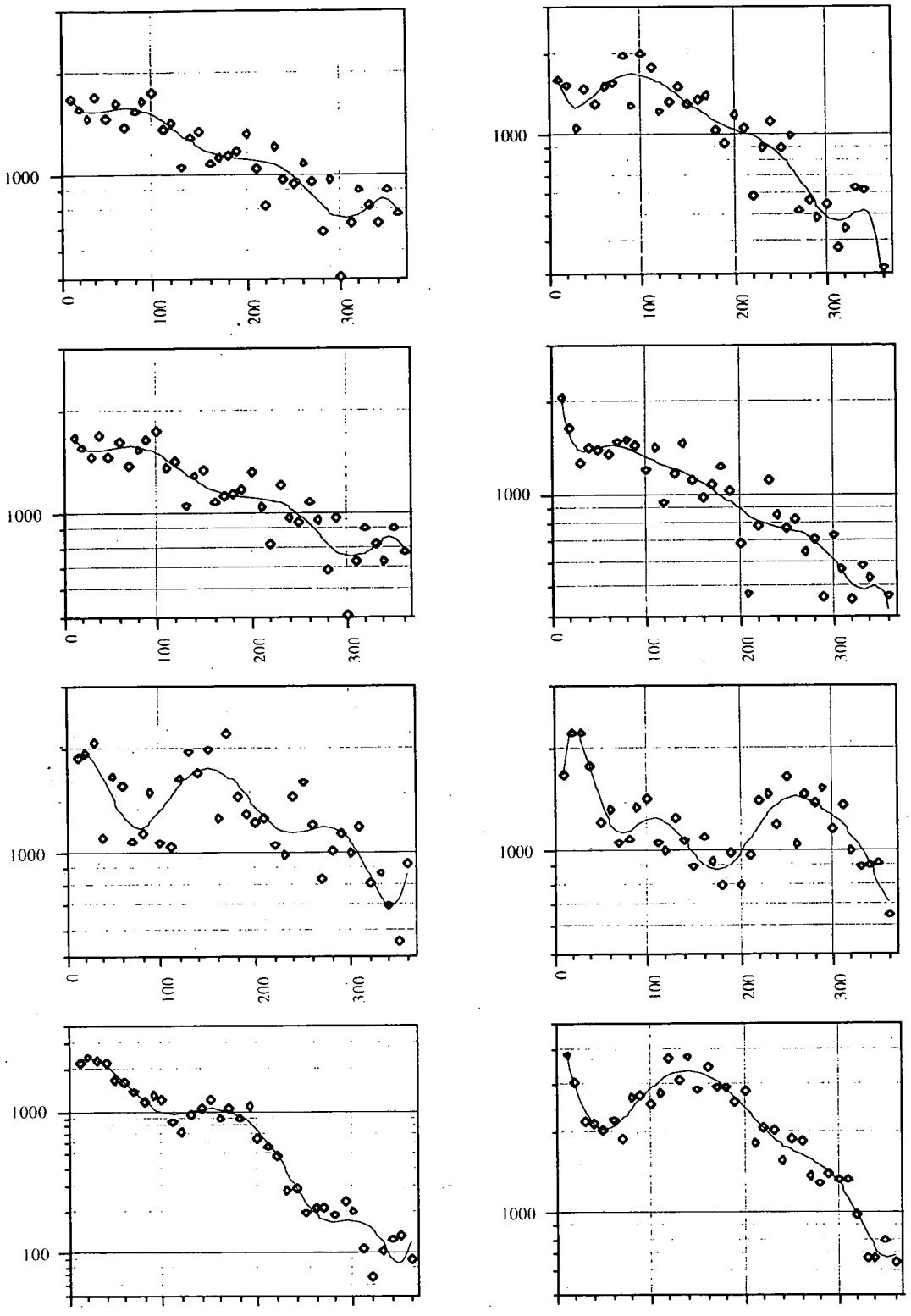


Figure 3.8. Fluctuations of light production by single cells of JM101/pZH6 after stopping *luxAB* induction.

Axes as on figure 3.3.

attempt was made to decrease the plasmid copy number in order to reduce the possibility of expression artifacts due to the high copy number of *ftsZ* promoters. For this purpose the pZH5 plasmid was put in the MM38Δ*pcnB* strain. As pZH5 is pBR322 based, this was expected to decrease the plasmid copy number to about 5 copies per cell. However, this resulted in such a dramatic decrease in light production (over 100-fold when measured in liquid cultures), that single cell imaging appeared to be absolutely impossible. Cells were only becoming visible after accumulating photons for over 60 minutes. The result of this experiment shows that, even with the improved luciferase, the resulting light output is insufficient for looking at the expression of chromosomal genes, at least in the case of those with medium strength promoters.

3.6. Discussion

The experiments performed here have shown that it is possible to detect single cells of *Escherichia coli* expressing bacterial luciferase from a cell division gene promoter placed on a multicopy plasmid. However, the system employed appeared to have serious limitations, the most important of which was the sensitivity issue - the Hamamatsu Argus 50 system was only sensitive enough to accumulate sufficient signal from *ftsZ* promoters on a multicopy plasmid by summing photon accumulation over 5-10 minute periods. As *ftsZ* is considered to be the gene with highest expression levels among all cell division genes, it is unlikely that the system may be useful for any other division related gene. In addition, the usability of the system is seriously hampered by the necessity of using a multicopy plasmid vector for carrying a fusion expressing luciferase as this could interfere with the normal regulation of a promoter under study. In the end, it is questionable how good bacterial luciferase is as a reporter for monitoring quantitative measurements on a single

cell level, because the light production appeared to fluctuate irregularly during the cell cycle, which could be due to the dependence of the luciferase reaction on the state of cell metabolism. This certainly wasn't a problem when large populations (or even not so large groups of cells) were observed, but it is really difficult to separate any cell-cycle dependent variations in *ftsZ* expression from the highly fluctuating background, especially taking into account that the predicted variation in *ftsZ* expression levels could be as low as twofold. In addition, it is possible that some aspect of cell metabolism that is crucial for the luciferase reaction is cell cycle dependent, as the observed fluctuations sometimes matched the cell cycle parameters. If this is true then luciferase is useless as a reporter for studying cell division.

To summarise, in the course of this research bacterial luciferase appeared to be not suitable for testing the expression of cell division genes from their native promoters. Design of more sensitive CCD cameras could change this situation. On the other hand, some other recently appeared approaches are probably preferable for this sort of studies. GFP fusions allow direct visualisation of the events during cell cycle *in vivo*, whereas recent advances in immunodetection microscopy can also answer some of the questions the idea of this research was intended to address.

CHAPTER 4

TOPOLOGICAL CHARACTERISATION OF RODA PROTEIN.

4.1. Introduction.

The *mrd* gene cluster located at 14.5 minutes on the *Escherichia coli* chromosome encodes several genes involved in the maintenance of the rod shape of *E. coli* cells (Tamaki *et al.*, 1980; Stoker *et al.*, 1983). Cells carrying mutations in either *rodA* or *pbpA* grow as spheres under non-permissive conditions and eventually lyse if grown in rich media. However, increasing the ppGpp level by introduction of additional mutations or by growing mutants in poor media eliminates the lethal effect and enables the mutants to grow and divide as spheres (Vinella *et al.*, 1992). RodA together with the product of *pbpA* controls probably the most important modification to the 'minimal' cell cycle (Donachie, 1992). Apart from a possible physiological significance (maintenance of an approximately constant cell surface to volume ratio) this morphogenetic system somehow ensures that division is linked to the completion of each round of chromosome replication. If this system is knocked out, cell division is delayed and hence the cells are large and polyploid (Begg and Donachie, 1985). Therefore, RodA and PBP2 can be viewed as true cell division proteins necessary to maximise the number of viable cells per chromosome (Donachie *et al.*, 1995). There is also a possibility, supported by some experimental evidence, that the 'normal' cell cycle requires periodic switching of the peptidoglycan synthesising machinery from 'elongation mode' controlled by PBP2 to the 'division mode' depending on PBP3 action, which may result from changes in levels of preferred peptidoglycan precursors (Begg *et al.*, 1990; Markiewicz *et al.*, 1982). Indeed, it has been shown that in spherical *E. coli* derivatives peptidoglycan synthesis only occurs at the time of septation and the composition of peptidoglycan appeared to be different (Signoretto *et al.*, 1996). This supports the idea that the activity of a specific transpeptidases, PBP2 and PBP3, leads to

formation of minor amounts of altered peptidoglycan, possibly forming special "primer" sites (Wientjes and Nanninga, 1991) necessary for elongation or division.

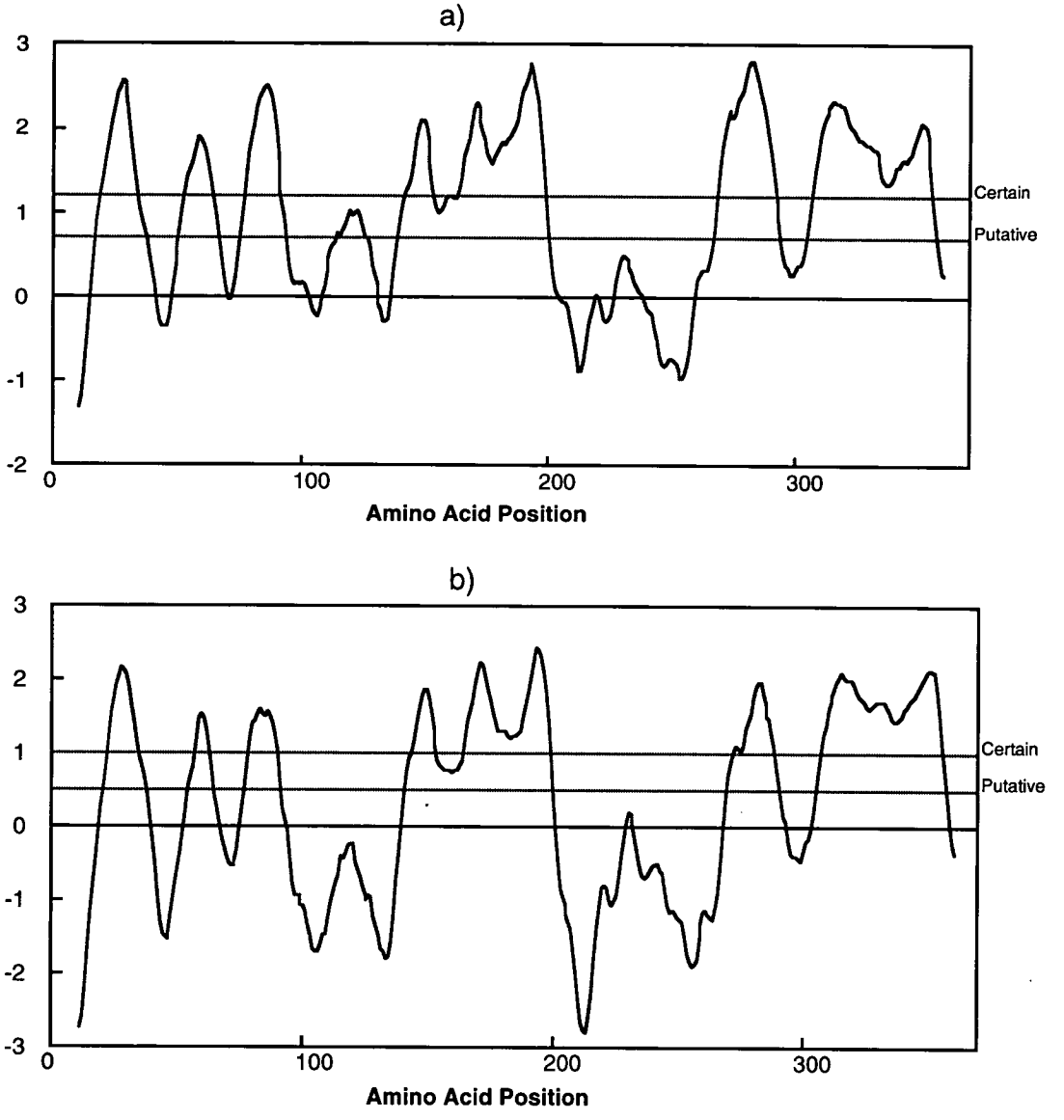


Figure 4.1. *The hydrophobicity profile of RodA protein built with Kyte and Doolittle (a) and GES (b) scales by the TopPredII programme (Claros & von Heijne, 1994).*
Dotted lines show the cut-offs for certain and "putative" transmembrane segments.

Very little is known about the biochemical function of RodA, although there is indirect evidence that it may be required for the

enzymatic activity of PBP2 (Ishino *et al.*, 1986) implying that these proteins might interact in some way. The amino acid sequence suggests that RodA is located in the cytoplasmic membrane and the hydrophobicity profile (Kyte & Doolittle, 1982) shows that RodA has several hydrophobic regions separated by short (with one exception) hydrophilic areas (Figure 4.1.). The protein is therefore likely to belong to the group of helix-bundle membrane proteins. The only long hydrophilic region of approximately 60 residues is located in the second half of the protein. Depending on the arrangement of the transmembrane helices, this hydrophilic region may be periplasmic or cytoplasmic. Determining the topology of the arrangement of membrane helices is important for understanding RodA action, because the presence of the hydrophilic part of the protein in the periplasm would be expected to be necessary for any interactions with the peptidoglycan synthesising enzymes, in particular PBP2.

According to current knowledge, in the majority of multispanning membrane proteins a bundle of apolar, sometimes slightly amphiphilic, transmembrane α-helices, each 17-25 residues long, is oriented perpendicular to the plane of the membrane. Although the precise 3D structure has been determined for only very few helix-bundle proteins, due to difficulties in crystallising them, remarkable progress has been made in understanding how the aminoacid sequence determines the membrane topology of these proteins (von Heijne, 1994). "Topology", for membrane proteins means the secondary structure of the membrane embedded domain and is defined by the location of the transmembrane segments in the amino acid sequence, and by the overall membrane orientation of the molecule. The most important event during the biogenesis of an integral membrane protein is very likely to be the insertion of its transmembrane segments into the lipid bilayer. Once this insertion has been achieved, the core structure of a molecule is defined (and it is this structure that is represented by the protein topology). What

then remains, is for transmembrane helices to assemble into a membrane-embedded helix bundle and for the polar segments exposed outside the bilayer to fold into their proper tertiary structures. Thus, at least for polytopic membrane proteins with most of their mass buried within the bilayer in the form of helices, a good part of the structure will be known, if the transmembrane organisation of the chain can be determined experimentally or calculated from the aminoacid sequence.

Because it is not usually possible to crystallise membrane proteins and therefore determine their 3D-structure, alternative approaches are used to evaluate the secondary structure of these proteins. For prokaryotes protein fusions with suitable compartment-specific reporters are the most widely used tool. Among these reporters β-lactamase and alkaline phosphatase are active only in the periplasm and β-galactosidase is active only in the cytoplasm.

Based on the information from the few proteins with known 3D structures as well as from more numerous proteins with experimentally determined topology, several approaches have been made to predicting the topology of helix bundle proteins. Typical methods for the prediction of membrane-spanning regions assume the transmembrane segments to be helices that are completely exposed to lipid and completely traverse the membrane in the direction normal to its surface. The simplest analytic scheme is to generate a hydropathy plot for a given sequence, with the segments being centred at the peaks of the plot. Average residue hydrophobicity calculated in a window of 17-22 residues using a suitable hydrophobicity scale provides a criterion for finding transmembrane domains. Plots based on smaller windows could then be used to determine the most probable end points of the segments (Kyte and Doolittle, 1982). Next, a "positive-inside" rule

(von Heijne, 1986; von Heijne & Gavel, 1988) is applied to determine the orientation of the predicted helices. Short (up to approximately 60 residues) periplasmic loops are translocated independently of the general secretion pathway machinery and this translocation is less efficient when positively charged residues are present in the given loop. On the other hand, this restriction does not apply to the longer loops, which are efficiently translocated in a Sec-dependent manner. Correspondingly, short periplasmic loops rarely have positively charged residues and longer loops have an arginine + lysine content close to the average values (Andersson & von Heijne, 1993). Combining prediction of transmembrane helices based on the hydropathy plots with the positive inside rule allows to suggest the correct membrane topology for many proteins.

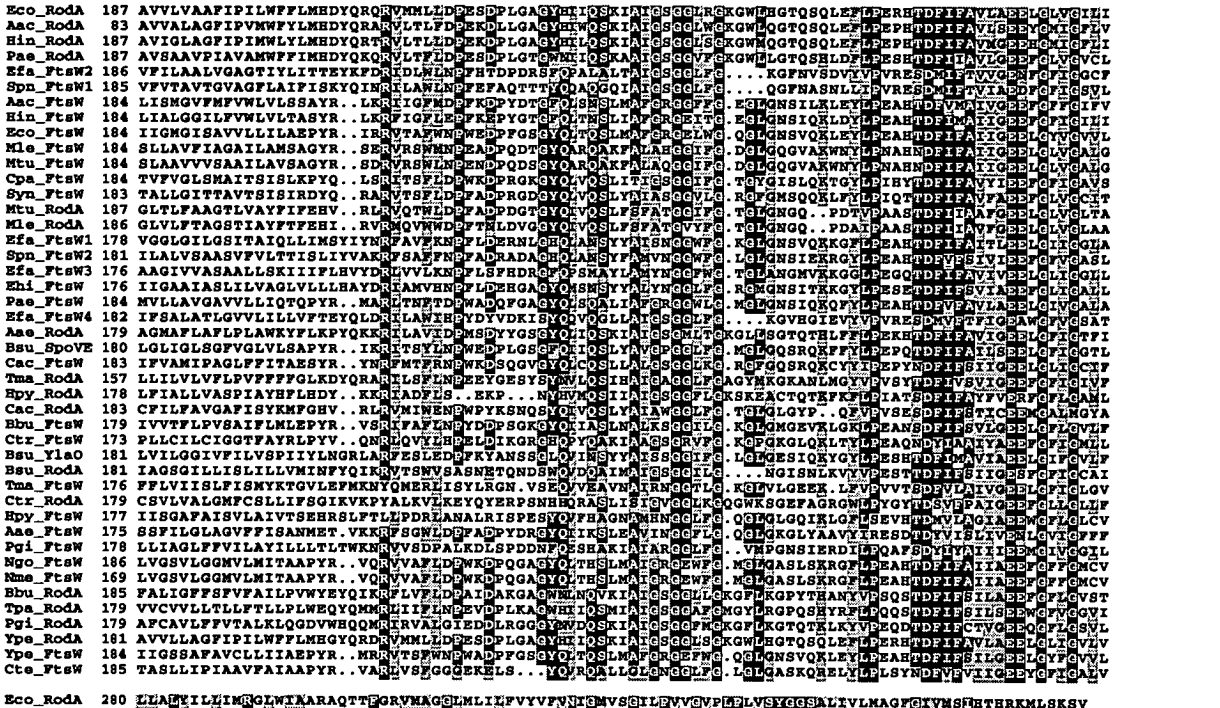
Although these methods are remarkably efficient, especially when compared with attempts to predict full 3D structures, available algorithms can correctly predict the presence and location of 90-95% of transmembrane helices (Rost *et al.*, 1995; von Heijne, 1994). As good as these numbers are, they mean that prediction of all transmembrane segments in a multispanning membrane protein with, say, 10 transmembrane helices, has a good chance of being wrong. Therefore, theoretical predictions, although helpful, can sometimes be insufficiently reliable and always need experimental testing.

4.2. Computer analysis of RodA topology.

Attempts to predict RodA topology using simple methods based on hydrophobicity profiles gave conflicting results for different hydrophobicity scales. When the TopPred programme (Claros and von Heijne, 1994) was used with the two most popular hydrophobicity scales, KD and GES (Engelman *et al.*, 1986; Kyte and

Doolittle, 1982), 9 transmembrane segments for RodA were predicted in approximately the same locations (Figure 4.1.). KD scale also detected one "putative" segment - slightly amphiphilic and a bit short for a typical transmembrane helix, and the predicted topology having the highest difference in the distribution of positively charged residues included this additional segment.

Several methods of topology prediction utilise additional information from homologous sequences (if such sequences are available) to achieve better performance. Therefore all sequences showing similarity to RodA were extracted from nucleotide databases and in some cases from released sequences from ongoing genome projects. Similarities with GenBank sequences were detected using gapped BLAST2 searching programmes (Altschul *et al.*, 1997) Sequences similar to RodA were detected in incomplete genomes using programmes from the FASTA package (Pearson *et al.*, 1997). About 50 sequences could be detected this way at the time of writing. Wherever possible annotated sequences of interest were extracted from the databases. In cases where only unannotated DNA sequence was available, the longest possible ORF with homology to RodA was taken for the purpose of this study. A multiple sequence alignment was then constructed by the ClustalX programme with default parameters (except that the threshold for delaying divergent sequences during multiple alignment was lowered to 35%). An iterative approach was used for the construction of the alignment. The original alignment was used to build a phylogenetic tree with the help of the Protpars programme from the PHYLIP package (Felsenstein, 1989) and the result was used as a guide tree for the next ClustalX alignment. The process was repeated until no changes were introduced into the alignment after the next iteration. The final alignment (Figure 4.2.) was refined by hand (mostly on the gap boundaries).


Eco_RodA 187 AVVLVAAFIPILWFFLMHEDYQRQRVMMLLDPESDPLGAGYHIIQSKIAIGSGGLRGKGWLHGTQSQLEFLPERHTDFIFAVLAEELGLVGILI
Aac_RodA 83 AVVALAGFIPVMWFYLMHDYQRARVLTLFDPEKDLLGAGYHIWQSKIAIGSGGLWGKGWLQGTQSQLEFLPEPHTDFIFAVISEELGLVGMIGFLV
Hin_RodA 187 AVIGLAGFIPIMWLYLMHDYQRTRVLTLLDPEKDPLGAGYHILQSKIAIGSGGLSGKGWMQGTQSQLEFLPEPHTDFIFAVMGEELGMIGFLI
Pae_RodA 187 AVSAAVPIAVAMWFFIMHDYQKQRVLTFLDPESDPLGTGWHIIQSKAAIGSGGVFGKGWLLGTQSSLDFLESEHTDFIFAVLGEELGLVGVCL
Efa_FtsW2 186 VFILAALVGAGTIYLITTEYKFDRIDLWLNPFHTDPDRSFQPALALTAIGSGGLFG....KGFNVSDVLYPVRESDMIFTVVGENFGFIGGCF
Spn_FtsW1 185 VFVTAVTGVAGFLAIPISKYQINRILAWLNPFEFAQTTTYQAQGQIAIGSGGLFG....QGFNASNLLIPVRESDMIFTVIAEDFGFIGSVL
Aac_FtsW 184 LISMGVFMFVWLVLSSAYR..LKRIIGFMDPFKDPYDTGFQLSMSLMAFGRGGFFG.EGLGNSILKLEYLPEAHTDFVMAIVGEEFGFFGIFV
Hin_FtsW 184 LIALGGILFVWLVLTASYR..LKRFIGFLEPFKEPYGTGFQLTNSLIAFGRGEITG.EGLGNSIQKLDYLPEAHTDFIMAIIGEEFGFIGILI
Eco_FtsW 184 IIGMGISAVVLLILAEPYR..IRRVTAFWNPWEDPFGSGYQLTQSLMAFGRGELWG.QGLGNSVQKLEYLPEAHTDFIFAIIGEELGLVGVVL
Mle_FtsW 184 SLLAVFIAGAILAMSAGYR..SERVRSWWNPEADPQDTGYQARQAKFALAHGGIFG.DGLGQGVAKWNYLPNAHNDFIFAIIGEELGLVGALG
Mtu_FtsW 184 SLAAVVVSAAILAVSAGYR..SDRVRSWLNPENDPQDSGYQARQAKFALAQGGIFG.DGLGQGVAKWNYLPNAHNDFIFAIIGEELGLVGALG
Cpa_FtsW 184 TVFVGLSMAITSISLKPYQ..LSRITSFLDPWKDPRGKGYQLVQSLITIGSGGIFG.TGYGISLQETGYLPIHYTDFIFAVIEEFGFIGAVS
Syn_FtsW 183 TALLGITTAVTSISIRDYQ..RARVTSFLDPFADPRGDGYQLVQSLYAIASGGVLG.RGFGMSQQKLFYLPIQITPDFVFAVLAEELGLVGCIT
Mtu_RodA 187 GLTLFAAGTLVAYFIFEHV..RLRVQTWLDPFADPDGTGYQVVQSLFSFATGGVFG.TGLGNGQ..PDTVPAASTDFIIAAFGEELGLVGLTA
Mle_RodA 186 GLVLFTAGSTIAYYFTEHI..RVRMQVWWDPFTNLDVGGYQVQSLFSFATGVVFG.TGLGNGQ..PDAIPAASTDFIIAAFGEELGLVGLAA
Efa_FtsW1 178 VGGLGILGSITAIQLLIMSYIYNRFAVFKNPFLDERNLGHQLANSYYAISNGGWFG.KGLGNSVQKKGFLPEAHTDFIFAITLEELGIIGGLA
Spn_FtsW2 181 ILALVSAASVFVLTTISLIYVAKRFSAFNPFADRADACGQLANSYFAMVNGGWFG.LGLGNSIEKRGYLPEAHTDFVPSVIEEFGFVGASL
Efa_FtsW3 176 AAGIVVASAALLSKIIIFLHVYDRLVVLKNPFLSFHDRGPQPSMAYLAMYNGGFWG.TGLANGMVKKGGLPEGGTDFIFAVIVEELGLIGGLL
Ehi_FtsW 176 IIGAAIASLILVAGLVLLLBAYDRLMVEMPFLDERGAGYQLMSMSYYALYNGGLFG.RGMGNSITKKGYLPESETDFIFSVIAEEFGLIGALL
Pae_FtsW 184 MVLLAVGAVVLLIQTQPYR..MARLTNFTDPWADQFGAGYQLSQALIAFGRGGWLG.MGLGNSIQKQFYLPEAHTDFVFAVLAEELGIVGALA
Efa_FtsW4 182 IFSALATLGVVLILLVFTEYQLDRILAWIEPYDYVDKISYQVVQGLLAIGSGGLFG....KGVEGIEVVYPVRESDMVFTFIGEAWGFVGSAT
Aae_RodA 179 AGMAFLAFLPLAWKYFLKPYQKKRILAVIDPMSDYYGSGYQLIQSKIAIGSGMLTGKGLLSGTQTHLFFLPEKHTDFIFAVIGEELGFIGTFI
Bsu_SpoVE 180 LGLIGLSGFVGLVLSAPYR..IKRITSYLNPWEDPLGSGFQIIQSLYAVGPGGLFG.MGLGQSRQKFFYLPEPQTDFIFAIISEELGFIGGTL
Cac_FtsW 183 IFVAMIPAGLFFITAESYR..YNRFMTFRNPWKDSQGVGYQLCQSLLALGSGGLKG.KGFGQSRQKCYYLPEPYNDFIFSIIGEELGLIGCIF
Tma_RodA 157 LLILVLVFLPVFFFFGLKDYQRARILSFLNPEEYGESYSYMVLQSIHAIGAGGLFGAGYMKGKANLMGYVPVSYTDFIVSVIGEEFGFIGIVF
Hpy_RodA 178 LFIALLVASPIAYHFLHDY..KKRIADFLS..EKP...NYHVMQSIIAIGSGGFLGKSKEACTQTEFKFLEIATSDFIFAYVFREFGFLGAML
Cac_RodA 183 CFILFAVGAFISYKMFGHV..RLRVMIWENPWPYKSNQSYQIVQSLYAIAWGGLFG.TGLGLGYP..QFVPVSESDFIFSTICEEFGALMGYA
Bbu_FtsW 179 IVVTFLPVSAIFLMLEPYR..VSRIFAFLNPYDPPSGKGYQIASLWALKSGGILG.KGLGMGEVKLGKLPEAHSDFIFSVIGEELGFLGVLF
Ctr_FtsW 173 PLLCILCIGGTFAYRLPYV..QNRLQVYLHPELDIKGRGHQYQAKIAAGSGRVFG.KQFGKGLQKLTYLPEAQNDYIFAAIVAEELGFIGMLL
Bsu_YlaO 181 LVILGGIVFILVSPIIYLNGRLARFESLEDPKYANSSGLQVINSYYAISSGGIFG.LGLGESIQKYGYLPESHTDFIMAVIAEELGIFGVLF
Bsu_RodA 181 IAOSGILLISLILLVMINFYQIRRVTSNVSASNETQNDSWQVDQAIMAFGSGGILG....NGISNLKVVYPESTTDFIFSIIGESFGFIGCAI
Tma_FtsW 176 FFLVIISLFISMYKTGVLEFMKNYQMERLISYLRGN.VSEQVVEAVNAIRNGGTLG.KGLVLGEEK.LFVPVVTSDFVLAIVGEELGFIGLGV
Ctr_RodA 179 CSVLVALGMFCSLLIFSGIKVKPYALKVLKEYQYERPSNHHQRASLISIGVGGLKGQGWKSGEGAFGRGWLFYGYTDSVFPAIGEEFGLIGLLF
Hpy_FtsW 177 IISGAFAISVLAIVTSEHRSLFTLLPDRLANALRISPESYQVFHAGNFMHNGGLFG.QGLQLGQQIKLGFLSEVHTDMVLAGIAEEVGFLGLCV
Aae_FtsW 175 SSFILGLAGVFFISANMET.VKKRFSGWLDPFADPYDRGYQIKSLEAVINGGFLG.QGLGKGLYAAVYIRESDTDYVISLIVENLGVIGFFF
Pgi_FtsW 178 LLIAGLFFVILAYILLLTLTWKNRVVSDPALKDLSPDDNFQSEHAKIAIARGGLFG..VMPGMSIERDIFFQAFSDYIYAIIIEENGIVGGIL
Ngo_FtsW 186 LVGSVLGGMVLMITAAPYR..VQRVVAFLDPWKDPQGAGYQLTHSLMAIGRGEWFG.MGLGASLSKRGFLPEAHTDFIFAIIAEELGFFGMCV
Nme_FtsW 169 LVGSVLGGMVLMITAAPYR..VQRVVAFLDPWKDPQGAGYQLTHSLMAIGRGEWFG.MGLGASLSKRGFLPEAHTDFIFAIIAEELGFFGMCV
Bbu_RodA 185 FALIGFFSFVFAILPVWYEYQIKRFLVYDSPLDAIPAKGAQWNLNQVKIAIGSGGLLGKGFLKGPYTHANYVPSQSTDFIFSILAEEFGFLGVST
Tpa_RodA 179 VVCVVLLTLLFTLLPLWEQYQMMRLIIFLNPEVDPLKAGWHIQSMIAIGSGGAFGMGYLRGPQSEYRFLPQQSTDFIFSILSEEWGFVGGVI
Pgi_RodA 179 AFCAVLFFVTALKLQGDVWHQQMRIRVALGIEDDLRGGGYWVDQSKIAIGSGGFMGKGFLKGTQTQLKYVPEQDTDFIFCTVQEEFGFLGSVL
Ype_RodA 181 AVVLLAGFIPILWFFLMHEGYQRDRVMMLLDPESDPLGAGYHIIQSKIAIGSGGLSGKGWLHGTQSQLEFLPERHTDFIFAVLAEELGLIGVVV
Ype_FtsW 184 IIGSSAFAVCLLIIAEPYR..MRRVTSFWNPWADPFGSGYQLTQSLMAFGRGEFWG.QGLGNSVQKLEYLPEARTDFIFSILGEELGYFGVVL
Cte_FtsW 185 TASLLIPIAAVFAIAAPYR..VARLVSFGGGEKELS...YQVRQALLGLGNGGLFG.LGLGASKQRELYLPLSYNDFVFVIIGEEYGFIGALV

Eco_RodA 280 LLAFTILLIMGLWIAARAQTTFGRVMAGGLMLILFVYVFVNICMVSGILPVVGVPLFLVSYGGSALIVLMAGFGIVMSHETHRKMLSKSV
Aac_RodA 176 LLAIYLFIVAGLMIGVNAQSAGRIIVGALTLIFFVYVFVNICMVSGILPVVGVPLFLVSYGGTSYVAIMAGFGLIMSLETHKEHFLKGT
Hin_RodA 280 LMAIYLFIVLGLMIAVNAQTSGRIIAGATTLIFFVYVFVNICMVSGILPVVGVPLFLVSYGGTSYVAIMASFGLLMSLETHKSQFMKR.
Pae_RodA 280 LLVFLLLISLGLVITAQAQTLVGKLLAGGTTMTFFVYVFVNICMVSGLLPVVGVPLFFISYGGTSLVTLLSGFGVLMSLETHRKWIAQ..
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Spn_FtsW1 274 VIALFYLMLIYLMLKITLKSNNQIYTYISTGIIMMLLFHIFEIGAVTGLLPLTGIPLLFIQGGGSAIISNLIGVGLLLSMSYQTNLAEEKS
Aac_FtsW 274 IIILLGLLVFFAMKIQRELEQRKGFLASGISFWIFFQGFVLGMALGMLPTKGITFPLVSYGGSSIIS.....................
Hin_FtsW 274 VILLLGLLIFFAMKIQRELEQRRGFFALGIGFWIFFQGFVLGMALGMLPTKGLTFPLVSYGGSSIIMSATIGILLRRIDHENRLFRIGQ
Eco_FtsW 274 AALMVFFVAFFAMSIQRKIDHRFSGFLACSIGIWFSFQALVVGAAAGMLPTKGLTLPLISYGGSSLLIMSTAIMMLLRIDYETRLEKAQA
Mle_FtsW 274 LLGLFGLFAYTGMRIASRSADPFLRLLTATTTMWVLGQAFIIGYVIGVLPVTGQLPFISAGGFSAAAILFMIGIMANAARHEPEAVAAL
Mtu_FtsW 274 LLGLFGLFAYTGMRIASRSADPFLRLLTATTTLWVLGQAFIIGYVIGVLPVTGQLPLISAGGFSTAATLSLIGIIANAARHEPEAVAAL
Cpa_FtsW 274 LLLLIIFYFLLVITVILKTNHPVLRLVQCSAIILLMIQTLLMAVATGIFPTTGQLPLFFSYGGNALLANLINCSFLIRLALETKDR....
Syn_FtsW 273 FLAFLGLFTTMGLRVAMRCRHRVKRLIGLGVVIFLVGQSLLITGVASGALPTTGLPLFFSYGGSSSCLSSLVLAGLLVRVARESNEAEVIP
Mtu_RodA 275 ILMGTTIVIIFGLRTAIATRDSFGKLLAAGISSTLAIQLFIVVGGVTRLIPLTGLTTPWMSYGGSSSLLANYVLLAILARISHGARRPLRTR
Mle_RodA 274 LLMGTTIVIVFGLRTAIATRDSFGKLLAAGLASTLAIQLFIVSGGVTTLIPLTGLTTPWMSYGGSSSLLANYVLLAILARISSARRPLRSR
Efa_FtsW1 270 ILGLLMFMIAFIILVGVRSKKPFNSLMCIGIGTMLLIQVFIVVGGITGIIPLTGITFPLLSQGGSNSLLIISIAVAFVLNLSADETRQKLEN
Spn_FtsW2 273 ILALLFFMILFIILVGIRAENPFNAMVALGVGGMMLVQVFVVIGGISGLLPSTGVTFPFLSQGGNSLLVLSVAVAFVLNLDASEKRAKLYR
Efa_FtsW3 268 LLFLLLFLAASILRSSCVIKNHCYGLFLLGVGTLILAQTAIIGGVLGLIPMTGIPLFFVSYGGSFSYLIFSVALGIVIKILIANERRQLNGQ
Ehi_FtsW 268 VLFLLFLLCMGIFQKSTKQKNQQANLLLIGVGTWILVQTSIIGSILGLIPMTGVPLFFVSYGGTSYLILSFAIGLALNISSRQVKEKNKQ
Pae_FtsW 274 TVALFVFVSLRALYIGIWAKQFSAYVAYGLAFLWIGQFLIIGVNVGLLPTKGLTLPFLSYGGSSLVICCACLGMLLRIEWERRTHLGSE
Efa_FtsW4 271 VVFLYFYFLFYQVIVAGLRSNSRGCMYICVALIFSLVFQTVEIGAVIGLLPLKGQGGTSLVMAITSLGFVKGREAA.....
Aae_RodA 272 LCSLFLLLFLRLIQYHEMAQRLSEKLFIAGTFSLILFQFTVTLMTMGMFVVCIPLFFVSVGGSATITFSAMIGVTQYLYKKYRYPYMKL
Bsu_SpoVE 270 ILLLFSVLLWRGIRIALGAPDLYGSFVAVGIISMIAIQVMIIGVVTGLIPVTGITLFFLSYGGSSLLTLMLMAVGVLLNVSRYSR......
Cac_FtsW 273 IIILFIVLIQRGIKIAMNAKDTYGMLLAVGITSVIAVQAIIIAVVTGSMPVTGVPLFFISYGGSSSLTFNLIAMGILLNISSQGN.....
Tma_RodA 250 LLSLFGLLFFEVSRWILNVKDEYWEILMVSACGLIWFHVFEVVSMNLGLLPVTGVPLFFISYGGTSTLVFSILVGLILKGIALARVEKK.
Hpy_RodA 264 LFAIYIGLSLHLFFYLFENSDWSLKIVALGISILIFVYSSVIAMTLGLAPVVGIPLLFISYGGSSSFITFNILFGILENILLAFRYIFGYNS
Cac_RodA 271 IMIGYFLLFYICMRAAVRAEDNISRLLAVGYSAMIGAEVIILVGGVTGMIPLTGITLFLISAGGSSMIMIFVALGILQGKISEGG.....
Bbu_FtsW 269 AISGFFLFFVFGYFFAIHSNSRSKFFIAFISSLAIFLQSMNIIIAIGLFPTGINLPFFSAGGSSIIVTMALSGLLSNVSKNLSN.....
Ctr_FtsW 263 LILLYMGFIYSGYVIAMRASLLSGAALAISITVIIGMQAFILGVVSGLLPSKGVNLPFFSQGGSSSLIANMCGMGLLLRMCDEENQQNRIG
Bsu_YlaO 273 VIFLLGFVVIKGFYIARKCEDPGSLLAIGSSNIAIQSFNILGVSGLIIPLTVTLPFISYGGSSLVLLLGSMGILANISMFVKYSENKK
Bsu_RodA 270 VVIMFFFLIYILLVVIIDKPFNRLASFFCVGYTALIVIHTFQIIGMNIGIMPVTGIPLLFVSYGGSSSLTSTLIGFGIVYNASVQLTKYRSYL
Tma_FtsW 266 VLFSFYGLVSSLVKVATKHTVPSVRTFISGFAILIMLQVMTVGVISGILPVTVTLLFVSYGGSSSLLSIMIGFGIVGNMILESER.....
Ctr_RodA 272 VLWLFYNLVCFGCRTVAVAVDDFGRFLAGVTVNLVMHVLIVSMMSGLLPITGVPVLIVSYGGSSSVISTMASLGILQSHTYSRRFAK.....
Hpy_FtsW 269 CFILFSVLIVLIFRIANRLKEPKYSLFCVGVLLISFSLVIAFGVGSGILPVKGLAVFFLSYGGSSSLLANCIATGLVLKLARYTK.....
Aae_FtsW 266 ILSLQFLFALSLFKYAVRIYGMYEKIILQVALNFLYSVFVVYAMALNIIPPKGIALFFISYGVGNLLSNMIMLGIVGSHYRRNSDVLN..
Pgi_FtsW 269 IPLAYFVLFFLAQIAQRTASRFEGMLLMGFGLLYLLQAMFFIVASGFTLVTGQTELLISKGGTSYLITSLAFGIMMSIRRIALNKENG
Ngo_FtsW 276 LIFCYGWLVVRAFSIGKQLGLTLNAYIASGIGIWIGIQSFFIGVNIGALPTKGLTLPLLSMGVGGSSVFFMLISMMLLLRIDYENRQKMRGY
Nme_FtsW 259 LIFCYGWLVVRAFSIGKQLGLTLNAYIASGIGIWIGIQSFFIGVNIGALPTKGLTLPLLSMGVGGSSVFFMLISMMLLLRIDYENRRKMRGY
Bbu_RodA 278 IIIDFFFLFFRFLIIMNKSQDRYMALVISGLGLLFFHTSFVVGMSLGVLPITGIPFFLSYGGSSTITFFLAMSFYFNISIVAM.....
Tpa_RodA 272 VFGLFLLLFFLHTSIMSHVDDLYGKLIASGVLGMFLFHFVVVGMTMGIMPITGIPLLLSYGGSSLWTAMIATGLLMSLNARQ.....
Pgi_RodA 272 LLIGYATLIIRIVALAERQTKVISRVYGHSVAAILLFHLSIVGMVIGVLPVTGIPLFFVSYGGSTLLIFIFLRLDADRDC.....
Ype_RodA 274 LLGLYLCLIMRGLVIAANAQTTFGRVMVGGLMLILFVYVFVNICMVSGILPVVGVPLFLVSYGGSALVVLMAGFGIVDVDTYSSKMVI...
Ype_FtsW 274 AALMVFFVAFRAMSNNSKIGQRRSGFLACSIGIWFSFQALDVVGAAAGMLPTKGLTLPLISYGGSSLIIMSTAIVLLRRIDFETRLAKAQA
Cte_FtsW 272 IILLFSGLFACGIIIAKHAPDLFGRYVAIGVTFAIVFFAFIIIAVACHLMPTTGVALFFISYGGTALLFNSLGIGLLVSLSRYRKKVETIE

Figure 4.2. *Multiple sequence alignment of RodA homologues.* The alignment is built with ClustalX and shaded picture produced by MacBoxshade programmes. Positions with a high percentage of identical or similar residues are shaded. For the purpose of predicting the topology of RodA, positions with gaps in RodA were removed. Protein names from the original annotations were used where possible. If annotation was not available (or was wrong), an attempt was made to distinguish RodA and FtsW orthologues based on sequence similarity and operon structure. Where this was not possible, proteins were named "FtsW" and numbered if more than one paralogue was present in a given species.
Species name abbreviations:

Aae - *Aquifex aeolicus*
Aac - *Actinobacillus actinomycetemcomitans*
Bbu - *Borrelia burgdorferi*
Bsu - *Bacillus subtilis*
Cac - *Clostridium acetobutylicum*
Cpa - *Cyanophora paradoxa*
Cte - *Chlorobium tepidium*
Ctr - *Chlamydia trachomatis*
Eco - *Escherichia coli*
Efa - *Enterococcus faecalis*
Ehi - *Enterococcus hirae*
Hin - *Haemophilus influenzae*
Hpy - *Helicobacter pylori*
Mtu - *Mycobacterium tuberculosis*
Mle - *Mycobacterium leprae*
Ngo - *Neisseria gonorrhoeae*
Nme - *Neisseria meningitidis*
Pae - *Pseudomonas aeruginosa*
Pgi - *Porphyromonas gingivalis*
Spn - *Streptococcus pneumoniae*
Syn - *Synechocystis sp.*
Tma - *Thermotoga maritima*
Tpa - *Treponema pallidum*

Complete genomic sequences were accessed through GenBank. Unfinished genome sequences were obtained from the corresponding institutions – The Institute for Genomic Research (Cte, Efa, Pgi, Spn), Oklahoma University (Aac, Ngo), Sanger Centre (Mle and Nme) and Genome Therapeutics Corporation (Cac)

The actual predictions of the possible membrane topologies of the RodA protein were made with the help of several computer programs. These included TopPred II (Claros and von Heijne, 1994) for methods based on hydrophobicity profiles (Engelman *et al.*, 1986; Kyte and Doolittle, 1982) and two other approaches using additional information from multiple sequence alignments - PredictProtein (Rost *et al.*, 1996) and TMAP (Persson and Argos, 1994) . A "positive inside rule" (von Heijne, 1992) was then applied to establish a possible membrane topology for the protein. Results from all these methods appeared to be in a good agreement with the BlaM fusion data in predicting ten hydrophobic regions within the RodA protein that could serve as membrane spanning domains (Table 4.1).

Segment No	KD	GES	PHD	TMAP
1	18 - 38	18 - 38	19 - 40	14 - 34
2	48 - 68	49 - 69	48 - 71	48 - 68
3	76 - 96	73 - 93	73 - 99	74 - 94
4	110 - 130	-	112 - 127	113 - 133
5	137 - 157	137 - 157	140 - 155	140 - 160
6	160 - 180	160 - 180	163 - 177	-
7	183 - 203	183 - 203	185 - 205	174 - 194
8	272 - 292	271 - 291	264 - 296	266 - 294
9	305 - 325	305 - 325	304 - 328	307 - 327
10	341 - 361	341 - 361	336 - 359	339 - 359

Table 4.1. *Boundaries of transmembembrane segments predicted in RodA by several computer programmes.* KD and GES are the predictions made by TopPred with the corresponding hydrophobicity scales. TopPred predicts two possible topologies with the Kyte and Doolittle scale. In the second one (not shown here) the fourth hydrophobic segment is ignored.

As noted above (Figure 4.1.), simple predictions based on the hydrophobicity profile had trouble with the fourth transmembrane

segment. However, both methods based on multiple sequence alignments detected it. TMAP disagreed with the other programmes in splitting the highly hydrophobic region between amino acids 137 and 205 in two transmembrane segments instead of three because it required a minimum of 20 amino acids for a transmembrane segment to be considered functional. Against the possibility of two transmembrane segments in this region is the fact that there are several conserved charged residues in this area that would appear right on the segment junctions if there were three membrane spanning domains here.

Final topology predictions from these methods differed in the number of transmembrane segments found. All predictions with 10 transmembrane segments suggested a location of the N terminus of RodA in the cytoplasm, whereas in the case of 9 membrane segments the N terminus was always predicted to be periplasmic. In addition, in the prediction in which the fourth putative transmembrane segment was omitted, a medium sized (about 40 aminoacid residues) cytoplasmic loop was predicted instead.

To resolve the uncertainties of these predictions, topological models of RodA protein were tested experimentally.

4.3. Construction of RodA-BlaM fusions.

Fusion with different topology probes is at present the most commonly used technique for determining protein topology. Three proteins are routinely used as topology probes - β-galactosidase, β-lactamase and alkaline phosphatase. The last two proteins have their signal peptides removed and rely on protein export signals from the N-terminally fused protein in question to manifest their activity as they are only active in the periplasm. LacZ is only active in the cytoplasm; therefore it can also be used as a topology probe, but in

the opposite manner. From information in the literature, β-lactamase seemed to be the most attractive candidate for a topology probe because there have been no problems reported with toxicity of fusion proteins (in contrast to the other two reporters) or leaky expression of cytoplasmic fusions (alkaline phosphatase). In addition, β-lactamase has the advantage over other reporters that fusions appearing to be in the "wrong" compartment (cytoplasm) could be distinguished from out-of-frame fusions by plating cells on ampicillin-containing agar at high cell density. In such conditions some cells will lyse releasing β-lactamase and allowing the rest to grow (Broome-Smith *et al.*, 1990).

The pJBS633 plasmid (Broome-Smith & Spratt, 1986) was chosen as vector for the construction of BlaM fusions (Figure 4.3.). This plasmid allows cloning into the *tet* resistance gene, thus placing a gene of interest under *tet* promoter-control if necessary, and blunt-end ligation to '*blaM* (5' truncated gene with the sequence coding for the signal peptide deleted) at the PvuII site. Two approaches were used for the construction of RodA-BlaM fusions. First, two fusions were made using suitable sites within *rodA*. This produced two plasmids. pZH24 has an *Nhe*I-*Eco*O109I fragment of the *rodA* gene from pADD5 cloned into the *Nhe*I-PvuII sites of pJBS633. Sticky ends produced by *Eco*O109I were filled in with Klenow polymerase for blunt end ligation. pZH25 has an *Nsp*I-*Ssp*I fragment from pADD11 cloned into the *Sph*I-*Pvu*II sites of pJBS633. This gave fusions to BlaM after amino acids 75 and 321 in RodA.

To create more fusions, the complete *rodA* gene with flanking sequences was cloned in pJBS633 as an *Nhe*I-*Sal*I fragment to give plasmid pZH28. pZH28 was then restricted with *Bam*HI downstream of *rodA* and subjected to nuclease *Bal*31 digestion followed by treatment with the Klenow fragment of DNA polymerase I, restriction with PvuII and religation. This produced a series of deletions in *rodA*

starting from the 3' end. These deletion plasmids were transformed into XL1-Blue cells and recipients were selected on kanamycin plates. In-frame fusions were first selected by patching the recombinant clones onto ampicillin plates (20 μg ml^{-1}). The size of *rodA* remaining on the fusion plasmids was first estimated using PCR and fusions widely spread across RodA were retained for further analysis. The exact positions of the selected in-frame *rodA::blaM* fusions were determined by DNA sequencing. 87 fusions were sequenced in total and 53 of these appeared to be unique.

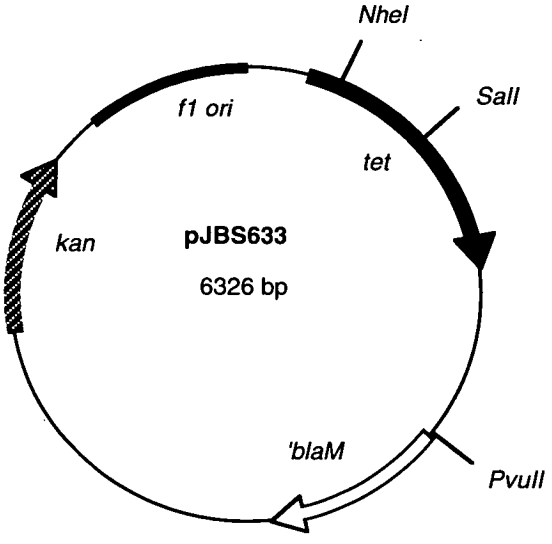

Figure 4.3. *The pJBS633 plasmid used for the construction of RodA-BlaM fusions.*

4.4. Levels of ampicillin resistance determined by RodA-BlaM fusions.

Minimal inhibitory concentrations (MICs) for ampicillin for 52 independent *rodA::blaM* fusions were measured. As the level of single cell ampicillin resistance is expected to correlate linearly with the amount of periplasmic β-lactamase (Jaurin *et al.*, 1982), the different

MICs detected in the fusion carrying clones should reflect the efficiency with which the N-terminal RodA segment of each fusion mediates the export of BlaM to the periplasm. Two main classes of fusions were observed - one with high (40-320 μg ml^{-1}) and one with low (1-5 μg ml^{-1}) resistance to ampicillin (the MIC for the XL-1 strain carrying the original pJBS633 is about 1 μg ml^{-1}), presumably reflecting the cytoplasmic and periplasmic locations of the corresponding parts of the RodA protein (Figure 4.4.).

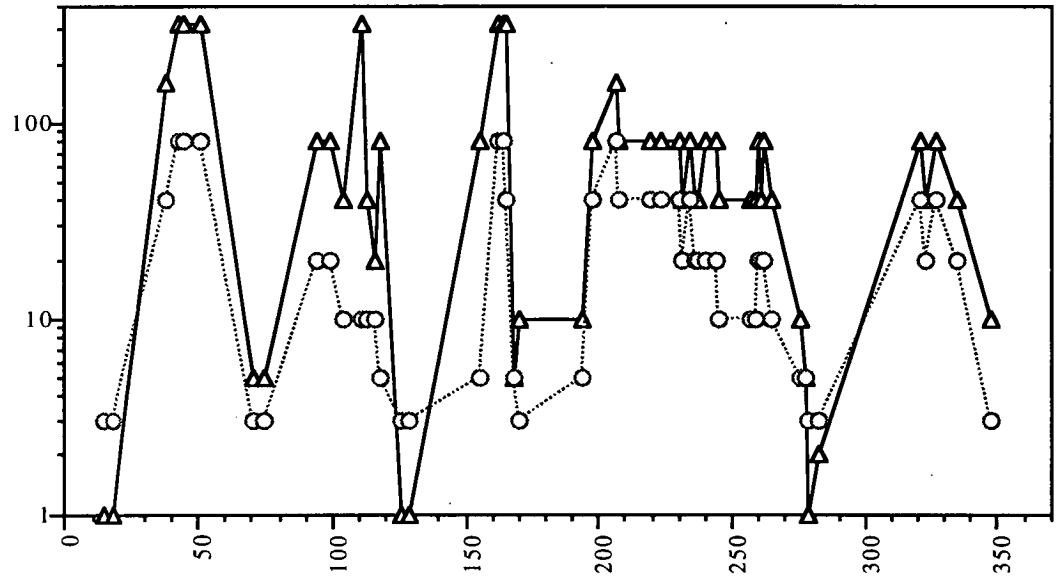

Figure 4.4. *Minimal inhibitory ampicillin concentrations for XL1-Blue (triangles) and JM109 (circles) cells with different RodA::BlaM fusion plasmids.*
Horizontal axis- number of the last RodA amino acid remaining in the fusion. Vertical axis - ampicillin MIC, μg ml^{-1}

Interestingly, MIC levels varied slightly between different strains. For strain JM109, they were lower than in XL-1 Blue by a factor of two on average, but that difference was more pronounced for fusion junctions in certain locations. The most significant difference appeared to be for residues 94-118 with MICs of 10-20 μg ml^{-1} in JM109 and 80-320 μg ml^{-1} in XL-1 Blue (Figure 4.5.). The MIC for the JM109 strain with the original pJBS633 plasmid was

higher - around 2.5 μg ml^{-1} *versus* 1 μg ml^{-1} in XL-1 Blue. The general trend is that the difference in ampicillin resistance levels between the two strains is more pronounced for fusions located at the ends of putative transmembrane segments. That is, where the last hydrophobic segment in the fusion is truncated, the fusion is likely to have a disproportionately higher ampicillin resistance level (characteristic of a periplasmic fusion) in XL-1 Blue than in strain JM109.

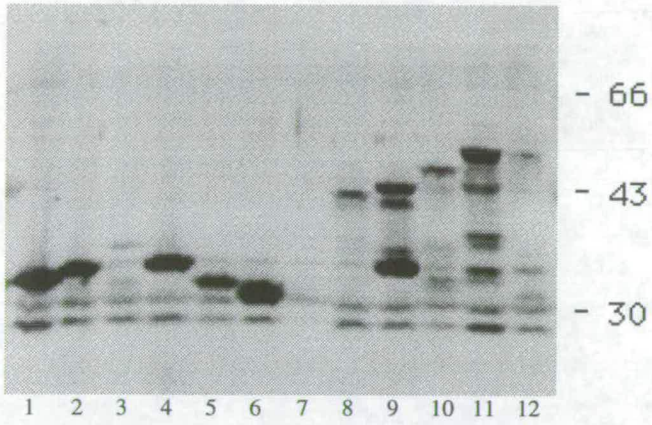

Figure 4.5. *Western blot analysis of RodA-BlaM fusions.*
Fusions to the following residues of RodA protein: 18 (lane 1), 43 (lane 2), 71 (lane 3), 111 (lane 4), 129 (lane 5), 162 (lane 6), 170 (lane 7), 194 (lane 8), 207 (lane 9), 245 (lane 10), 262 (lane 11) and 278 (lane 12). Protein samples were prepared from XL1-Blue strain harbouring the corresponding plasmids.

To check if the observed differences in the levels of ampicillin resistance are indeed determined by the different locations of the BlaM parts of the fusion proteins rather than by their relative stabilities, protein samples were subjected to a western blot analysis with anti β-lactamase antibody. Some variations in the signal intensities of the protein bands on Western blots were observed (Figure 4.5.). These could be explained either by the different stabilities of the fusion proteins *in vivo* or by problems during sample preparation. The first explanation does not seem to be likely because the observed amounts of proteins on western blots did not correlate

with the ampicillin resistance levels, and in many cases were exactly the opposite. In addition, no ampicillin resistant fusions were located in the regions predicted to be cytoplasmic by computer methods and, out of the small number of ampicillin sensitive fusions, none were located in the putative periplasmic loops. Therefore, although the real reasons for the observed differences in the amounts of proteins detected by western blotting are not clear, it is likely that the differences in the MICs conferred by different fusions do reflect the real location of the BlaM parts of the fusion proteins. Indeed, the fact that all putative periplasmic fusions appeared to be stable, whereas some of the predicted cytoplasmic fusions were not, may be taken as indirect evidence that periplasmic proteins are immune to degradation *in vivo*, whereas some cytoplasmic fusion proteins are accessible to protein degrading systems.

Also notable is the anomalous mobility of some of the hybrid proteins. This is not surprising because anomalous mobility on SDS-PAGE gels is typical for highly hydrophobic proteins and FtsW, a protein similar to RodA, also has a mobility higher than expected (Khattar *et al.*, 1994). Fusions to the extreme N terminal part of RodA and also after the first transmembrane segment move at the expected size. However, as more transmembrane segments are added, the mobility of the proteins increases, and a protein with over 160 extra amino acid residues has almost the same mobility as the wild type β-lactamase. Adding slightly more of the RodA polypeptide significantly decreases the mobility of longer fusions. Interestingly, the problematic fusions that are poorly visible on Western blots are at the position where the trend changes from increasing to decreasing mobility on PAGE.

4.5. The topological model of RodA protein.

The amount of experimental data combined with the computer predictions is sufficient to present a topological model of RodA protein. Fusion data argue in favour of ten transmembrane segments and strongly support the existence of the fourth hydrophobic region in RodA as a transmembrane segment.

Fusions to N-terminal amino acids (14 and 18) are cytoplasmic and fusions to amino acids after the first hydrophobic segment (positions 38-51) are exported to the periplasm. Therefore the N terminus of RodA is located in the cytoplasm and must be followed by a transmembrane segment with approximate coordinates 19-39. Fusions to positions 71-75 and 94-118 show that the next two hydrophobic regions also act as transmembrane helices. Ampicillin sensitive fusions to residues 126 and 129 show that the fourth stretch of hydrophobic residues, although short, is sufficient to keep the BlaM part of the fusion in the cytoplasm. This resolves the difference between computer predictions regarding this segment. The second cytoplasmic loop is followed by a rather long hydrophobic region (139-204). Most fusions to aminoacids in this region are ampicillin resistant, but ampicillin sensitive fusions to positions 168,170 and 194 suggest the presence of one cytoplasmic loop, hence there are three transmembrane segments in this region. Then follows a large hydrophilic region (residues 205-259). All fusions to this region are ampicillin resistant, confirming the existence of a large periplasmic loop here. Fusions to residues 276-283 are ampicillin sensitive, and should correspond to a cytoplasmic loop. And the presence of the fifth periplasmic loop is supported by ampicillin resistant fusions to residues 288-335. Finally, the fusion closest to the C terminus (to residue 348) has low ampicillin resistance (especially in JM101 strain - 3 mg ml^{-1}) showing that the C-terminus of RodA is cytoplasmic.

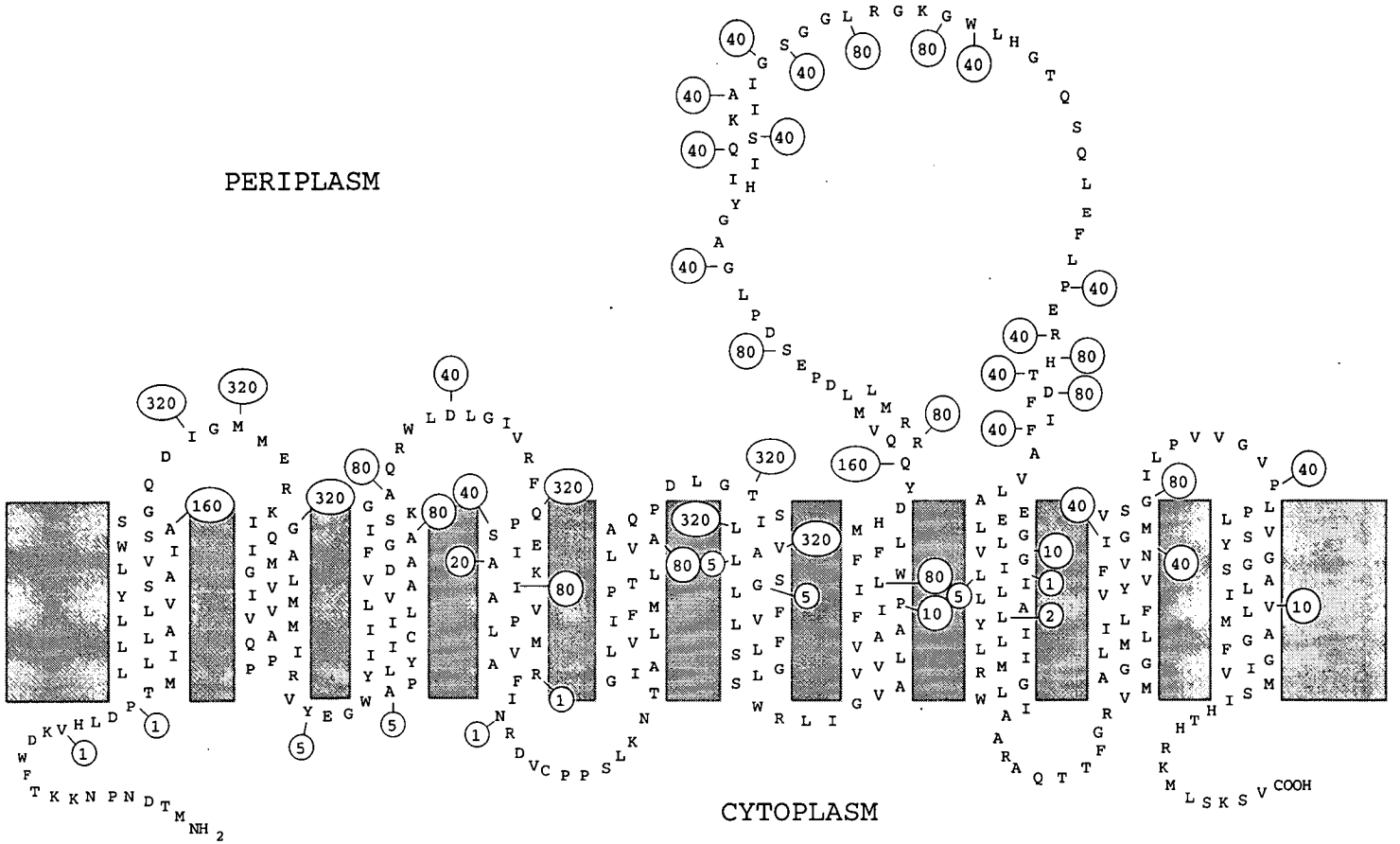

Figure 4.6. *The proposed topology of RodA protein.*
The drawing is schematic and only tries to show the topology of RodA rather than its real structure. Circled numbers are ampicillin MICs of corresponding fusions in the XL-1 Blue strain.

Taking these considerations into account, the topological model of RodA protein is shown in Figure 4.6. As the protein fusion technique is not precise enough to determine the boundaries of the transmembrane segments exactly, the positioning of the ends of the transmembrane domains shown in the figure is based on the computer analysis.

4.6. Determining the site of mutation in the *rodA52*^{ts} allele.

As the location of a mutation in a protein combined with the mutant phenotype may be helpful in elucidating its function, the position of the mutation in *rodA52*^{ts}, (the only RodA missense allele available in the lab) was found by sequencing. The gene was first amplified by PCR using primers flanking the coding sequence:

5' GCCATACAAGAATTCGCAAGTGG 3'

5' CATGCCGCAAGCTTTCCTGCC 3'.

Several precautions were taken to reduce the chance of an amplification error. A high fidelity thermostable Vent DNA polymerase was used. Amplified DNA fragments from two separate PCRs were used in two independent ligation reactions with plasmid pUC19. DNA was prepared from several recombinant clones obtained in each ligation, mixed in equal proportions and used for dideoxy chain termination sequencing with two primers mentioned above as well as five other internal *rodA* primers:

5' CACGCTGCTGGTGGCT 3'

5' TTGATGTTCCCAGGTCAG 3'

5' TTCTCCCCGAACGCCAT 3'

5' CGCCAGTACCGCGAAG 3'

5' CTGGCTGGACCTCGG 3'.

Two G->A mutations appeared to be present in the sequence at positions 827 and 977 leading to amino acid substitutions Gly_{276}->Asp and Ser_{326}->Asn. As the *rodA52*^{ts} allele was the result of nitrosoguanidine treatment, the presence of two mutations is not really surprising. Both mutations are located in the conserved C terminal part of the protein. From the topology perspective, the first mutation probably introduces the more drastic change, substituting a neutral residue for the negatively charged one, whereas the second mutation simply exchanges polar residues. As Gly_{276} is the fifth residue within the eighth transmembrane segment, introducing a charged residue here may destabilise the α-helical structure of this segment and thus render the protein non-functional at 42°. Sequence alignment of all available RodA/FtsW homologues shows that this residue is located within a highly conserved region and that this particular glycine residue is strictly conserved in almost all homologous sequences (Figure 4.2.). The second mutation, located at the C-terminal end of the next transmembrane segment, should not have a significant effect on the helical structure in this region because it substitutes a polar residue for another polar one and because this position is less conserved amongst RodA homologues (eight different residues occur at this position, although they are hydrophobic in most cases and none of them is asparagine). On the other hand, a Ser to Asn substitution is not considered to be very conservative and may still have some impact on the functionality of RodA protein.

4.7. RodA-like proteins in *E. coli* and other bacteria.

At the time of writing the growing family of known RodA homologues (ortho- and paralogues) consisted of about 50 complete and several partial sequences. Unfortunately, the functions are

known only for four of these proteins - two from *E. coli* (RodA and FtsW) and two from *B. subtilis* - SpoVE, a protein involved in sporulation (Ikeda *et al.*, 1989) and RodA (Henriques *et al.*, 1998). The only clear RodA and FtsW equivalents can be found in *H. influenzae* and *Actinobacillus actinomycetemcomitans*. The sequences from *Neisseria* are also sufficiently similar to that of *E. coli* FtsW. In several cases possible RodA and FtsW correspondence can be established on the basis of the operon structure, assuming that the RodA homologue located within the equivalent of the *mra* cluster is an orthologue of FtsW and another protein, located elsewhere (often paired with a PBP), is an orthologue of RodA. Of course, these assumptions may be misleading in a species with more than two RodA-like proteins. For example, *spoVE* in *B. subtilis* is located in the equivalent of the *E. coli mra* operon, yet *spoVE* mutants are only defective in sporulation and their vegetative growth seems to be normal.

As these proteins are from quite different bacteria, it is not surprising that not all proteins show good relations to other members of the group (Figure 4.7.). Still there are clusters of clearly similar proteins. The largest of these groups includes Eco_FtsW and seven homologues from other Gram negative bacteria, mostly enterobacteria: Nme_FtsW, Ngo_FtsW, Ype_FtsW, Aac_FtsW, Hin_FtsW, Pae_FtsW (abbreviations are as in Figure 4.2.). Another group includes five RodA orthologues: Ype_RodA, Aac_RodA, Hin_RodA, Pae_RodA. The next two groups include proteins from Gram positive bacteria: Bsu_YwcF, Efa_FtsW4, Efa_FtsW2, Spn_FtsW1 and Bsu_YlaO, Efa_FtsW1, Efa_FtsW3, Spn_FtsW2, Ehi_FtsW. It is tempting to speculate that one of these groups consists of FtsW paralogues, while another one consists of RodA paralogues, but the levels of similarity between the proteins from Gram negative and Gram positive bacteria are too low to say this for sure. Also notable are two cases of clustering of proteins from quite

distant bacteria - FtsW proteins from *H. pylori* and *T. maritima* and RodA proteins from *Mycobacteria* and *C. acetobutylicum*.

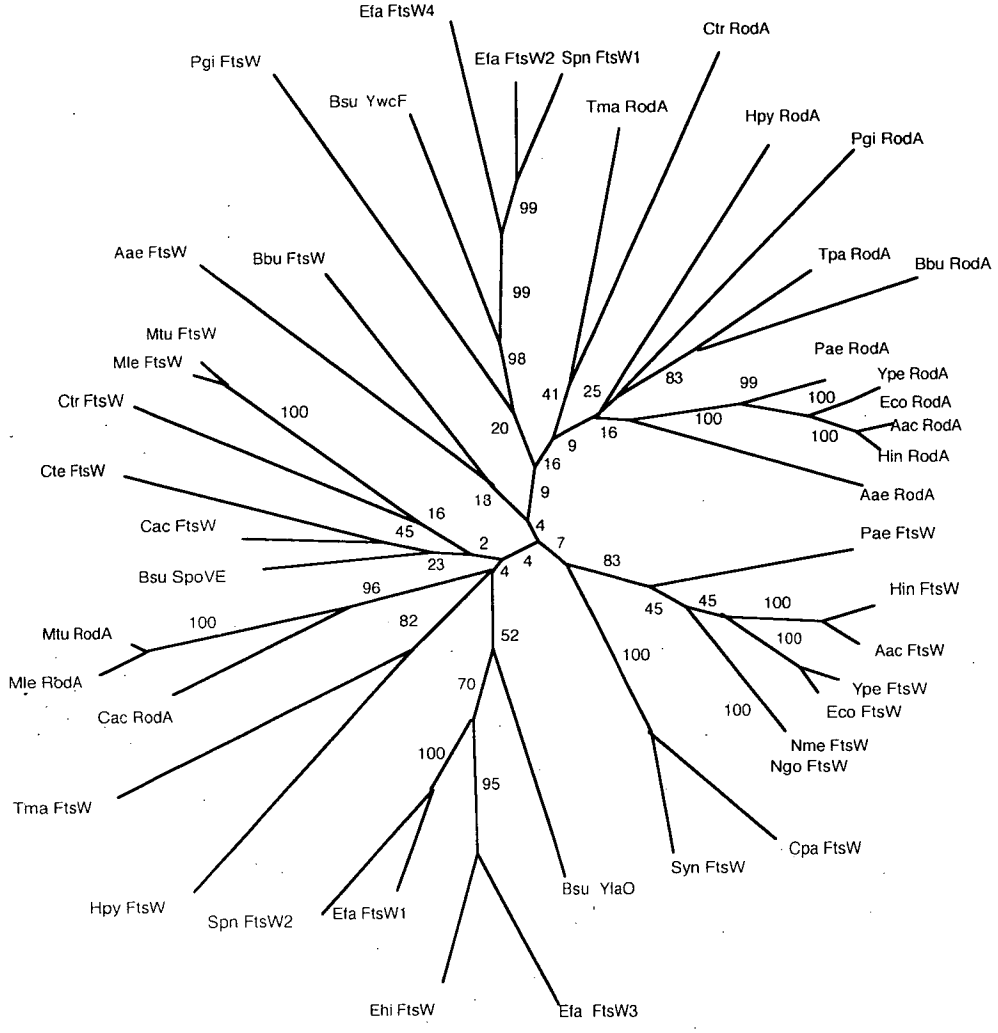

Figure 4.7. *Phylogenetic tree of the RodA/FtsW protein family.*
The alignment shown in Figure 4.2. was used as input for a phylogeny programme. Positions containing gaps in any of the sequences were removed. Also discarded was the part of the alignment corresponding to the first three transmembrane segments of RodA. The tree was built with the Protpars programme from the PHYLIP (Felsenstein, 1989) package. Branch lengths were calculated by the Puzzle programme (Strimmer & von Haeseler, 1996) using maximum likelihood. Numbers shown are PHYLIP bootstrap values. Species name abbreviations are as in Figure 4.2.

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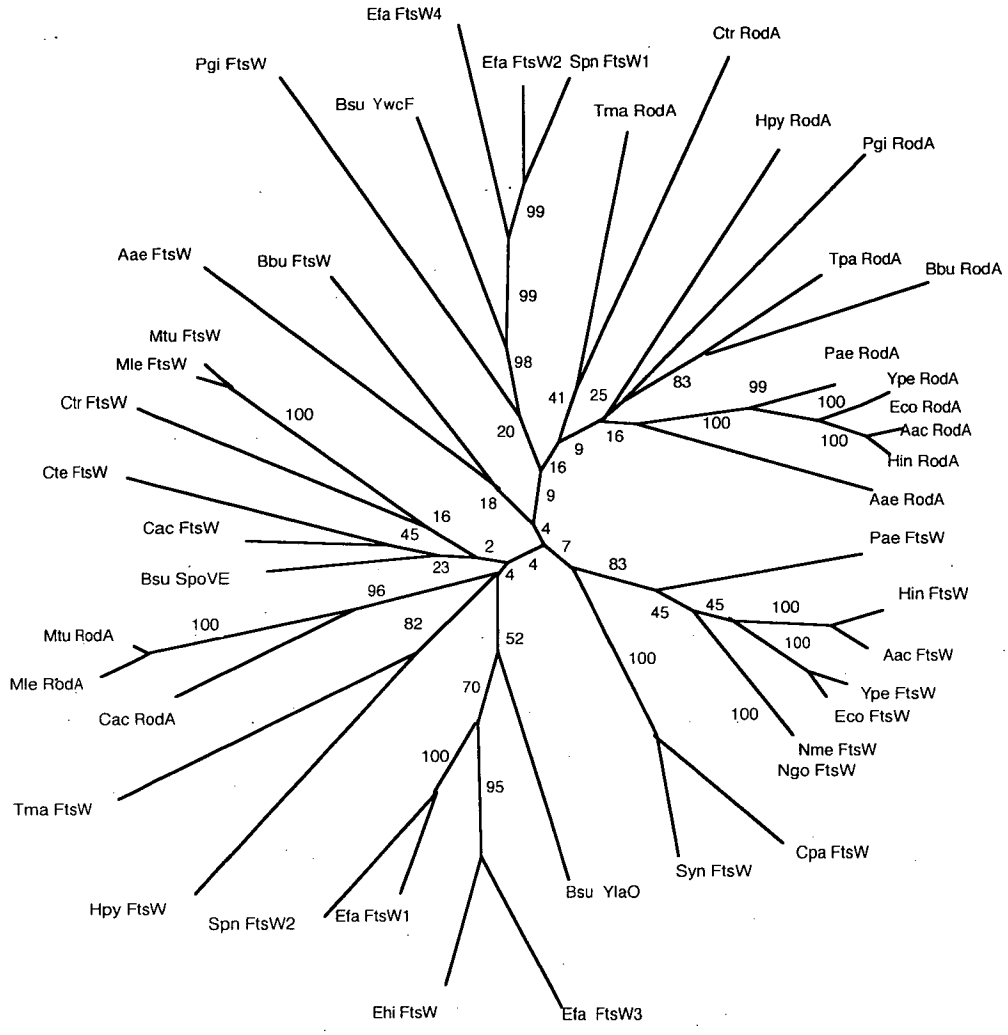


Figure 4.7. *Phylogenetic tree of the RodA/FtsW protein family.*
The alignment shown in Figure 4.2. was used as input for a phylogeny programme. Positions containing gaps in any of the sequences were removed. Also discarded was the part of the alignment corresponding to the first three transmembrane segments of RodA. The tree was built with the Protpars programme from the PHYLIP (Felsenstein, 1989) package. Branch lengths were calculated by the Puzzle programme (Strimmer & von Haeseler, 1996) using maximum likelihood. Numbers shown are PHYLIP bootstrap values. Species name abbreviations are as in Figure 4.2.

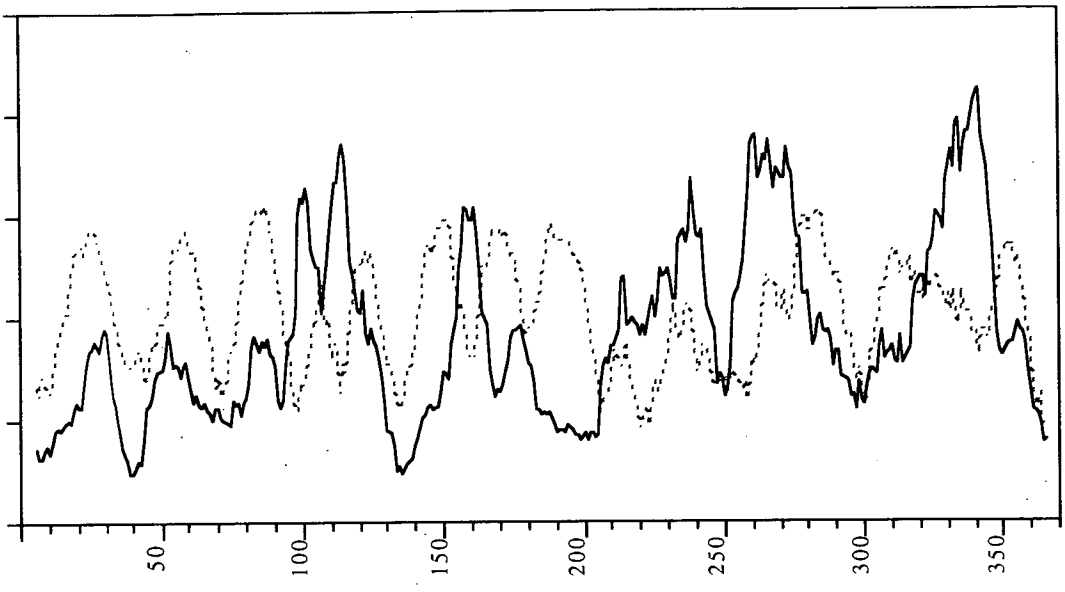


Figure 4.8. *Similarity across the multiple sequence alignment of RodA homologues.*
The plot is based in the alignment shown on Figure 4.2. Numbers of identical residues or hydrophobicity values (KD scale) were summed at a given position and then averaged across a window of 9 residues.
Solid line - similarity between RodA orthologues. Hydrophobicity profile (KD scale) is shown as the dotted line for comparison.
Horisontal scale – amino acid positions. Vertical scale – relative similarity or hydrophobicity.

The overall similarity within this group of proteins is not particularly high - around 30% identical residues, less for some proteins. The conservation across the length of the homologous proteins is not even. C termini are generally better conserved, and the hydrophobic regions seem to be significantly less conserved than the rest of the proteins (Figure 4.8.). Protein regions encompassing the equivalents of the first three transmembrane segments of RodA show almost no conservation apart from the presence of the number of hydrophobic residues required to form membrane spanning domains. In the remaining parts of the proteins, hydrophilic residues in the loop regions tend to be more conserved with the exception only of the fourth transmembrane segment, and even in this region the two residues that are most conserved are charged . The five longest conserved regions correspond approximately to residues 95-121,

155-164, 223-245, 253-280 and 317-347 of the RodA protein. The third and fourth conserved regions are located in the large hydrophilic region suggesting that this part has an important role in the function of RodA and orthologous proteins. Also remarkable is the high degree of conservation just before and inside the fourth hydrophobic segment.

Despite the low level of sequence similarity in the hydrophobic areas, hydrophobicity profiles of these proteins resemble each other well enough to allow us to speculate that the majority of these proteins may have the same topology as RodA from *E. coli*; *i.e.* 10 transmembrane segments, one large periplasmic loop and both ends of the protein located in the cytoplasm. There are, however, notable exceptions to this generalisation, inasmuch as several proteins have additional hydrophobic (possibly transmembrane) regions. Interestingly, all proteins with additional transmembrane segments are putative RodA equivalents, the topology of FtsW proteins seems to be more conservative. RodA proteins from two spirochetes have two transmembrane segments inserted after the seventh transmembrane domain (segment numbering as in Eco_RodA). RodA from *Porphyromonas gingivalis* even has four segments inserted in the same location. RodA from two *Mycobacteria* and *C. acetobutylicum* have two segments added to the N-terminus. Also, FtsW from *A. aeolicus* has a long hydrophilic piece inserted between the first and the second transmembrane segments. These changes, however, should have little impact on the overall topology of the proteins. That is, the parts of the proteins that have similarity to Eco_RodA, should have the same topological position, because the introduction of an even number of transmembrane helices into existing structure does not change the orientation of the remaining parts of a protein provided the distribution of the positive charge is not changed drastically. Addition of an odd number of segments in the middle of a protein is, however, different as it would require

reorientation of some of the preexisting segments. It is therefore not surprising that such an example is not found. However, in at least one case there is a hydrophobic segment added to the N terminus of a RodA homologue: and, interestingly, this is found in another protein from *E. coli*, FtsW.

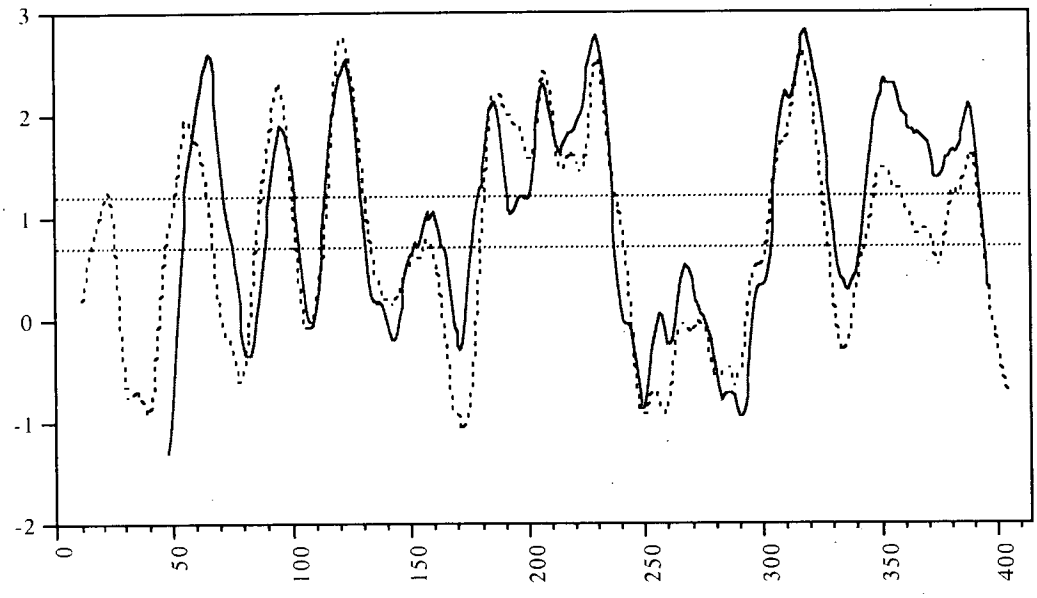


Figure 4.9. *Comparison of the hydrophobicity profiles of RodA (solid line) and FtsW (dotted line) proteins.* Profiles were drawn by TopPredII with KD scale. Coordinates are for FtsW protein: the RodA profile was shifted horizontally to align hydrophobicity peaks with FtsW.

Overall, FtsW seems to be remarkably similar to RodA and hydrophobicity profiles of these two proteins are almost indistinguishable (Figure 4.9.). There is, however, a striking difference between the sequences of the genes coding for these two proteins. There are two possible translation start sites of FtsW separated by 90 nucleotides. These 90 nucleotides, if translated, would add one extra transmembrane segment preceded by four positively charged residues. As the beginning of this extra segment has a strong positive charge, the longer FtsW protein is likely to be oriented with its N terminus located in the cytoplasm, and the

orientation of the next three transmembrane segments would therefore have to be the opposite to that of RodA protein. This, in turn, would mean that the most probable topology for the protein would exclude the next transmembrane segment (which is the one 'questionable' in RodA), forming a cytoplasmic loop with the rest of the protein oriented in the same way as RodA. Another model would have the opposite orientation of the first transmembrane segment and orientation of the remaining ten segments identical to that of RodA. The first model is preferable according to the "positive inside" rule, as the charge differences for the two models are 19 and 8. (Two related sequences, FtsW from *Synechocystis sp.* and *Cyanophora paradoxa* may have an additional N-terminal transmembrane segment, but in this case there is no strong positive charge in front and, hence, no topological problem.)

Neither of the FtsW start codons has a perfect ribosome binding sequence upstream, but the sequence near the second start codon is probably better suited for this purpose. Nevertheless, it has been shown that a longer protein can be expressed from a construct with an improved ribosome binding sequence in front of the first start codon and that this protein is functional, although toxic if overexpressed. Moreover, only the larger protein seemed to be detectable "*in vivo*" in wild-type cells (Khattar *et al.*, 1994). Therefore, in order to check whether the shorter protein is also functional, an attempt was made to complement an *ftsW*^{ts} mutation by a shorter variant of the FtsW protein.

A set of primers was designed to amplify a 5'-truncated *ftsW* gene, capable of coding for the shorter FtsW protein with the N-terminal transmembrane segment removed. The first primer included the sequence immediately upstream of the second possible start codon of *ftsW* and an *Nde*I site overlapping the start codon. This restriction site was introduced with the aim of improving the

ribosome binding site. This, however, proved to be unnecessary. The PCR product, produced using primers

5' ATGAATTCAAGGGCTGGCATATGGGCTCGC 3'

5' TCACCATTAAAAGCTTTCCTTGAC 3',

was cloned into pUC18 as an *EcoR*I-*Hind*III fragment to give pZH31. An *ftsW201* strain carrying this plasmid was able to divide at the restrictive temperature, even without IPTG induction of the *lacZ* promoter in front of the *ftsW* ORF. Although filaments were still present in the culture at high temperature, the complementation was better than with plasmid pJF118HEW producing the longer FtsW protein. This proves that the shorter FtsW protein is functional, at least to the extent of complementing a *ts* mutation. As the shorter version of FtsW protein, judging from the amino acid sequence, should be virtually identical to RodA in terms of topology, it would probably be correct to say that functional FtsW has the same topology as RodA. And if the longer protein really is the one that is active *in vivo*, it must have the same topology as the short one (and RodA). That means the only long hydrophilic part of the protein is located in the periplasm, making the previously suggested direct interaction with FtsZ (Khattar *et al.*, 1994) or the role of FtsW in stabilising the FtsZ ring (Khattar *et al.*, 1997) hardly possible.

A recent report, (Wang *et al.*, 1998) noted again that only a longer variant of FtsW is detectable *in vivo* using Western analysis. Although this does not exclude the possibility of existence of the short protein *in vivo*, it does stress the necessity of assessing the topology of the longer FtsW variant. A possible problem here is that antiserum was raised to a peptide starting from amino acid 34 in FtsW, which would be No 4 in the shorter protein – so it is possible that this region could have a different conformation in the two proteins with the one in the shorter variant not being detectable with this antibody. In addition, there was a step of affinity purification involved, presumably utilising the long FtsW - this would specifically

select against antibodies to the shorter form of FtsW, if the antigenic properties of that region in the two FtsW variants are different.

4.8. Discussion.

The evidence presented here shows that RodA in *E. coli* is an integral inner membrane protein with ten transmembrane segments and one large periplasmic loop. On the basis of its homology with RodA and very similar hydrophobicity profile, it is possible to suggest that FtsW has essentially the same membrane topology and that both proteins are likely to have very similar activities. Although the data discussed above do not show directly what these activities might be, the membrane topology of these proteins may give a hint to the answer. This type of integral membrane structure is usually a feature of membrane transporters or channels, and has only been observed in proteins involved in membrane transport. Indeed, some weak homologies of RodA with different classes of membrane transporters were detected during database searches. This makes RodA and FtsW good candidates for the function of the proposed membrane transporter that translocates bactoprenol linked peptidoglycan precursors to the periplasm, which is to date the only step in peptidoglycan synthesis with as yet unidentified participants. There is one additional feature of the RodA topology that favours this function. The fourth suggested transmembrane region in RodA is not highly hydrophobic and is short for a typical transmembrane segment. The previous segment is not very efficient (when compared to others) as an export signal for BlaM. What this might mean is that this pair of hydrophobic segments is not permanently inserted in the membrane, but can instead flip and flop between the cytoplasm and the membrane. Interestingly, these unusual characteristics of the fourth segment are well conserved among the homologous proteins.

Two charged residues within the segment are almost absolutely conserved in the homologous proteins. This suggests that the "unusual" composition of the fourth transmembrane segment is important for the function of these proteins. As these two residues are separated by two others, they appear to be located on the same side of the α-helix, an ideal location for interaction with a hydrophilic compound. Therefore the fourth transmembrane segment might be the part of RodA that contacts the hydrophilic part of the peptidoglycan precursor molecule, *N*-acetylglucosaminyl-*N*-acetylmuramyl(pentapeptide)-pyrophosphate undecaprenol, and possible switching of these two segments between integral membrane and cytoplasmic states might facilitate the transfer of the hydrophilic part of the specific substrate molecule through the membrane.

There is some evidence that RodA acts together with PBP2, which is encoded by another gene (*pbpA*) in the *mrd* operon (Ishino *et al.*, 1986) and it has been suggested that FtsW and PBP3 could form a similar pair (Matsuhashi *et al.*, 1990). Indirect support for this idea is provided by the similar operon organisation of these genes. Both pairs of membrane proteins are thought to be present in *E. coli* in very low amounts, and it is possible that both RodA and FtsW might act as transporters in close proximity to their corresponding PBPs, providing them with the necessary substrates. RodA/PBP2 and FtsW/PBP3 might even physically interact, forming parts of the multienzyme complexes responsible for the synthesis of peptidoglycan specific for either elongation or division (Ehlert & Höltje, 1996). It has been shown that apart from N-terminal membrane anchoring and C-terminal transpeptidase domains PBP3 has a large periplasmic domain of an unknown function (Goffin *et al.*, 1996; Nguyen-Disteche *et al.*, 1998). The amino acid sequence similarity suggests the same structure for PBP2. It is not known what the function of the middle domains in these PBPs is, but the presence of this domain is required for the functionality of at least

PBP3 (Goffin *et al.*, 1996). It is therefore tempting to suggest that this domain of PBP2/3 is required for the interaction with the corresponding substrate transporter, and the large periplasmic loop present in RodA and FtsW proteins might be the site of this interaction.

It is currently accepted that RodA protein is only needed for the maintenance of the rod shape. If this is really so, it would be logical to expect only rod-shaped bacteria to have RodA orthologues. As the similarity amongst RodA/FtsW-like proteins is low, it is often impossible to tell which of the paralogous proteins present in a given species is RodA and which is FtsW. Still, the very fact of the presence of more then one homologue in a bacterium could be indirect evidence that both RodA and FtsW are present. A rod-shaped bacterium needs at least two proteins (both RodA and FtsW), but a coccal one needs only one (FtsW). This seems to be true for at least three coccal bacteria: two *Neisseria* species and *Synechocystis sp.* These three bacteria have only one protein of the pair, and at least for *Neisseria* it is possible to say with all certainty that the protein in question is FtsW - not only there is a significant sequence similarity, but the protein is located in an exact copy of the *mra* cluster. There are, however, two other coccal bacteria that do have more than one RodA/FtsW homologue - *S. pneumoniae* and *E. faecalis*. An important difference between these two species and *Neisseria* and *Synechocystis* is that *S. pneumoniae* and *E. faecalis* form cell chains - it is therefore possible to say that their cells have polarity, just as rod-shaped cells do. These coccal bacteria are similar to rod-shaped bacteria in that the division planes are parallel to each other as opposed to perpendicular positioning of the division planes in "true" coccal forms like *rodA* mutants of *E. coli* (Begg & Donachie, 1998). The polarity in these cocci may be determined not by the cell shape, but by the sites of contacts between the neighbouring cells. Hence, the function of possible RodA proteins in these bacteria might be in

maintaining cell polarity rather than elongating the cell wall. How could this be achieved? One possible explanation would be that RodA in these proteins performs exactly the same function as it does in rod-shaped bacteria, *i.e.* controls the elongation of lateral cell wall, but that it does so much less efficiently. The result would be the synthesis of a minute amount of "lateral" cell wall. This amount would not be sufficient to turn the spherical cells into rods, but it would be sufficient to create "special" areas of peptidoglycan, such that septum formation would only be possible there. This would ensure that division planes in daughter calls would be parallel and that the original asymmetry would be maintained. This explanation is supported by a demonstration that certain cocci can elongate and become rod-shaped if treated with antibiotics inhibiting septation (Lleo *et al.*, 1990).

CHAPTER 5

ROLES OF FTSA AND FTSZ IN ACTIVATION OF DIVISION SITES

5.1. Introduction.

E. coli cells made spherical by *rodA* or *pbpA* mutations are unstable when allowed to grow fast, e.g. in LB medium (Rodriguez & de Pedro, 1990). The cells of *rodA* and *pbpA* temperature sensitive mutants increase in size after the temperature shift and eventually lyse after several divisions (Vinella *et al.*, 1992). This makes construction of knockout mutations in these genes difficult. The isolation of an amber mutant in the *rodA* gene was only possible in a suppressor strain where some RodA was still produced (Begg *et al.*, 1990). Moving the amber mutation into suppressor-free background proved to be extremely difficult, but Ken Begg in this lab found that *rodA* null strain can be easily maintained on minimal medium. He further isolated a stable variant, KJB24, that had somewhat smaller cells but was able to grow in rich medium. Some time later Ken found that increased stability is explained by the presence of a second copy of transposon Tn5 (which was used as linked marker to transduce the $rodA^{am}$ mutation to suppressor-free background). This copy of Tn5 mapped in 0-2 minute region of the chromosome. The insertion also appeared to confer a distinct phenotype. When this copy of Tn5 was transduced into W3110, it caused minicell production, reduction in the mean cell length from 3.9 μm to 2.8 μm and an increase in the proportion of visibly constricted cells from 14% to 29%. Therefore an attempt was made to investigate this phenomenon further to find out the reason for the increased stability of the *rodA* null mutant

5.2. The suppression of lethality of *rodA* mutations in rich medium is due to the insertion of Tn5 in *ddlB* .

To determine the position of the Tn5 insertion it was first cloned from the chromosome of the W3110 strain carrying the "wee cell" Tn5 insertion as an *Eco*RI fragment in pUC19. About 300 bases were sequenced from each side of the cloned fragment and this sequence was compared to that of the *E.coli* chromosome. Both sequenced fragments appeared to belong to the *ddlB* gene located in the *mra* cluster at 2 minutes. The precise position of the Tn5 insertion is after base 211 of the *ddlB* ORF (Figure 5.1). The orientation of the transposon in the chromosome was then determined by PCR with primers on ether side of *ddlB* and one primer matching the sequence of the *kan* gene of Tn5. The orientation of the Tn5 insertion was such that transcription of the *kan* gene opposed that of *ddlB*.

```
CGCGGCGGTGAAGATGGTAC CTGACTCTTATACACAAGTC
ddlB <-'  '-> Tn5
```

Figure 5.1. The sequence of the *ddlB::Tn5* junction.

To confirm that this insertion of Tn5 is indeed responsible for the suppression of lethality in *rodA* strains, the *ddlB::Tn5* insertion was transduced into JM101 *rodA*^{ts}. The introduction of this insertion made this *rodA*^{ts} strain stable at the restrictive temperature: spherical cells became a bit smaller and didn't lyse when grown in rich medium.

The location of the insertion was surprising at first as it was not immediately obvious how the inactivation of the *ddlB* gene could lead to the observed phenotypic effect. *ddlB* codes for D-Ala-D-Ala ligase, an enzyme that is essential for peptidoglycan synthesis, so its inactivation is expected to be lethal. The original *ddlB* mutant was a temperature sensitive lethal, supporting this expectation. A second

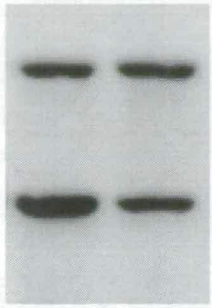
gene with the D-Ala-D-Ala ligase activity, *ddlA*, was discovered later in *E. coli* (Zawadzke *et al.*, 1991), but it was not known whether this gene is essential, although in experiments *in vitro* D-Ala-D-Ala ligase activity of the *ddlA* product was higher than that of DdlB. The viability of the Tn5 insertion within the *ddlB* gene demonstrates that *ddlB* is not essential and suggests that *ddlA* can supply sufficient amounts of D-Ala-D-Ala ligase. It is therefore not clear why the original *ddl* mutation was lethal. One possibility would be for this mutant to have a secondary ts mutation in one of the downstream cell division genes.

Another important inference from the *ddlB::Tn5* insertion is that a polar insertion within the *mra* gene cluster does not prevent expression of essential downstream genes, even 'though promoters located in the beginning of this cluster are thought to be required for full transcription of all the genes in the cluster (Flärdh *et al.*, 1998).

5.3. The *ddlB::Tn5* insertion causes overproduction of FtsZ protein.

It is known that the transcription of *ftsQ*, *ftsA* and *ftsZ* genes is dependent on the promoters located far upstream (reviewed in (Vicente *et al.*, 1998)). Even though the largest amount of FtsZ, the product of the most distal of the three genes, was initially thought to result from transcription from the promoters located within *ftsA* and *ftsQ* (Flärdh *et al.*, 1997; Garrido *et al.*, 1993), transcription originating from the promoters located further upstream also appeared to be important for the viability of *E. coli* cells(Flärdh *et al.*, 1998; Mengin-Lecreulx *et al.*, 1998). Therefore it would be predicted that the insertion of Tn5 within *ddlB* would result in decreased expression of the downstream genes. Therefore the amount of FtsZ

protein was measured in W3110 *ddlB::Tn5* cells. Surprisingly, the level of FtsZ in these cells was found to be increased (Figure 5.2), although the higher levels of FtsZ fitted the observed phenotype better and were in accordance with the earlier described phenotype of strains overexpressing *ftsQAZ* from a multicopy plasmid (Ward & Lutkenhaus, 1985). The level of FtsZ in the *ddlB::Tn5* strain was increased 5-6 fold on average.



1 2

Figure 5.2. *Western blot analysis of FtsZ in W3110 ddlB ::Tn5 (lane 1) and W3110 (lane 2) strains.*
Upper bands correspond to GroEL, lower bands - FtsZ.

It was not immediately obvious how the insertion of a transposon within *ddlB* could cause such an increase in the amounts of the product of at least one of the downstream genes. Rather, a decrease in production was expected. One possible reason for the observed effect could be the presence of a strong promoter reading out of Tn5. It is indeed known that Tn5 contains at least one strong promoter: the one driving transcription of the kanamycin phosphotransferase gene. This promoter, however, initiates transcription in the opposite direction to transcription of the *mra* operon. There is also a slight chance of a promoter having been created at the transposon-*ddlB* junction, but no promoter consensus sequence could be recognised in the junction sequence. Another possible mechanism for the observed increase in expression of the genes downstream of the Tn5 insertion would be some sort of

suppressor mutation compensating for the presumably lethal decrease of transcription of the essential cell division genes *ftsQAZ*. This suppressing mutation, however, must be located very close to the site of the Tn5 insertion, presumably right after it, because this copy of Tn5, together with its characteristic phenotype, could be transduced to another strain with normal frequency. A more attractive explanation could be promoter occlusion in that portion of the *mra* operon. Promoter occlusion happens when several promoters are present in front of an ORF, and transcription from the upstream promoter prevents the initiation of transcription from the downstream one (Adhya & Gottesman, 1982; Hausler & Somerville, 1979). This could happen even if the upstream promoter is a relatively weak one. So if there is a promoter in front of the site of Tn5 insertion causing the occlusion of the (stronger) downstream promoters, the insertion could release them from that sort of repression and cause the observed increase in the production of FtsZ protein. Indeed, the presence of promoters upstream of the *ddlB* gene has been known for some time and these have been recently shown to be responsible for two thirds of the transcription normally reaching *ftsZ* (Flärdh *et al.*, 1998). Insertion of Tn5 would interrupt transcription coming from these promoters and activate promoters located downstream. Indirect support for this mechanism of increased FtsZ production is provided by the fact that minicell production is pronounced in a strain carrying the *ddl::Tn5* insertion only in the late exponential and early stationary phases of culture growth. This most likely indicates that the increased levels of FtsZ protein production only occur at high cell densities. The first two promoters located downstream from the site of Tn5 insertion are SdiA and RpoS dependent, which means they are only induced in the stationary phase (Sitnikov *et al.*, 1996), and the observed growth phase dependency of FtsZ overexpression exactly corresponds to what could be expected if one (or both) of these two promoters was

activated after transcription coming from the upstream promoters was blocked.

After this work was completed, an insertion of the omega interposon into the *ddlB* gene was described (Flärdh *et al.*, 1998). As the omega interposon contains strong transcriptional terminators and was inserted 88 bases upstream of the site of Tn5 insertion described here, omega insertion would be expected to have a phenotype similar to that of Tn5 insertion. This, however, appeared not to be so - FtsZ transcription was decreased rather than increased. This discrepancy can be due to all measurements of *ftsZ* transcription being done in early log phase, whereas all measurements of FtsZ protein levels described here were done in the early stationary phase.

5.4. Effects of increased FtsA and FtsZ levels on the phenotype of wild type cells.

As mentioned in 5.1, the *ddlB::Tn5* insertion had a distinct effect on the phenotype of the otherwise "wild type" strains. As the *ddlB::Tn5* insertion results in increased levels of FtsZ (at least), these results are not really surprising and correspond in principle to the phenotype of strains with the pZAQ plasmid which carries additional copies of *ftsQAZ* (Ward and Lutkenhaus, 1985). As the pZAQ derivative with the *ftsZ* gene deleted didn't give this phenotype, Ward and Lutkenhaus attributed it to the increased expression of FtsZ protein alone. In our experiments, however, this didn't appear to be a complete explanation because the phenotype of strains with pSUZ, overproducing FtsZ alone, was clearly different - minicells were produced, but cells retained their normal length.

To determine which genes in pZAQ are necessary for the increased division capacity, frameshift mutations (potentially causing

the least alteration to the transcription of the cloned genes) were introduced into *ftsQ*, *ftsA* or *ftsZ* in pZAQ. To achieve this, pZAQ was cut with an enzyme within the coding frame of a gene of interest (*Bst*BI, *Bgl*II, and *Mlu*I for *ftsZ*, *ftsA*, and *ftsQ*, respectively), sticky ends were filled in with the Klenow fragment of DNA polymerase I and re-ligated to give a two or four base pair insertion, causing a frameshift in the desired gene. This produced three plasmids, each with one gene inactivated - *ftsQ* in pZAQ*, *ftsA* in pZA*Q and *ftsZ* in pZ*AQ (Figure 5.3). The inactivation of the desired gene was confirmed first by restriction analysis and then by DNA sequencing.

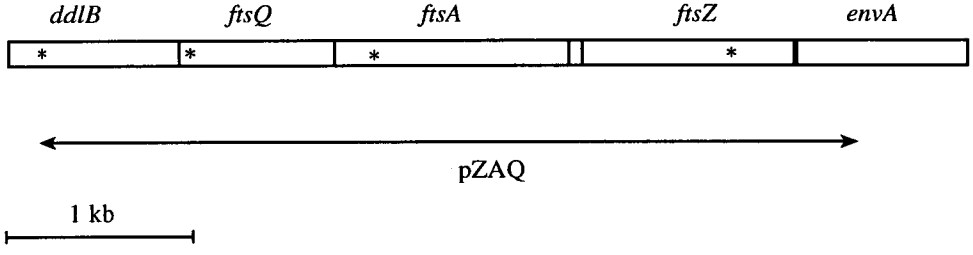


Figure 5.3. *Scheme of the ddlB-envA region.*
Coding regions of the genes are shown as boxes. Asterisks denote the sites of insertions in each gene (2-4 bases in *ftsQAZ* and Tn5 in *ddlB*). Arrowheads indicate the ends of the chromosomal fragment cloned in pZAQ.

As it was possible that a frameshift in one of the genes could lead to the altered expression of the others, the presence of active products was first tested in complementation tests with temperature-sensitive mutants *ftsZ84*, *ftsA13*, *ftsA22* and *ftsQ1*. In all three cases the plasmids were able to complement two mutants out of three confirming that only the gene in which the frameshift was introduced had been inactivated. To completely remove the possibility of unexpected phenotypic effects due to different levels of gene expression in these plasmids, the amounts of FtsZ and FtsA proteins were tested in Western blot measurements (Figures 5.4 and 5.5). The

results of these experiments show that the relative levels of FtsA and FtsZ do not change significantly in these plasmids unless the frameshift is in the gene itself (with the possible exception of slightly lower FtsA level in pZAQ*). In addition, Figure 5.5 demonstrates that amount of FtsA is indeed increased as the result of *ddlB::Tn5* insertion. Interestingly, the amounts of FtsZ and FtsA proteins produced by strains with pZAQ and its derivatives were close to these in *ddlB::Tn5* strain (Table 5.1 and Table 5.2).

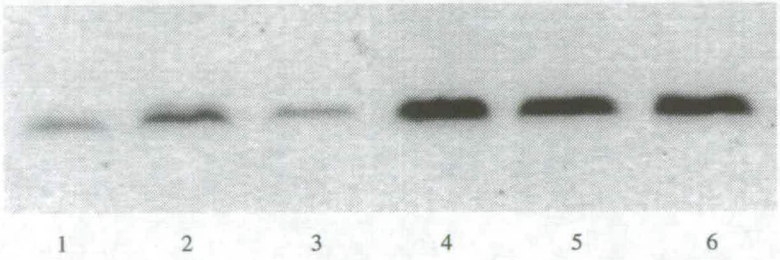


Figure 5.4. *Western blot analysis of FtsZ in JM101 strain with plasmids pZ*AQ (lane 1), pSUZ (lane 2), pBR322 (lane 3), pZA*Q (lane 4), pZAQ* (lane 5), pZAQ (lane 6).*

The plasmids with frameshifts described above were used to determine which genes are necessary for minicell production and early central divisions. This confirmed the earlier report that pZAQ reduces cell length at division and induces the production of minicells (Ward and Lutkenhaus, 1985). Contrary to that report, however, it appeared that, whereas overexpression of FtsZ alone was sufficient to induce minicell production, it did not increase the frequency of central divisions. For central divisions to occur at shorter cell lengths (*i.e.* earlier in the cell cycle), overproduction of both FtsA and FtsZ was necessary (Table 5.3.). Overproduction of FtsQ appeared to make no changes to the observed phenotypes.

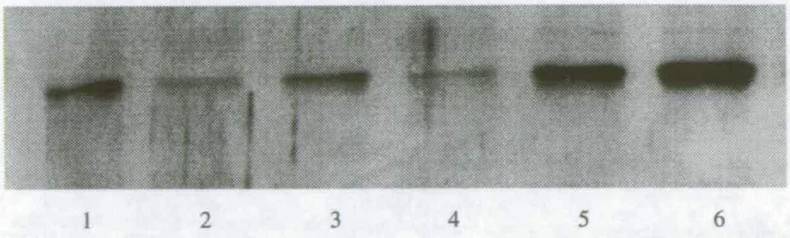


Figure 5.5. *Western blot analysis of FtsA overproducing strains W3110 ddlB::Tn5 (lane 1), JM101/pBR322 (lane 2), JM101/pZAQ* (lane 3), JM101/pZA*Q (lane 4), JM101/pZ*AQ (lane 5) and JM101/pZAQ (lane 6).*

Strain	FtsZ amount
JM101/pZ*AQ	1.2
JM101/pSUZ	3.2
JM101/pBR322	1
JM101/pZA*Q	7.4
JM101/pZAQ*	6.4
JM101/pZAQ	6.9

Table 5.1. *Relative amounts of FtsZ protein.*
Calculated by measuring intensity of bands on scanned films of the western blot shown on figure 5.2. Values corrected for loading relative to GroEL and normalised to the value for JM101/pBR322.

To check which of the three genes whose expression is potentially increased in a *ddlB::Tn5* strain is necessary for the stability of *rodA* strains in rich media, Ken Begg introduced three pZAQ derivatives with frameshifts into a freshly constructed strain with *rodA(am)* mutation in a *sup*^{o} strain (W3110). Only pZAQ* was able to suppress the lethality of *rodA* mutation, hence, increased amounts of both FtsA and FtsZ are necessary for this suppression to occur.

Strain	FtsA amount
W3110 *ddlB*::Tn5	2.9
JM101/pBR322	1
JM101/pZAQ*	2.4
JM101/pZA*Q	0.6
JM101/pZ*AQ	4.8
JM101/pZAQ	6.3

Table 5.2. *Relative amounts of FtsA protein.*
Calculated by measuring intensity of bands on the western blot shown on figure 5.5. Values corrected for loading relative to GroEL and normalised to the value for JM101/pBR322.

Strain	mean cell length, μm	constrictions, %	
		central	polar
JM101	4.8	16	0
JM101/pZAQ	3.4	20	12
JM101/pZAQ*	3.9	22	10
JM101/pZA*Q	5.1	13	7
JM101/pZ*AQ	4.3	17	0

Table 5.3. *Phenotypic characteristics of JM101 strain with pZAQ plasmid and its frameshift derivatives.*
The measurements shown here and those presented in Table 5.4 were done by Ken Begg.

5.5. Simultaneous overexpression of FtsA and FtsZ is necessary and sufficient to increase the number of central divisions in *minB* mutants.

minB mutants form the same number of septa as normal *E. coli* cells, but half of these are made in the wrong place: at the cell poles, because the reuse of these potential division sites is not blocked. A *minB* population consists of a mixture of minicells and rod-shaped cells of variable lengths, indicating that this mutant does not have enough "division potential" to form septa at all available division sites (Donachie and Begg, 1996; Teather *et al.*, 1974). Bi and Lutkenhaus (1990) achieved division at all potential division sites in *minB* strain by transforming it with pZAQ; as the inactivation of *ftsZ* gene in the plasmid prevented these additional divisions, they concluded that FtsZ alone is limiting in *minB* strain and providing additional copies of *ftsZ* is sufficient to restore "missed" divisions.

As the increased amounts of both FtsA and FtsZ seem to be responsible for the increased frequency of central divisions in normal cells and for the stability of fast growing *rodA* strains indicating increased "division potential" of the cells overproducing these two proteins, it looked worthwhile to check if in another situation with division deficiency, *i.e.* in the *minB* mutant, higher levels of FtsA are necessary in addition to higher levels of FtsZ for the restoration of divisions in central sites and bringing the length of *minB* mutant cells back to normal. Therefore both the *ddlB::Tn5* insertion and the set of pZAQ derivatives described above were introduced into the *minB* strain. The introduction of pZAQ or *ddlB::Tn5* insertion restored central divisions in the *minB* mutant. As with the wild type and *rodA* cells, the presence of intact copies of both *ftsA* and *ftsZ* was necessary to achieve additional central divisions (Table 5.4).

These results demonstrate once again that, contrary to the conclusions of the earlier work (Bi & Lutkenhaus, 1990) but in

accordance with the published data, FtsA and FtsZ together determine the division potential of *Escherichia coli* cells.

STRAIN	mean cell length, μm
P678	3.9
P678-54 (*minB*)	6.7
P678-54/ pZAQ	4.7
P678-54/ pZAQ*	4.1
P678-54/ pZA*Q	8.1
P678-54/ pZ*AQ	6.5
P678-54 *ddlB::Tn5*	4.4

Table 5.4. *Cell lengths of a minB strain and its derivatives.* Minicells are excluded from the calculations.

5.6. Division potential and a model for FtsA/FtsZ action.

The experiments described above show different situations in which increasing the amounts of both FtsA and FtsZ led to the same phenotypic effect: a higher frequency of central divisions. In case of *rodA* mutants this results in the reduction of the average cell volume and allows the cells to grow successfully in rich media, avoiding lysis which would otherwise occur as the result of the inability of the steadily enlarging cells to complete divisions. In another situation in which division capacity is less than the number of potential division sites, *i.e.* in the *minB* mutant, increase in FtsA and FtsZ levels reduces the average cell length to normal by allowing extra central divisions to occur. And if the levels of those two proteins are increased in wild type cells, these cells get shorter because of a reduction in the period between chromosome segregation and division, and minicells are also produced.

These experiments clearly demonstrate that in three cases the amounts of only two of the many proteins involved in cell division, FtsA and FtsZ, are limiting. It appears that other components of the division machinery are in excess, although for many of these proteins only few molecules are present per cell. The amounts of FtsA and FtsZ seem to be precisely maintained at the levels exactly required for the approaching division to occur. Therefore it is the amounts of these two proteins that determine the "division potential" of *E. coli* cells. This suggests that the amounts of FtsZ and FtsA may be responsible for determining the right moment for the cell to divide. FtsZ and FtsA levels (or the levels of their active conformations) are insufficient for one division to occur immediately after another, and the cells are only able to divide when the concentrations of these two proteins finally reach the required level. This explains how small alterations in the concentrations of these proteins lead to defects in cell division.

While the result of the simultaneous overexpression of both FtsA and FtsZ is the same in all cases studied here and could be attributed to increase in the division potential, the consequences of overexpressing FtsZ alone are a bit more difficult to explain. If both FtsA and FtsZ are necessary for normal divisions, why are additional divisions at cell poles possible when only the concentration of FtsZ is increased? There is data arguing for FtsA molecules being used in central divisions only once (Donachie *et al.*, 1979). This may be due to FtsA remaining sequestered at cell poles after the completion of division (Tormo & Vicente, 1984). If these sequestered FtsA molecules remain functional, then providing additional FtsZ may be sufficient for polar divisions to occur. The situation, however, is complicated by the existence of Min proteins known to be inhibitors of polar division.

MinC and MinD proteins act together as an unspecific inhibitor of cell division, while MinE allows division to proceed at the cell

centre (de Boer *et al.*, 1992; de Boer *et al.*, 1989)]. Studies on overexpression of MinCD led to the suggestion that it is an antagonist of FtsZ (Bi & Lutkenhaus, 1990), which was later supported by the observation that MinCD overexpression inhibits the formation of the Z ring (Bi and Lutkenhaus, 1993). However, use of a yeast two-hybrid system failed to disclose any interactions between FtsZ and MinCD, although interaction between the components of the Min system was detected (Huang *et al.*, 1996). Overexpressed MinC protein can inhibit division on its own and this inhibition is insensitive to MinE, therefore it was suggested that MinC is the actual inhibitor of division, and MinD is required to activate MinC and to provide the sensitivity to MinE (de Boer *et al.*, 1992). If the components of the Min system are indeed the direct inhibitors of Z ring formation, then polar divisions resulting from the overexpression of FtsZ could only be due to FtsZ titrating the inhibitor from the polar sites. In this case the difference in the phenotypes of a wild type strain overexpressing FtsZ and a *min* mutant could not be explained, because the FtsZ overexpression should effectively remove the MinCD inhibition, mimicking the result of *minB* mutation and leading to the same division deficiency: missed divisions and longer cells. As overexpressing FtsZ in wild type cells does not lead to missed central divisions and cells retain their normal lengths, the inhibitory effect of MinCD on FtsZ must be indirect.

A more likely scenario for the action of Min proteins would be that FtsA rather than FtsZ is the immediate target of MinCD inhibitor and FtsA is not inactivated, but simply immobilised at poles after division. In wild type cells, each pole after division would have half of the original quantity of FtsA. These FtsA molecules would be unable to move to the site of next division, and FtsZ concentration would be insufficient for any divisions to occur. New FtsA molecules would be made together with FtsZ and, once the concentration of FtsZ required for division was reached, division would occur at the cell centre,

possibly because by that time the amount of free FtsA available for central division would be double that of FtsA sequestered at each pole. If the concentration of FtsZ is increased, polar divisions can occur, which suggests that amounts of FtsA equal to half of those present at the time of central septation are sufficient for division. Central divisions in this case may be limited by the available amount of "free" FtsA, which should be close to zero immediately after division. However, if FtsA requirements for central divisions are the same as for the polar ones, overproducing FtsZ should lead to earlier septation, as lower concentrations of FtsA are apparently sufficient for polar divisions. This does not happen, indicating that either FtsA requirements for central divisions are higher than for the polar ones, or some other mechanism may determine the preference for the central division site. Min proteins are the obvious candidates for this role.

In *minB* mutants FtsA would not be sequestered at poles, and all divisions would depend on "free" FtsA. In this case both polar and central division would draw FtsA from the same cytoplasmic pool and hence use up a "quantum" of FtsA, as well as FtsZ, and these proteins would have to be resynthesised for the next division to occur. Therefore a polar division would result in one central division being skipped, which would eventually lead to cells of variable lengths being present in the populations of *minB* strains. Overexpressing FtsZ in a *minB* strain would have no phenotypic effect as divisions in this case are limited by FtsA availability.

Although the above suggestion on the interplay of FtsA and Min proteins is largely speculative, some data indirectly supporting it exists. It has been reported that the phosphorylation state of FtsA correlates with its intracellular location: cytoplasmic FtsA is phosphorylated, whereas membrane-bound FtsA is not (Sánchez *et al.*, 1994). It is not known, what cellular function these different forms of FtsA perform, because the *ftsA* mutants that are non-

phosphorylatable (*ftsA*102 and *ftsA*104) can still complement several *ftsA* mutant alleles, including the amber mutation *ftsA16* (Sánchez *et al.*, 1994). It is tempting to suggest that dephosphorylation may be the mechanism of immobilising the "used" FtsA molecules at the cell poles, in which case all newly made FtsA molecules may be phosphorylated to be able to join the forming "divisome", or "septator", at the central division site (divisome structure and role are discussed in Chapter 1). Dephosphorylation of FtsA could make the association of FtsA with other components of the divisome more stable and even irreversible, sequestering the protein at cell poles after division. This also implies that, when the FtsZ ring disassembles, not only FtsA, but other divisome components ("subassemblies": Nanninga, 1998) may remain attached to the cell poles after division so that an additional supply of FtsZ molecules alone will be sufficient for new divisions to occur at cell poles. The Min system may be responsible for the changes in the phosphorylation state of FtsA. In particular, MinD could be involved here as it is an ATP binding protein, and its ATPase activity has been demonstrated to be necessary for division inhibition (de Boer *et al.*, 1991), but additional protein(s) may be required. Interestingly, trancient (oscillating) polar localisation of MinD was recently demonstrated (Raskin and de Boer, 1999). This gives further, although indirect, support to the possibility of MinD involvement in sequestering FtsA at poles.

Although the exact mechanism of interaction of FtsA, FtsZ and Min proteins still remains unclear and additional experiments are required to check the hypothesis suggested above, the experimental results do demonstrate that both FtsA and FtsZ are required for increased division potential in wild type, *rodA* and *minB* cells of *E. coli.*. These results support an earlier suggestion (Tormo and Vicente, 1984) that FtsA is sequestered at poles after division. FtsA probably cannot be sequestered at poles in a *minB* mutant. The Min system

cannot act as the direct inhibitor of FtsZ. It could, however, have a role in polar sequestration of FtsA.

REFERENCES

Addinall, S. G., Bi, E. & Lutkenhaus, J. (1996). FtsZ ring formation in *fts* mutants. *J. Bacteriol.* **178**, 3877-3884.

Addinall, S. G., Cao, C. & Lutkenhaus, J. (1997). FtsN, a late recruit to the septum in *Escherichia coli*. *Mol. Microbiol.* **25**, 303-309.

Addinall, S. G., Cao, C. & Lutkenhaus, J. (1997). Temperature shift experiments with an *ftsZ84*(Ts) strain reveal rapid dynamics of FtsZ localization and indicate that the Z ring is required throughout septation and cannot reoccupy division sites once constriction has initiated. *J. Bacteriol.* **179**, 4277-4284.

Addinall, S. G. & Lutkenhaus, J. (1996). FtsA is localized to the septum in an FtsZ-dependent manner. *J. Bacteriol.* **178**, 7167-7172.

Addinall, S. G. & Lutkenhaus, J. (1996). FtsZ-spirals and -arcs determine the shape of the invaginating septa in some mutants of *Escherichia coli*. *Mol. Microbiol.* **22**, 231-237.

Adhya, S. & Gottesman, M. (1982). Promoter occlusion: transcription through promoter may inhibit its activity. *Cell* **29**, 939-944.

Aldea, M., Garrido, T., Hernandez-Chico, C., Vicente, M. & Kushner, S. R. (1989). Induction of a growth-phase-dependent promoter triggers transcription of bolA, an Escherichia coli morphogene. *EMBO J.* **8**, 3923-3931.

Aldea, M., Hernandez-Chico, C., de la Campa, A. G., Kushner, S. R. & Vicente, M. (1988). Identification, cloning, and expression of bolA, an ftsZ-dependent morphogene of Escherichia coli. *J. Bacteriol.* **170**, 5169-5176.

Altschul, S. F., Madden, T. L., Schaffer, A. A., Zhang, J., Zhang, Z., Miller, W. & Lipman, D. J. (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res.* **25**, 3389-3402.

Andersson, H. & von Heijne, G. (1993). *Sec* dependent and *sec* independent assembly of *E.coli* inner membrane proteins: the topological rules depend on chain length. *EMBO J.* **12**, 683-691.

Ayala, J. A., Garrido, T., de Pedro, M. A. & Vicente, M. (1994). Molecular biology of bacterial septation. In *Bacterial cell wall*, pp. 73-101. Edited by a. R. H. J.-M. Ghuysen. Amsterdam, The Netherlands: Elsevier Biomedical Press.

Ballesteros, M., Kusano, S., Ishihama, A. & Vicente, M. (1998). The ftsQ1p gearbox promoter of Escherichia coli is a major sigma S- dependent promoter in the ddlB-ftsA region. *Mol. Microbiol.* **30**, 419-430.

Baquero, M.-R., Bouzon, M., Quintela, J. C., Ayala, J. A. & Moreno, F. (1996). *dacD*, an *Escherichia coli* gene encoding a novel penicillin-binding protein (PBP6b) with D,D-carboxypeptidase activity. *J. Bacteriol.* **178**, 7106-7111.

Begg, K. J., Dewar, S. J. & Donachie, W. D. (1995). A new *Escherichia coli* cell division gene, *ftsK*. *J. Bacteriol.* **177**, 6211-6222.

Begg, K. J. & Donachie, W. D. (1985). Cell shape and division in *Escherichia coli*: experiments with shape and division mutants. *J. Bacteriol.* **163**, 615-622.

Begg, K. J. & Donachie, W. D. (1998). Division planes alternate in spherical cells of *Escherichia coli*. *J. Bacteriol.* **180**, 2564-2567.

Begg, K. J., Takasuga, A., Edwards, D. H., Dewar, S. J., Spratt, B. G., Adachi, H., Ohta, T., Matsuzawa, H. & Donachie, W. D. (1990). The balance between different peptidoglycan precursors determines whether *Escherichia coli* cells will elongate or divide. *J. Bacteriol.* **172**, 6697-6703.

Bi, E. & Lutkenhaus, J. (1990). FtsZ regulates frequency of cell division in *Escherichia coli*. *J. Bacteriol.* **172**, 2765-2768.

Bi, E. & Lutkenhaus, J. (1990). Interaction between the *min* locus and *ftsZ*. *J. Bacteriol.* **172**, 5610-5616.

Bi, E. & Lutkenhaus, J. (1993). Cell division inhibitors SulA and MinCD prevent formation of the FtsZ ring. *J. Bacteriol.* **175**, 1118-1125.

Bi, E. F. & Lutkenhaus, J. (1991). FtsZ ring structure associated with division in *Escherichia coli*. *Nature* **354**, 161-164.

Bork, P., Sander, C. & Valencia, A. (1992). An ATPase domain common to prokaryotic cell cycle proteins, sugar kinases, actin and hsp70 heat shock proteins. *Proc. Natl. Acad. Sci. USA* **89**, 7290-7294.

Bowler, L. D. & Spratt, B. G. (1989). Membrane topology of penicillin binding protein 3 of *Escherichia coli*. *Mol. Microbiol* **3**, 1277-1286.

Boyle, D. S. (1997). Studies on the *ftsW* and *mraY* genes of *Escherichia coli*. Ph. D. Thesis. Edinburgh.

Boyle, D. S., Khattar, M. M., Addinall, S. G., Lutkenhaus, J. & Donachie, W. D. (1997). *ftsW* is an essential cell-division gene in *Escherichia coli*. *Mol. Microbiol.* **24**, 1263-1273.

Britton, R. A., Powell, B. S., Court, D. L. & Lupski, J. R. (1997). Characterization of mutations affecting the Escherichia coli essential GTPase era that suppress two temperature-sensitive dnaG alleles. *J. Bacteriol.* **179**, 4575-4582.

Britton, R. A., Powell, B. S., Dasgupta, S., Sun, Q., Margolin, W., Lupski, J. R. & Court, D. L. (1998). Cell cycle arrest in Era GTPase mutants: a potential growth rate- regulated checkpoint in *Escherichia coli*. *Mol. Microbiol.* **27**, 739-750.

Broome-Smith, J. K., Ioannidis, I., Edelman, A. & Spratt, B. G. (1988). Nucleotide sequences of the penicillin-binding protein 5 and 6 genes of *Escherichia coli*. *Nucleic Acids Res.* **16**, 1617.

Broome-Smith, J. K. & Spratt, B. G. (1986). A vector for the construction of translational fusions to TEM β-lactamase and the analysis of protein export signals and membrane protein topology. *Gene* **49**, 341-349.

Broome-Smith, J. K., Tadayyon, M. & Zhang, Y. (1990). Beta-lactamase as a probe of membrane protein assembly and protein export. *Mol. Microbiol* **4**, 1637-1644.

Brown, E. D., Vivas, E. I., Walsh, C. T. & Kolter, R. (1995). MurA (MurZ), the enzyme that catalyzes the first committed step in peptidoglycan biosynthesis, is essential in *Escherichia coli*. *J. Bacteriol.* **177**, 4194-4197.

Buddelmeijer, N., Aarsman, M. E. G., Kolk, A. H. J., Vicente, M. & Nanninga, N. (1998). Localization of cell division protein FtsQ by immunofluorescence microscopy in dividing and nondividing cells of *Escherichia coli*. *J. Bacteriol.* **180**, 6107-6116.

Burman, L. G. & Park, J. T. (1984). Molecular model for elongation of the murein sacculus of *Escherichia coli*. *Proc. Natl. Acad. Sci. USA* **81**, 1844-1848.

Cam, K., Rome, K., Krisch, H. M. & Bouché, J.-P. (1996). RNase E processing of essential cell division genes mRNA in *Escherichia coli*. *Nucleic Acids Res.* **24**, 3065-3070.

Carson, M., Barondess, J. & Beckwith, J. (1991). The FtsQ protein of *Escherichia coli*: membrane topology, abundance, and cell division phenotypes due to overproduction and insertion mutations. *J. Bacteriol.* **173**, 2187-95.

Chen, J. C., Weiss, D. S., Ghigo, J.-M. & Beckwith, J. (1999). Septal localization of FtsQ, an essential cell division protein in *Escherichia coli*. *J. Bacteriol.* **181**, 521-530.

Claros, M. G. & von Heijne, G. (1994). TopPred II: An improved software for membrane protein structure predictions. *Comput. Appl. Biosci.* **10**, 685-686.

Cooper, S. & Helmstetter, C. E. (1968). Chromosome replication and the division cycle of Escherichia coli B/r. *J. Mol. Biol.* **31**, 519-540.

Dai, D. & Ishiguro, E. E. (1988). *murH*, a new genetic locus in *Escherichia coli* involved in cell wall peptidoglycan synthesis. *J. Bacteriol.* **170**, 2197-2201.

Dai, K. & Lutkenhaus, J. (1991). *ftsZ* is an essential cell division gene in *Escherichia coli*. *J. Bacteriol.* **173**, 3500-3506.

Dai, K. & Lutkenhaus, J. (1992). The proper ratio of FtsZ to FtsA is required for cell division to occur in *Escherichia coli*. *J. Bacteriol.* **174**, 6145-6151.

Dai, K., Xu, Y. & Lutkenhaus, J. (1993). Cloning and characterization of *ftsN*, an essential cell division gene in *Escherichia coli* isolated as a multicopy suppressor of *ftsA12*(Ts). *J. Bacteriol.* **175**, 3790-3797.

Dai, K., Xu, Y. & Lutkenhaus, J. (1996). Topological characterization of the essential *Escherichia coli* cell division protein FtsN. *J. Bacteriol.* **178**, 1328-1334.

de Boer, P., Crossley, R. & Rothfield, L. (1992). The essential bacterial cell-division protein FtsZ is a GTPase. *Nature* **359**, 254-256.

de Boer, P. A., Crossley, R. E. & Rothfield, L. I. (1990). Central role for the *Escherichia coli minC* gene product in two different cell division-inhibition systems. *Proc Natl Acad Sci U S A* **87**, 1129-1133.

de Boer, P. A., Crossley, R. E. & Rothfield, L. I. (1992). Roles of MinC and MinD in the site-specific septation block mediated by the MinCDE system of *Escherichia coli*. *J. Bacteriol.* **174**, 63-70.

de Boer, P. A. J., Crossley, R. & Rothfield, L. I. (1989). A division inhibitor and a topological specificity factor coded for by the minicell locus determine the proper placement of the division site in *Escherichia coli*. *Cell* **56**, 641-649.

de Boer, P. A. J., Crossley, R. E., Hand, R. E. & Rothfield, L. I. (1991). The MinD protein is a membrane ATPase required for the correct placement of the *Escherichia coli* division site. *EMBO J.* **10**, 4371-4380.

de Jonge, B. L., Wientjes, F. B., Jurida, I., Driehuis, F., Wouters, J. T. & Nanninga, N. (1989). Peptidoglycan synthesis during the cell cycle of *Escherichia coli*: composition and mode of insertion. *J. Bacteriol.* **171**, 5783-5794.

Dewar, S. J., Begg, K. J. & Donachie, W. D. (1992). Inhibition of cell division initiation by an imbalance in the ratio of FtsA to FtsZ. *J. Bacteriol.* **174**, 6314-6316.

Dewar, S. J. & Donachie, W. D. (1993). Antisense transcription of the *ftsA-ftsZ* gene junction inhibits cell division in *Escherichia coli*. *J. Bacteriol.* **175**, 7097-7101.

Di Berardino, M., Dijkstra, A., Stüber, D., Keck, W. & Gubler, M. (1996). The monofunctional glycosyltransferase of *Escherichia coli* is a member of a new class of peptidoglycan-synthesising enzymes. *FEBS Lett.* **392**, 184-188.

Dijkstra, A. J. & Keck, W. (1996). Identification of new members of the lytic transglycosylase family in *Haemophilus influenzae* and *Escherichia coli*. *Microb. Drug Resist.* **2**, 141-145.

Di Lallo, G., Anderluzzi, D., Ghelardini, P. & Paolozzi, L. (1999). FtsZ dimerization in vivo. *Mol Microbiol* **32**, 265-274.

Doi, M., Wachi, M., Ishino, F., Tomioka, S., Ito, M., Sakagami, Y., Suzuki, A. & Matsuhashi, M. (1988). Determinations of the DNA sequence of the mreB gene and of the gene products of the mre region that function in formation of the rod shape of Escherichia coli cells. *J. Bacteriol.* **170**, 4619-4624.

Donachie, W. D. (1992). What is the minimum number of dedicated functions required for a basic cell cycle? *Curr. Opin. Genet. Dev.* **2**, 792-798.

Donachie, W. D., Addinall, S. & Begg, K. (1995). Cell shape and chromosome partitioning in procaryotes or, Why *E.coli* is rod-shaped and haploid. *BioEssays* **17**, 569-576.

Donachie, W. D. & Begg, K. J. (1996). "Division potential" in *Escherichia coli*. *J. Bacteriol.* **178**, 5971-5976.

Donachie, W. D., Begg, K. J., Lutkenhaus, J. F., Salmond, G. P., Martinez-Salas, E. & Vincente, M. (1979). Role of the *ftsA* gene product in control of *Escherichia coli* cell division. *J. Bacteriol.* **140**, 388-394.

Donachie, W. D. & Masters, M. (1966). Repression and the control of cyclic enzyme synthesis in *Bacillus subtilis*. *Nature* **209**, 476-479.

Doublet, P., van Heijenoort, J., Bohin, J. P. & Mengin-Lecreulx, D. (1993). The murI gene of Escherichia coli is an essential gene that encodes a glutamate racemase activity. *J. Bacteriol.* **175**, 2970-2979.

Draper, G. C., McLennan, N., Begg, K., Masters, M. & Donachie, W. D. (1998). Only the N-terminal domain of FtsK functions in cell division. *J. Bacteriol.* **180**, 4621-4627.

Edwards, D. H. & Donachie, W. D. (1993). Construction of a triple deletion of penicillin-binding proteins 4, 5 and 6 in *Escherichia coli*. In *Bacterial Growth and Lysis: Metabolism and Structure of the Bacterial*

Sacculus, pp. 369-374. Edited by J.-V. H. M.A. de Pedro, W. Löffelhardt. New York: Plenum Publishing Corp.

Ehlert, K. & Höltje, J. V. (1996). Role of precursor translocation in coordination of murein and phospholipid synthesis in *Escherichia coli*. *J. Bacteriol.* **178**, 6766-6771.

Ehlert, K., Höltje, J. V. & Templin, M. F. (1995). Cloning and expression of a murein hydrolase lipoprotein from *Escherichia coli*. *Mol. Microbiol.* **16**, 761-768.

Engel, H., Kazemier, B. & Keck, W. (1991). Murein-metabolizing enzymes from *Escherichia coli*: sequence analysis and controlled overexpression of the slt gene, which encodes the soluble lytic transglycosylase. *J. Bacteriol.* **173**, 6773-6782.

Engelman, D. M., Steitz, T. A. & Goldman, A. (1986). Identifying nonpolar transbilayer helices into amino acid sequences of membrane proteins. *Annu. Rev. Biophys. Biophys. Chem.* **15**, 321-353.

Erickson, H. P., Taylor, D. W., Taylor, K. A. & Bramhill, D. (1996). Bacterial cell division protein FtsZ assembles into protofilament sheet and minirings, structural homologs of tubulin polymers. *Proc. Natl. Acad. Sci. USA* **93**, 519-523.

Felsenstein, J. (1989). PHYLIP - Phylogeny Inference Package (Version 3.2). *Cladistics* **5**, 164-166.

Flärdh, K., Garrido, T. & Vicente, M. (1997). Contribution of individual promoters in the *ddlB-ftsZ* region to the transcription of the essential cell-division gene *ftsZ* in *Escherichia coli*. *Mol. Microbiol.* **24**, 927-936.

Flärdh, K., Palacios, P. & Vicente, M. (1998). Cell division genes *ftsQAZ* in *Escherichia coli* require distant *cis*-acting signals upstream of *ddlB* for full expression. *Mol. Microbiol.* **30**, 305-315.

Garcia-Lara, J., Shang, L. H. & Rothfield, L. I. (1996). An extracellular factor regulates expression of *sdiA*, a transcriptional activator of cell division genes in *Escherichia coli*. *J. Bacteriol.* **178**, 2742-2748.

Garrido, T., Sánchez, M., Palacios, P., Aldea, M. & Vicente, M. (1993). Transcription of *ftsZ* oscillates during the cell cycle of *Escherichia coli*. *EMBO J.* **12**, 3957-3965.

Ghigo, J. M., Weiss, D. S., Chen, J. C., Yarrow, J. C. & Beckwith, J. (1999). Localization of FtsL to the *Escherichia coli* septal ring. *Mol Microbiol* **31**, 725-37.

Glauner, B. & Höltje, J.-V. (1990). Growth pattern of the murein sacculus of *Escherichia coli*. *J. Biol. Chem.* **265**, 18988-18996.

Glauner, B., Höltje, J.-V. & Schwartz, U. (1988). The composition of the murein of *Escherichia coli*. *J. Biol. Chem.* **263**, 10088-10095.

Goffin, C., Fraipont, C., Ayala, J., Terrak, M., Nguyen-Disteche, M. & Ghuysen, J. M. (1996). The non-penicillin binding module of the tripartite penicillin binding protein 3 of *Escherichia coli* is required for folding and/or stability of the penicillin binding module and the membrane-anchoring module confers cell septation activity on the folded structure. *J. Bacteriol.* **178**, 5402-5409.

Goodell, E. W. (1985). Recycling of murein by *Escherichia coli*. *J. Bacteriol.* **163**, 305-310.

Goodell, E. W. & Higgins, C. F. (1987). Uptake of cell wall peptides by *Salmonella typhimurium* and *Escherichia coli*. *J. Bacteriol.* **169**, 3861-3865.

Gordon, G. S., Sitnikov, D., Webb, C. D., Teleman, A., Straight, A., Losick, R., Murray, A. W. & Wright, A. (1997). Chromosome and low copy plasmid segregation in *E. coli*: visual evidence for distinct mechanisms. *Cell* **90**, 1113-1121.

Guzman, L.-M., Barondess, J. J. & Beckwith, J. (1992). FtsL, an essential cytoplasmic membrane protein involved in cell division in *Escherichia coli*. *J. Bacteriol.* **174**, 7716-7728.

Guzman, L. M., Weiss, D. S. & Beckwith, J. (1997). Domain-swapping analysis of FtsI, FtsL, and FtsQ, bitopic membrane proteins essential for cell division in Escherichia coli. *J. Bacteriol.* **179**, 5094-5103.

Hale, C. A. & de Boer, P. A. (1997). Direct binding of FtsZ to ZipA, an essential component of the septal ring structure that mediates cell division in E. coli. *Cell* **88**, 175-185.

Hale, C. A. & de Boer, P. A. J. (1999). Recruitment of ZipA to the septal ring of *Escherichia coli* is dependent on FtsZ and independent of ftsA. *J. Bacteriol.* **181**, 167-176.

Hara, H., Yasuda, S., Horiuchi, K. & Park, J. T. (1997). A promoter for the first nine genes of the *Escherichia coli mra* cluster of cell division and cell envelope biosynthesis genes, including *ftsI* and *ftsW*. *J. Bacteriol.* **179**, 5802-5811.

Harz, H., Burgdorf, K. & Höltje, J. V. (1990). Isolation and separation of the glycan strands from murein of *Escherichia coli* by reversed-phase high-performance liquid chromatography. *Anal. Biochem.* **190**, 120-128.

Hausler, B. & Somerville, R. L. (1979). Interaction *in vivo* between strong closely spaced constitutive promoters. *J. Mol. Biol.* **127**, 353-356.

Henderson, T. A., Templin, M. & Young, K. D. (1995). Identification and cloning of the gene encoding penicillin-binding protein 7 of *Escherichia coli*. *J. Bacteriol.* **177**, 2074-2079.

Henriques, A. O., Glaser, P., Piggot, P. J. & Moran, C. P. J. (1998). Control of cell shape and elongation by the *rodA* gene in *Bacillus subtilis*. *Mol. Microbiol.* **28**, 235-247.

Höltje, J. (1996). A hypothetical holoenzyme involved in the replication of the murein sacculus of *Escherichia coli*. *Microbiology* **142**, 1911-1918.

Höltje, J.-V. (1993). "Three for one"a simple growth mechanism that guarantees a precise copy of the thin, rod-shaped murein sacculus of *Escherichia coli*. In *Bacterial growth and lysis*, pp. 419-426. Edited by J.-V. H. M. A. de Pedro, and W. Löffelhardt. New York: Plenum Press.

Höltje, J. V. (1996). Molecular interplay of murein synthases and murein hydrolases in *Escherichia coli*. *Microb. Drug Resist.* **2**, 99-103.

Höltje, J. V., Kopp, U., Ursinus, A. & Wiedemann, B. (1994). The negative regulator of β-lactamase induction is a N-acetyl-anhydromuramyl-L-amidase. *FEMS Microbiol. Lett.* **122**, 159-164.

Höltje, J. V., Mirelman, D., Sharon, N. & Schwarz, U. (1975). Novel type of murein transglycosylase in *Escherichia coli*. *J. Bacteriol.* **124**, 1067-1076.

Huang, J., Cao, C. & Lutkenhaus, J. (1996). Interaction between FtsZ and inhibitors of cell division. *J. Bacteriol.* **178**, 5080-5085.

Ikeda, M., Sato, T., Wachi, M., Jung, H. K., Ishino, F., Kobayashi, Y. & Matsuhashi, M. (1989). Structural similarity among *Escherichia coli* FtsW and RodA proteins and *Bacillus subtilis* SpoVE protein which function in cell division cell elongation and spore formation respectively. *J. Bacteriol.* **171**, 6375-6378.

Ikeda, M., Wachi, M., Jung, H. K., Ishino, F. & Matsuhashi, M. (1991). The *Escherichia coli mraY* gene encoding UDP-N-acetylmuramoyl-pentapeptide: undecaprenyl-phosphate phospho-N-acetylmuramoyl-pentapeptide transferase. *J. Bacteriol.* **173**, 1021-1026.

Ishino, F., Jung, H. K., Ikeda, M., Doi, M., Wachi, M. & Matsuhashi, M. (1989). New mutations *fts*-36, *lts*-33, and *ftsW* clustered in the *mra* region of the *Escherichia coli* chromosome induce thermosensitive cell-growth and division. *J. Bacteriol.* **171**, 5523-5530.

Ishino, F. & Matsuhashi, M. (1981). Peptidoglycan synthetic enzyme activities of highly purified penicillin-binding protein 3 in *Escherichia coli*: A

septum-forming reaction sequence. *Biochem. Biophys. Res. Commun.* **101**, 905-911.

Ishino, F., Park, W., Tomioka, S., Tamaki, S., Takase, I., Kunugita, K., Matsuzawa, H., Asoh, S., Ohta, T., Spratt, B. G. & Matsuhashi, M. (1986). The functional biosynthesis of bacterial cell wall peptidoglycan. 2. Peptidoglycan synthetic activities in membranes of *Escherichia coli* caused by overproduction of penicillin binding protein 2 and RodA protein. *J. Biol. Chem.* **261**, 7024-7031.

Jacobs, C., Joris, B., Jamin, M., Klarsov, K., Van Beeumen, J., Mengin-Lecreulx, D., van Heijenoort, J., Park, J. T., Normark, S. & Frere, J. M. (1995). AmpD, essential for both beta-lactamase regulation and cell wall recycling, is a novel cytosolic N-acetylmuramyl-L-alanine amidase. *Mol. Microbiol.* **15**, 553-559.

Jaurin, B., Grundström, T. & Normark, S. (1982). Sequence elements determining *ampC* promoter strength in *Escherichia coli*. *EMBO J.* **1**, 875-881.

Keck, W., van Leeuwen, A. M., Huber, M. & Goodell, E. W. (1990). Cloning and characterisation of mepA, the structural gene of the penicillin-insensitive murein hydrolase from *Escherichia coli*. *Mol. Microbiol.* **4**, 209-219.

Khattar, M. M., Addinall, S. G., Stedul, K. H., Boyle, D. S., Lutkenhaus, J. & Donachie, W. D. (1997). Two polypeptide products of the *Escherichia coli* cell division gene *ftsW* and a possible role for FtsW in FtsZ function. *J. Bacteriol.* **179**, 784-793.

Khattar, M. M., Begg, K. J. & Donachie, W. D. (1994). Identification of FtsW and characterization of a new ftsW division mutant of Escherichia coli. *J. Bacteriol.* **176**, 7140-7147.

Koch, A. L. & Doyle, R. J. (1985). Inside-to-outside growth and turnover of the wall of gram-positive rods. *J. Theor. Biol.* **117**, 137-157.

Korat, B., Mottl, H. & Keck, W. (1991). Penicillin-binding protein 4 of *Escherichia coli*: molecular cloning of the *dacB* gene, controlled overexpression, and alterations in murein composition. *Mol. Microbiol.* **5**, 675-684.

Kyte, J. & Doolittle, R. F. (1982). A simple method for displaying the hydropathic character of a protein. *J. Mol. Biol.* **157**, 105-132.

Labie, C., Bouché, F. & Bouché, J.-P. (1990). Suppression of both DicB- and MinD-dependent division inhibition by inactivation of the *minC* gene product. *J. Bacteriol.* **172**, 5852-5855.

Labischinski, H., Barnickel, G., Naumann, D. & Keller, P. (1985). Conformational and topological aspects of the three dimensional

architecture of bacterial peptidoglycan. *Ann. Inst. Pasteur/ Microbiol.* **136A**, 45-50.

Lange, R. & Hengge-Aronis, R. (1991). Growth phase-regulated expression of bolA and morphology of stationary- phase Escherichia coli cells are controlled by the novel sigma factor sigma S. *J. Bacteriol.* **173**, 4474-4481.

Liger, D., Masson, A., Blanot, D., van Heijenoort, J. & Parquet, C. (1995). Over-production, purification and properties of the uridine-diphosphate- N-acetylmuramate:L-alanine ligase from *Escherichia coli. Eur. J. Biochem.* **230**, 80-87.

Lindquis, S., Weston-Hafer, K., Shmidt, H., Pul, C., Korfmann, G., Erikson, J., Sanders, C., Martin, H. H. & Normark, S. (1993). AmpG, a signal transducer in chromosomal β-lactamase induction. *Mol. Microbiol.* **9**, 703-715.

Liu, G., Draper, G. C. & Donachie, W. D. (1998). FtsK is a bifunctional protein involved in cell division and chromosome localization in *Escherichia coli. Mol. Microbiol.* **29**, 893-903.

Liu, Z., Mukherjee, A. & Lutkenhaus, J. (1999). Recruitment of ZipA to the divison site by interaction with FtsZ. *Mol Microbiol* **31**, 1853-61.

Lockhart, A. & Kendrick-Jones, J. (1998). Interaction of the N-terminal domain of MukB with the bacterial tubulin homologue FtsZ. *FEBS Lett.* **430**, 278-82.

Lommatzsch, J., Templin, M., Kraft, A. R., Vollmer, W. & Höltje, J. V. (1997). Outer membrane localization of murein hydrolases: MltA, a third lipoprotein lytic transglycosylase in *Escherichia coli. J. Bacteriol.* **179**, 5465-5470.

Lowe, J. & Amos, L. A. (1998). Crystal structure of the bacterial cell-division protein FtsZ. *Nature* **391**, 203-206.

Lutkenhaus, J. (1993). *Escherichia coli* cell division. *Curr. Opin. Genet. Dev.* **3**, 783-788.

Lutkenhaus, J. (1993). FtsZ ring in bacterial cytokinesis. *Mol. Microbiol.* **9**, 403-409.

Ma, X., Erhardt, D. W. & Margolin, W. (1996). Colocalization of cell division proteins FtsZ and FtsA to cytoskeletal structures in living *Escherichia coli* cells by using green fluorescent protein. *Proc. Natl. Acad. Sci. USA* **93**, 12998-13003.

Ma, X., Sun, Q., Wang, R., Singh, G., Jonietz, E. L. & Margolin, W. (1997). Interactions between heterologous FtsA and FtsZ proteins at the FtsZ ring. *J. Bacteriol.* **179**, 6788-6797.

Markiewicz, Z., Broome-Smith, J. K., Schwarz, U. & Spratt, B. G. (1982). Spherical *Escherichia coli* due to elevated levels of D-alanine carboxypeptidase. *Nature* **297**, 702-704.

Marquardt, J. L., Seigele, D. A., Kolter, R. & Walsh, C. T. (1992). Cloning and sequencing of the *Escherichia coli murZ* and purification of its product, UDP-N-acetylglucosamine enolpyruvyl transferase. *J. Bacteriol.* **174**, 5748-5722.

Maruyama, I. N., Yamamoto, A. H. & Hirota, Y. (1988). Determination of gene products from the murE-murF region of Escherichia coli. *J. Bacteriol.* **170**, 3786-3788.

Matsuhashi, M. (1994). Utilization of lipid-linked precursors and the formation of peptidoglycan in the process of cell growth and division: membrane enzymes involved in the final steps of peptidoglycan synthesis and the mechanism of their integration. In *Bacterial cell wall*, pp. 55-71. Edited by a. R. H. J.-M. Ghuysen. Amsterdam: Elsevier Biomedical Press.

Matsuhashi, M., Wachi, M. & Ishino, F. (1990). Machinery for cell growth and division: penicillin-binding proteins and other proteins. *Res. Microbiol.* **141**, 89-103.

Meikle, A., Killham, K., Prosser, J. I. & Glover, L. A. (1992). Luminometric measurement of population activity of genetically modified *Pseudomonas fluorescens* in the soil. *FEMS Microbiol. Lett.* **99**, 217-220.

Mengin-Lecreulx, D., Ayala, J., Bouhss, A., van Heijenoort, J., Parquet, C. & Hara, H. (1998). Contribution of the P*mra* promoter to expression of genes in the *Escherichia coli mra* cluster of cell envelope biosynthesis and cell division genes. *J. Bacteriol.* **180**, 4406-4412.

Mengin-Lecreulx, D., Parquet, C., Desviat, L. R., Pla, J., Flouret, B., Ayala, J. A. & van Heijenoort, J. (1989). Organization of the *murE-murG* region of *Escherichia coli*: identification of the *murD* gene coding for the D-glutamic-acid-adding enzyme. *J. Bacteriol.* **171**, 6126-6134.

Mengin-Lecreulx, D., van Heijenoort, J. & Park, J. T. (1996). Identification of the *mpl* gene encoding UDP-*N*-acetylmuramate:L-alanyl-γ-D-glutamyl-*meso*-diamilnopimelate ligase in *Escherichia coli* and its role in recycling of cell wall peptidoglycan. *J. Bacteriol.* **178**, 5347-5352.

Mengin-Lecreulx, D. M., Texier, L., Rousseau, M. & van Hejeinoort, J. (1991). The murG gene of Escherichia coli codes for the UDP-N-acetyl glucosamine:N-acetyl-muramyl-(pentapeptide)-pyrophosphoryl-undecaprenol N- acetylglucosaminetransferase involved in the membrane steps of peptidoglycan synthesis. *J. Bacteriol.* **173**, 4625-4636.

Mukherjee, A. & Donachie, W. D. (1990). Differential translation of cell division proteins. *J. Bacteriol.* **172**, 6106-6111.

Nanninga, N. (1991). Cell division and peptidoglycan assembly in *Escherichia coli*. *Mol. Microbiol.* , 791-795.

Nanninga, N. (1998). Morphogenesis of *Escherichia coli*. *Microbiol. Mol. Biol. Rev.* **62**, 110-129.

Nanninga, N., Wientjes, F. B., de Jonge, B. L. & Woldringh, C. L. (1990). Polar cap formation during cell division in *Escherichia coli*. *Res Microbiol* **141**, 103-118.

Nguyen-Disteche, M., Fraipont, C., Buddelmeijer, N. & Nanninga, N. (1998). The structure and function of Escherichia coli penicillin-binding protein 3. *Cell. Mol. Life Sci.* **54**, 309-316.

Niki, H., Imamura, R., Kitaoka, M., Yamanaka, K., Ogura, T. & Hiraga, S. (1992). *E. coli* MukB protein involved in chromosome partition forms a homodimer with a rod-and-hinge structure having DNA binding and ATP/GTP binding activities. *EMBO J.* **11**, 5101-5109.

Niki, H., Jaffé, A., Imamura, R., Ogura, T. & Hiraga, S. (1991). The new gene *mukB* codes for a 177 kd protein with coiled-coil domains involved in chromosome partitioning of *E. coli*. *EMBO J.* **10**, 183-193.

Nogales, E., Wolf, S. G. & Downing, K. H. (1998). Structure of the αβ tubulin dimer by electron crystallography. *Nature* **391**, 199-203.

Osteryoung, K. W. & Vierling, E. (1995). Conserved cell and organelle division. *Nature* **376**, 473-474.

Palacios, P., Vicente, M. & Sánchez, M. (1996). Dependency of Escherichia coli cell-division size, and independency of nucleoid segregation on the mode and level of ftsZ expression. *Mol. Microbiol.* **20**, 1093-1098.

Park, J. T. (1993). Turnover and recycling of the murein sacculus in oligopeptide permease-negative strains of *Escherichia coli*: indirect evidence for an alternative permease system and for a monolayered sacculus. *J. Bacteriol.* **175**, 7-11.

Park, J. T. (1996). The murein sacculus. In *Escherichia coli and Salmonella: cellular and molecular biology, 2nd ed.*, pp. 48-57. Edited by R. C. I. F. C. Neidhardt, J. L. Ingraham, E. C. C. Lin, K. B. Low, B. Magasanik, W. S. Reznikoff, M. Riley, M. Schaechter, and H. E. Umbarger. Washington, D.C.: American Society for Microbiology.

Park, J. T. & Burman, L. G. (1985). Elongation of the murein sacculus of *Escherichia coli*. *Ann Inst Pasteur/Microbiol* **136A**, 51-58.

Park, J. T., Raychaudhuri, D., Li, H., Normark, S. & Mengin-Lecreulx, D. (1998). MppA, a periplasmic binding protein essential for import of the bacterial cell wall peptide L-alanyl-gamma-D-glutamyl-meso-diaminopimelate. *J. Bacteriol.* **180**, 1215-1223.

Parquet, C., Flouret, B., Mengin-Lecreulx, D. & van Heijenoort, J. (1989). Nucleotide sequence of the *murF* encoding the UDP-MurNAc-pentapeptide synthase of *Escherichia coli*. *Nucl Acids Res* **269**, 277-280.

Pearson, W. R., Wood, T., Zhang, Z. & Miller, W. (1997). Comparison of DNA sequences with protein sequences. *Genomics* **46**, 24-36.

Persson, B. & Argos, P. (1994). Prediction of transmembrane segments in proteins utilising multiple sequence alignments. *J. Mol. Biol.* **237**, 182-192.

Pichoff, S., Vollrath, B., Touriol, C. & Bouche, J.-P. (1995). Deletion analysis of gene *minE* which encodes the topological specificity factor of cell division in *Escherichia coli*. *Mol. Microbiol* **18**, 321-329.

Pogliano, J., Pogliano, K., Weiss, D. S., Losick, R. & Beckwith, J. (1997). Inactivation of FtsI inhibits constriction of the FtsZ cytokinetic ring and delays the assembly of FtsZ rings at potential division sites. *Proc. Natl. Acad. Sci. USA* **94**, 559-564.

Pucci, M. J., Discotto, L. F. & Dougherty, T. J. (1992). Cloning and identification of the Escherichia coli murB DNA sequence, which encodes UDP-N--acetylenolpyruvylglucosamine reductase. *J. Bacteriol.* **174**, 1690-1693.

Raskin, D. M. & de Boer, P. A. (1997). The MinE ring: an FtsZ-independent cell structure required for selection of the correct division site in *E. coli*. *Cell* **91**, 685-694.

Raskin, D. M. & de Boer, P. A. (1999). Rapid pole-to-pole oscillation of a protein required for directing division to the middle of escherichia coli [In Process Citation]. *Proc Natl Acad Sci U S A* **96**, 4971-6.

RayChaudhuri, D. (1999). ZipA is a MAP-Tau homolog and is essential for structural integrity of the cytokinetic FtsZ ring during bacterial cell division. *Embo J* **18**, 2372-2383

Robinson, A. C., Kenan, D. J., Hatfull, G. F., Sullivan, N. F., Spiegelberg, R. & Donachie, W. D. (1984). DNA sequence and transcriptional organization of essential cell division genes *ftsQ* and *ftsA* of *Escherichia coli*: evidence for overlapping transcriptional units. *J. Bacteriol.* **160**, 546-55.

Robinson, A. C., Kenan, D. J., Sweeney, J. & Donachie, W. D. (1986). Further evidence for overlapping transcriptional units in an

Escherichia coli cell envelope-cell division gene cluster: DNA sequence and transcriptional organization of the *ddl ftsQ* region. *J. Bacteriol.* **167**, 809-817.

Rodriguez, M. C. & de Pedro, M. A. (1990). Initiation of growth in *pbpAts* and *rodAts* mutants of *Escherichia coli*. *FEMS Microbiol. Lett.* **60**, 235-239.

Romeis, T. & Höltje, J. V. (1994). Penicillin-binding protein 7/8 of *Escherichia coli* is a D,D-endopeptidase. *Eur. J. Biochem.* **224**, 597-604.

Romeis, T. & Höltje, J. V. (1994). Specific interaction of penicillin binding protein 3 and protein 7/8 with soluble lytic transglycosylase in *Escherichia coli*. *J. Biol. Chem.* **269**, 21603-21607.

Rosenberg, A. H., Lade, B. N., Chui, D.-S., Lin, S.-W., Dunn, J. J. & Studier, F. W. (1987). Vectors for selective expression of cloned DNAs by T7 RNA polymerase. *Gene* **56**, 125-135.

Rost, B., Casadio, R., Fariselli, P. & Sander, C. (1995). Prediction of helical transmembrane segments at 95% accuracy. *Prot. Sci.* **4**, 521-533.

Rost, B., Fariselli, P. & Casadio, R. (1996). Topology prediction for helical transmembrane proteins at 86% accuracy. *Prot. Sci.* **7**, 1704-1718.

Sánchez, M., Valencia, A., Ferrandiz, M.-J., Sander, C. & Vicente, M. (1994). Correlation between the structure and biochemical activities of FtsA, an essential cell division protein of the actin family. *EMBO J.* **13**, 4919-4925.

Shockman, G. D. & Höltje, J. V. (1994). Microbial peptidoglycan (murein) hydrolases. In *Bacterial cell wall*, pp. 131-166. Edited by J.-M. Ghuysen & R. Hakenbeck. Amsterdam: Elsevier Science Publishing.

Signoretto, C., Di Stefano, F. & Canepari, P. (1996). Modified peptidoglycan chemical composition in shape-altered *Escherichia coli*. *Microbiology* **142**, 1919-1926.

Sitnikov, D. M., Schineller, J. B. & Baldwin, T. O. (1996). Control of cell division in *Escherichia coli*: regulation of transcription of *ftsQA* involves both RpoS and SdiA-mediated autoinduction. *Proc Natl Acad Sci U S A* **93**, 336-341.

Smith, R. W., Masters, M. & Donachie, W. D. (1993). Cell division and transcription of ftsZ. *J. Bacteriol.* **175**, 2788-2791.

Spratt, B. G. (1977). Properties of the penicillin-binding proteins of *Escherichia coli* K12. *Eur. J. Biochem.* **72**, 341-352.

Stoker, N. G., Broome-Smith, J. K., Edelman, A. & Spratt, B. G. (1983). Organization and subcloning of the *dacA-rodA-pbpA* cluster of cell shape genes in *Escherichia coli*. *J. Bacteriol.* **155**, 847-853.

Strimmer, K. & von Haeseler, A. (1996). Quartet puzzling: a quartet maximum likelihood method for reconstructing tree topologies. *Mol. Biol. Evol.* **13**, 964-969.

Tamaki, S., Matsuzawa, H. & Matsuhashi, M. (1980). Cluster of the *mrdA* and *mrdB* genes responsible for the rod shape and mecillinam sensitivity of *Escherichia coli*. *J. Bacteriol.* **141**, 52-57.

Teather, R. M., Collins, J. F. & Donachie, W. D. (1974). Quantal behavior of a diffusible factor which initiates septum formation at potential division sites in *E. coli*. *J. Bacteriol.* **118**, 407-413.

Tétart, F. & Bouché, J.-P. (1992). Regulation of the expression of the cell cycle gene *ftsZ* by DicF antisense RNA. Division does not require a fixed number of FtsZ molecules. *Mol. Microbiol.* **6**, 615-620.

Tomioka, S., Nikaido, T., Miyakawa, T. & Matsuhashi, M. (1983). Mutation of the *N*-acetylmuramyl-L-alanine amidase gene of *Escherichia coli*. *J. Bacteriol.* **156**, 463-465.

Tormo, A., Ayala, J. A., Depedro, M. A., Aldea, M. & Vicente, M. (1986). Interaction of *ftsA* and *pbp3* proteins in the *Escherichia coli* septum. *J. Bacteriol.* **166**, 985-992.

Tormo, A. & Vicente, M. (1984). The *ftsA* gene product participates in formation of the *Escherichia* coli septum structure. *J. Bacteriol.* **157**, 779-784.

Trusca, D., Scott, S., Thompson, C. & Bramhill, D. (1998). Bacterial SOS checkpoint protein SulA inhibits polymerization of purified FtsZ cell division protein. *J. Bacteriol.* **180**, 3946-3953.

Tsui, H. C., Zhao, G., Feng, G., Leung, H.-C. E. & Winkler, M. E. (1994). The *mutL* repair gene of *Escherichia coli* K-12 forms a superoperon with a gene encoding a new cell-wall amidase. *Mol. Microbiol.* **11**, 189-202.

Ueki, M., Wachi, M., Jung, H. K., Ishino, F. & Matsuhashi, M. (1992). *Escherichia coli mraR* gene involved in cell growth and division. *J. Bacteriol.* **174**, 7841-7843.

van Heijenoort, J., Parquet, C., Flouret, B. & van Heijenoort, Y. (1975). Envelope-bound N-acetylmuramyl-L-alanine amidase of *Escherichia coli* K12. Purification and properties of the enzyme. *Eur. J. Biochem.* **58**, 611-619.

Vicente, M., Gomez, M. J. & Ayala, J. A. (1998). Regulation of transcription of cell division genes in the *Escherichia coli dcw* cluster. *Cell. Mol. Life Sci.* **54**, 317-324.

Vinella, D., D'Ari, R. & Bouloc, P. (1992). Penicillin binding protein 2 is dispensable in *Escherichia coli* when ppGpp synthesis is induced. *EMBO J.* **11**, 1493-1501.

von Heijne, G. (1986). The distribution of positively charged residues in bacterial inner membrane proteins correlates with the transmembrane topology. *EMBO J.* **5**, 3021-3027.

von Heijne, G. (1992). Membrane protein structure prediction. Hydrophobicity analysis and the positive-inside rule. *J. Mol. Biol.* **225**, 487-494.

von Heijne, G. (1994). Membrane proteins: From sequence to structure. *Annu. Rev. Biophys. Biomol. Struct.* **23**, 167-192.

von Heijne, G. & Gavel, Y. (1988). Topogenic signals in integral membrane proteins. *Eur. J. Biohem.* **174**, 671-678.

von Rechenberg, M., Ursinus, A. & Höltje, J. V. (1996). Affinity chromatography as a means to study multi-enzyme-complexes involved in murein synthesis. *Microb. Drug Resist.* **2**, 155-157.

Wachi, M., Doi, M., Okada, Y. & Matsuhashi, M. (1989). New mre genes mreC and mreD, responsible for formation of the rod shape of Escherichia coli cells. *J. Bacteriol.* **171**, 6511-6516.

Wachi, M., Doi, M., Tamaki, S., Park, W., Nakajima-Iijima, S. & Matsuhashi, M. (1987). Mutant isolation and molecular cloning of mre genes, which determine cell shape, sensitivity to mecillinam and amount of penicillin-binding proteins in Escherichia coli. *J. Bacteriol.* **169**.

Wachi, M., Doi, M., Ueda, T., Ueki, M., Tsuritani, K., Nagai, K. & Matsuhashi, M. (1991). Sequence of the downstream flanking region of the shape-determining genes mreBCD of Escherichia coli. *Gene* **106**, 135-136.

Wachi, M. & Matsuhashi, M. (1989). Negative control of cell division by mreB, a gene that functions in determining the rod shape of Escherichia coli cells. *J. Bacteriol.* **171**, 3123-3127.

Walker, A. J., Jassim, S. A. A., Holah, J. T., Denyer, S. P. & Stewart, G. S. A. B. (1992). Bioluminescent *Listeria monocytogenes* provide a rapid assay for measuring biocide efficacy. *FEMS Microbiol. Lett.* **91**, 251-256.

Wang, L., Khattar, M. K., Donachie, W. D. & Lutkenhaus, J. (1998). FtsI and FtsW are localized to the septum in *Escherichia coli*. *J. Bacteriol.* **180**, 2810-2816.

Wang, L. & Lutkenhaus, J. (1998). FtsK is an essential cell division protein that is localized to the septum and induced as part of the SOS response. *Mol. Microbiol.* **29**, 731-470.

Wang, X., De Boer, P. A. J. & Rothfield, L. I. (1991). A factor that positively regulates cell division by activating transcription of the major cluster of essential cell division genes of *Escherichia coli*. *EMBO J.* **10**, 3363-3372.

Wang, X., Huang, J., Mukherjee, A., Cao, C. & Lutkenhaus, J. (1997). Analysis of the interaction of FtsZ with itself, GTP, and FtsA. *J. Bacteriol.* **179**, 5551-5559.

Ward, J. E. j. & Lutkenhaus, J. (1985). Overproduction of FtsZ induces minicell formation in *Escherichia coli*. *Cell* **42**, 941-949.

Weiss, D. S., Chen, J. C., Ghigo, J.-M., Boyd, D. & Beckwith, J. (1999). Localization of FtsI (PBP3) to the septal ring requires its membrane anchor, the Z ring, FtsA, FtsQ, and FtsL. *J. Bacteriol.* **181**, 508-520.

Weiss, D. S., Pogliano, K., Carson, M., Guzman, L. M., Fraipont, C., Nguyen-Disteche, M., Losick, R. & Beckwith, J. (1997). Localization of the Escherichia coli cell division protein Ftsl (PBP3) to the division site and cell pole. *Mol. Microbiol.* **25**, 671-681.

Wientjes, F. B. & Nanninga, N. (1991). On the role of the high molecular weight penicillin binding proteins in the cell cycle of *Escherichia coli*. *Res Microbiol* **142**, 333-344.

Wijsman, H. J. W. (1972). The characterization of an alanine racemase mutant of Escherichia coli. *Genet. Res.* **20**, 269-277.

Wild, J., Henning, J., Lobocka, M., Walczak, W. & Klopotowski, T. (1985). Identification of the *dadX* gene coding for the predominant isozyme of alanine racemase in *Escherichia coli* K12. *Mol. Gen. Genet.* **198**, 315-322.

Wu, L. J. & Errington, J. (1994). *Bacillus subtilis* SpoIIIE protein required for DNA segregation during asymmetric cell division. *Science* **264**, 572-575.

Yamanaka, K., Ogura, T., Niki, H. & Hiraga, S. (1996). Identification of two new genes, mukE and mukF, involved in chromosome partitioning in Escherichia coli. *Mol. Gen. Genet.* **250**, 241-251.

Yi, Q. M., Rockenbach, S., Ward, J. E., Jr. & Lutkenhaus, J. (1985). Structure and expression of the cell division genes *ftsQ*, *ftsA* and *ftsZ*. *J. Mol. Biol.* **184**, 399-412.

Yu, X. C., Tran, A. H., Sun, Q. & Margolin, W. (1998). Localization of cell division protein FtsK to the Escherichia coli septum and

identification of a potential N-terminal targeting domain. *J. Bacteriol.* **180**, 1296-1304.

Zawadzke, L. E., Bugg, T. D. H. & Walsh, C. T. (1991). Existence of two D-alanine:D-alanine ligases in *Escherichia coli*: cloning and sequencing of the *ddlA* gene and purification and characterization of the DdlA and DdlB enzymes. *Biochemistry* **30**, 1673-1682.

Zhao, C.-R., de Boer, P. A. J. & Rothfield, L. I. (1995). Proper placement of the *Escherichia coli* division site requires two functions that are associated with different domains of the MinE protein. *Proc. Natl. Acad. Sci. USA* **92**, 4313-4317.

Zhou, P. & Helmstetter, C. E. (1994). Relationship between ftsZ gene expression and chromosome replication in Escherichia coli. *J. Bacteriol.* **176**, 6100-6106.