Characterisation of a novel cell division operon in *E. coli* K-12

by

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'It goes on one at a time
it starts when you care
to act. It starts when you do
it again after they said no.
It starts when you say we
and you know who you mean, and each
day you mean one more.'

From 'The Low Road'
by Marge Piercy

I would like to dedicate this thesis with much love

to my parents, grandparents and brother, Paul,

for only ever asking me to be happy,

but most of all to Jay.

CONTENTS

Summary

Several genes essential for morphogenesis of *E. coli* are located in the 76-minute region of the chromosome. These include the cell division genes, *ftsE* and *ftsS*, and the heat shock control gene *rpoH(htpR)*. A λ transducing phage λGS22, which complemented mutations in *ftsE* and *ftsS* but not htp^R, was used to investigate the transcriptional organisation of the genes in this region. A 4.5 kb *Hin*dIII fragment was subcloned from λpGS22 into multicopy plasmid vectors, and was shown to complement mutations in *ftsE*. The DNA sequence of this fragment revealed four major open reading frames (ORFs), three of which were transcribed in the same direction and showed several features characteristic of an operon. Using transposon mutagenesis and deletion analysis, the second gene in this operon was determined to be *ftsE*. The previously unassigned mutation carried by strain OV32 was cloned and shown to map in the third gene in the operon. This novel cell division gene was designated *ftsX*. Both the *ftsE* and *ftsX* genes were shown to be co-transcribed. The *fts* gene products were identified in maxicell experiments. Interestingly, the ORF1 (*ftsY*) gene product was twice the predicted relative molecular mass. *In vitro* and *in vivo* insertion mutagenesis proved conclusively that *ftsY* encoded this protein. All four proteins were localised to the inner membrane of the cell, although some FtsE and FtsY protein was also found in the cytoplasmic fraction. It is possible that the proteins are associated with each other in the membrane as part of the 'septalsome'. The FtsE protein is particularly interesting because it shows strong homology with a small family of inner membrane-located proteins known to have nucleotide binding activity. The proteins also share strong homology at another separate domain of unknown function.

In conclusion, a novel cell division operon of three genes has been identified, and sequence, protein and localisation studies have indicated that the proteins could be associated with each other in the membrane to function in cell division.

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Declaration

This thesis has been composed by myself and has not been used in any previous application for a degree. The results presented here were obtained by myself, except for the DNA sequencing of the 4.5 kb *Hin*dIII fragment of pDG1, which was determined by G. Hatfull and G. Salmond at the MRC Unit, Cambridge. All sources of information have been specifically acknowledged by means of reference.

Deborah Gill

Abbreviations

bp	base pair
DDA	double Difco agar
EDTA	diaminoethane tetraacetic acid
h	hour(s)
kb	kilobase
IPTG	β-D-thiogalactopyranoside
min	minute(s)
m.o.i.	multiplicity of infection
MNNG	N-methyl-N-nitro-N'-nitrosoguanidine
Mr	relative molecular mass
NAM	N-acetyl muramic acid
NB	nutrient broth
PAGE	polyacrylamide gel electrophoresis
PEG	polyethylene glycol
pfu	plaque-forming units
PG	peptidoglycan
ppGpp	guanosine-5'-diphosphate-3'-diphosphate
PPO	2,5-diphenyloxazole
pppGpp	guanosine-5'-triphosphate-3'-diphosphate
sec	second(s)
SDS	sodium dodecylsulphate
TEMED	N,N,N',N'-Tetramethylethylenediamine
Tris	Trizma-base
UV	ultra-violet

Antibiotic abbreviations are listed in table 2:5(b)

Restriction enzyme abbreviations

A1	*Ava*I
A2	*Ava*II
Bg	*Bgl*II
Bm	*Bam*HI
E1	*Eco*RI
E5	*Eco*RV
H3	*Hin*dIII
Kp	*Kpn*I
Ps	*Pst*I
P1	*Pvu*I
P2	*Pvu*II
Sa	*Sal*I
Sm	*Sma*I
Sp	*Sph*I
St	*Stu*I
Ss	*Sst*I
Xh	*Xho*I

Chapter 1

Introduction

Cell division is a fundamental biological process and as such has been investigated for many years. Organisms with relatively simple cell cycles such as yeast and *Escherichia coli* have been used in the hope that principles derived from such studies will be applicable to the division and differentiation of more complex organisms. Despite the simple cell cycle of *E. coli*, the control of cell division in this organism still requires the spatial and temporal integration of many cellular activities.

In order to present the diverse background information relevant to the understanding of the analysis of cell division, this chapter has been divided into three parts each representing a principal area of investigation. Furthermore, each area is often characterised by a particular experimental approach. Part I reviews the main findings obtained from the analysis of the *E. coli* cell cycle and discusses the attempts to obtain a working model for cell division. These findings have been important in providing a basis for further experiments to investigate the link between growth and division as described in Part II, and the more recent, molecular and genetic analysis of cell division mutants, discussed in Part III. The development of all these areas, however, has largely been concurrent.

A critical discussion of all the studies involved is clearly beyond the scope of this chapter and many reviews have analysed the data in depth (Pritchard, 1974; Daneo-Moore & Shockman, 1977; Slater & Schaechter, 1977; Donachie, 1979; Helmstetter *et al.*, 1979; Nanninga *et al.*, 1982; Donachie *et al.*, 1984; Vicente, 1984).

I The analysis of cell cycle events in *E. coli*

1:1 The *E. coli* cell cycle

One of the first approaches used to study cell division, was the analysis of various physiological parameters throughout the cell cycle in an attempt to understand the relationships between them. This included the measurement of cell shape and size, to elucidate patterns of growth.

In shape, the typical *E. coli* cell is roughly cylindrical with hemispherical poles, and under conditions of steady growth rate, the cell elongates without much change in diameter (Marr *et al.*, 1966; Donachie & Begg, 1970). After approximately doubling in length, the cylindrical cell then divides by transverse fission into two cells of about equal length (Donachie *et al.*, 1976). Concurrent with this process is the replication of the chromosome and subsequent segregation of the nucleoids (Woldringh, 1976; Zusman *et al.*, 1973). The septum or crosswall forms between the two sister nucleoids, so that each cell contains a copy of the chromosome. In order for this to be achieved the assembly of cell surface polymers must be highly regulated to achieve the precise timing and position of septum formation.

The cell cycle of *E. coli* growing with a generation time 'T', can normally by resolved into two distinct periods, namely 'C' the time taken to replicate the chromsome and 'D', the period between termination of chromosome replication and cell division (Cooper & Helmstetter, 1968). The duration of each period has been measured and in *E. coli*

B/r, DNA replication (C) takes 40 minutes at 37°C, from initiation to completion (Helmstetter & Cooper, 1968). Thus, C is dependent on temperature and availability of DNA precursors, but is largely independent of growth rate (Cooper & Helmstetter, 1968). Completion of DNA replication was found to be an essential prerequisite for division (Clark, 1968; Helmstetter & Pierucci, 1968; Jones & Donachie, 1973), and division usually occurs about 20 minutes later (Cooper & Helmstetter, 1968; Helmstetter & Pierucci, 1976).

At fast growth rates, the time between successive divisions becomes shorter than the time taken to replicate the chromosome (Oishi *et al.*, 1964; Yoshikawa *et al.*, 1964) and thus initiation of a new round of DNA replication occurs before termination of those already in progress (Helmstetter & Cooper, 1968) Where generation time T is greater than C + D, there is a third period, 'B', between cell division and the next round of DNA replication (Helmstetter & Pierucci, 1976) and thus B, C and D periods have been suggested to correspond to G1, S and G2 phases in the cycles of eukaryotic cells (Cooper, 1979).

1:2 Theoretical models to explain the coupling of DNA replication and cell division

The relationships between growth, division and DNA replication have been studied extensively in an attempt to formulate a unifying model for the control of these events. Only the main findings are presented here but excellent reviews of early data include those by Slater and Schaechter (1974), Helmstetter *et al.* (1979) and Donachie, (1979). Recently a 'review of the reviews' was presented by Vicente (1984) which puts most

of the work presented here into perspective and restates the questions which remain to be answered.

The virtual absence of DNA-less cells within populations of *E. coli* indicates that the timing of DNA replication and cell division must be well regulated. The search for the 'signal' which coordinates DNA replication and division was the major aim of early work. It was first shown using synchronous populations of cells that termination of chromosome replication was a prerequisite for cell division (Clark, 1968; Helmstetter & Pierucci, 1968). Thus, if termination of a round of replication is prevented (by specific inhibition of DNA synthesis) then the cell division which would normally follow 20 minutes after that termination, is also prevented. It was thus proposed that termination of DNA replication was the crucial event in the timing of division (Zaritsky & Pritchard, 1973).

Donachie (1979) reviewed the reasons why the termination of DNA replication was unlikely to be the signal for division. For example, mutant strains of *E. coli* were isolated which were able to undergo continued cell division in the absence of DNA replication. Inouye (1969) described a temperature-sensitive DNA synthesis mutant in which the termination of replication was not required for triggering division at the restrictive temperature. Mutant strains carrying a *dnaA*(Ts) mutation were able to complete rounds of chromosome replication at the restrictive temperature, but were unable to initiate new ones. This mutation was shown to permit cell division for some time after the temperature-shift with the consequent production of many DNA-less cells of approximately normal size (Hirota *et al.*, 1968). Strains carrying *dna* mutations defective in the elongation of DNA, stop DNA synthesis

almost immediately, whereas those carrying *dna* mutations defective in the initiation of DNA, show a gradual decrease. DNA synthesis is eventually inhibited completely when a new initiation event is required. The *dnaA* mutation is defective in DNA synthesis initiation. A similar mutation *dnaC*(Ts) was described for *Salmonella typhimurium* (Spratt & Rowbury, 1971).

Donachie and co-workers (Donachie *et al.*, 1973; Donachie, 1979) then proposed that the initiation of replication created the requirement for termination of replication, explaining that if initiation did not take place, cell division could continue in the absence of all DNA synthesis. This also provided an explanation for the effects of introducing UV-damaged DNA into the cell, such as F plasmid or P1 prophage. This led to the proposal of the 'blocked replicon' model.

When DNA replication of certain plasmids such as ColI and P1 was prevented by UV-irradiation, host cell division was blocked even though replication of the host chromosome was apparently unimpaired (Monk, 1969; MacQueen & Donachie 1977). The UV-damaged DNA contains thymine dimers which prevent the progress of the replication fork although replication can be initiated. Subsequent division inhibition is temporary in strains which can repair DNA damage. This block to division is alleviated in strains which carry the *recA* mutation (Monk, 1969) which appears to uncouple division and DNA replication as part of the SOS response (see sections 1:13 and 1:14). This suggested that cell division of the host bacteria may also require completion of replication of certain plasmids if these plasmids were present in the cell. However, this termination is not usually sufficient to allow division and a further short period of *de novo* RNA and protein synthesis is also

required (Jones & Donachie, 1973).

In fact, a productive cell cycle in *E. coli* requires protein synthesis at three specific times. Firstly, protein synthesis is required for initiation of chromosome replication (Messer, 1972). Secondly, a 40 minute period of protein synthesis usually occurs concurrent with chromosome replication (Pierucci & Helmstetter, 1969), and finally, there is a short period of protein synthesis which must take place at, or subsequent to completion of chromosome replication (Jones & Donachie, 1973). The requirement for this last period of protein synthesis led to the concept of 'termination protein' which is synthesised as a consequence of chromosome replication. This study involved the synchronisation of *E. coli* cells by inhibiting protein synthesis for a period of time sufficient to allow completion of all rounds of replication. Further DNA synthesis was then prevented (by thymine deprivation or the addition of nalidixic acid) and protein synthesis allowed to resume. When the division block was eventually removed, division takes place unless protein synthesis is inhibited.

In 1963, Jacob *et al.*, proposed the 'replicon model', a positive control model to account for known facts of DNA replication. It was proposed that a replicon (unit of independent replication) should carry two specific determinants, namely a structural initiation gene and an operator which would act as the target site for initiation. Thus the product of the initiator gene would interact with the operator region to initiate replication. A mechanism to ensure that division was controlled, could be mediated via an 'inhibitor of initiation'. Thus, the inhibitor would be synthesised after each initiation event perhaps by locating the inhibitor gene near the origin of DNA replication.

Jacob *et al.*, also attempted to explain the mechanism of nucleoid segregation by comparing the cell envelope to the mitotic apparatus in eukaryotes. It was suggested that the origin of chromosomal replication could be attached to the cell membrane. Once the chromosome had been duplicated the envelope could grow at the site of attachment to segregate the chromosomes. More recently, the origin of replication has in fact been shown to be attached to the membrane and specifically to the outer membrane (Hendrickson *et al.*, 1982).

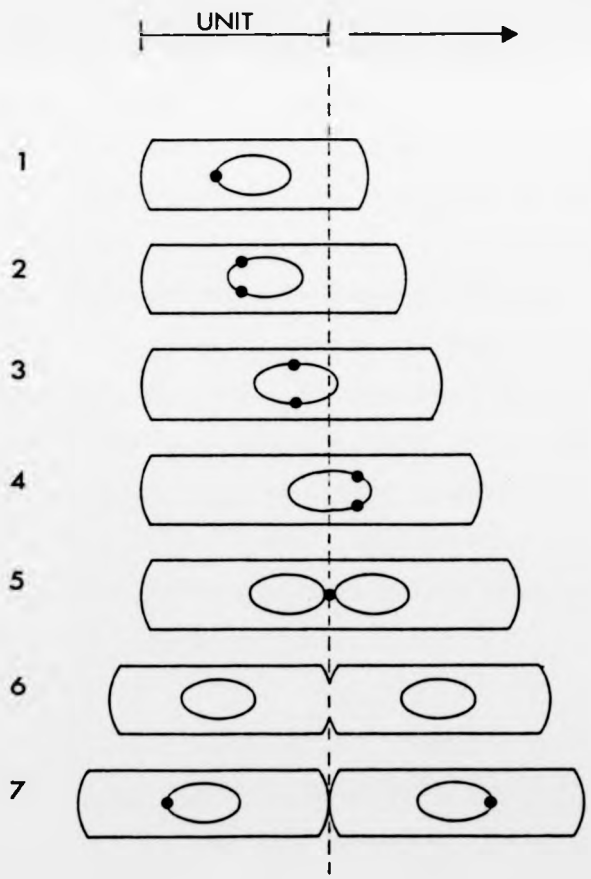
The mode of growth of the bacterial membrane was investigated by Donachie and Begg (1970) who proposed the concept of the 'unit cell' (figure 1:2). The unit cell was defined as the smallest cell of any given strain that can exist under any growth conditions. When the generation time of a cell is greater than 60 minutes the unit cell grows in one direction only from a single membrane growth site located at one pole, until the cell has reached twice its unit length. At this stage the growth site is located in the centre of the cell. It was proposed that, if these membrane growth sites were also the membrane attachment sites proposed by Jacob *et al.* (1963) then once the chromosome had been replicated, division at this site would ensure that sister chromosomes were segregated to sister cells (Donachie & Begg, 1970).

Donachie *et al.* (1976) showed that individual cells become committed to division when they reach a critical cell mass (initiation mass), but pointed out that the trigger for subsequent events was not cell mass (or length) *per se* but some co-incident event. This could be the synthesis of a series of division-specific proteins which might be concurrent with the initiation of chromosome replication (Pierucci & Helmstetter, 1969; Jones & Donachie, 1973).

Figure 1:2 The 'unit cell' model of growth.

When the generation time of the cell is greater than 60 minutes, the unit cell grows in one direction as indicated by the arrow. Stages 1-5 - polar growth of the cell and replication of chromosome; stage 6 - septum formation; stage 7 - cell separation.

Redrawn from Plakidou, (1983).



An alternative negative-control model for coupling of replication and division was proposed by Pritchard and co-workers, who investigated the relationship between chromosome replication and division by measuring changes in C and D periods under different conditions. The 'negative control model' (Pritchard *et al.*, 1969; Pritchard, 1974) was also based on ideas presented by Jacob *et al.*, (1963). This model proposed that chromosome replication would occur at any time, if it were not for the presence of an inhibitor locus, close to *oriC*, coding for an inhibitor or repressor. Replication of this locus early in chromosome replication resulted in a doubling in the concentration of the repressor which would then inhibit further initiations. It was suggested that this inhibitor would be diluted out, either by growth or by inherent instability. These workers also postulated that there was no specific division inhibitor and no fundamental difference between cell elongation and crosswall formation. Thus, according to this model, envelope growth occurs by addition of subunits in a manner similar to that described for *Streptococcus faecalis* (Higgins & Shockman, 1971; see section 1:5). The cell would extend in length at a constant rate which doubles once during the cycle at the end of chromosomal replication (Zaritsky & Pritchard, 1973; Pritchard, 1974; Zaritsky, 1975). Thus it was proposed that the envelope was laid down at the leading edge of a septum so that the cell is always ready to form a septum, but only does so when the rate of envelope synthesis doubles and the internal hydrostatic pressure falls rapidly enough, to allow a septum to form.

Slater and Schaechter (1974) summarised the relationship of DNA replication and cell division and concluded that it was highly complex, subject to change and affected by several facets of DNA metabolism including DNA repair and recombination. Helmstetter *et al.* (1969)

compared the positive- and negative-control models and decided that the crucial difference between them was whether or not chromosome replication and cell division were independent or interdependent. Vicente (1984) however, described the difference as being the nature of the signal required for septation. This may be the cell itself (Pritchard, 1974; Rosenberger *et al.*, 1978), or it may be the synthesis of individual molecules (Donachie, 1973). Tyson (1985) reviewed the mathematical evidence for various predictions which have been made about the control of cell cycle events. Statistical analysis of the experimental data and comparisons with the predicted results from various models showed that as yet, no one model accounts for all the observations made and concluded that a detailed analysis is now required for a single cell type in order to obtain such a unifying model. In whatever way one attempts to classify the models proposed to explain the coupling of DNA replication and cell division, it is obvious that further experiments using new approaches are required to distinguish between them.

1:3 Summary and appraisal of this work

Much of the work described earlier was aimed at determining the specific event in the cell cycle which can be correlated with division. This event has been proposed to be the termination of chromosomal replication (Zaritsky & Pritchard, 1973), the initiation of chromosomal replication (Donachie *et al.*, 1973), the duplication of an unregulated gene (Pritchard, 1974) or the attainment of a specific cell length (Donachie *et al.*, 1976).

Despite the extensive work and the enormous amount of data obtained using this approach, no unifying theory has finally emerged. There are probably several reasons for this. Rosenberger *et al*. (1978) suggested that these studies suffered from a "dearth of measurements and an absence of rigorous comparisons between predicted and experimental values, among contending models". For example, many of the models were based on findings from experiments designed to measure the duration of the C and D periods and the effects of interrupting these periods. However, the lengths of C and D periods were not found to be invariant, as was initially thought, (Marinouchi & Messer, 1972) and were found to differ between strains (Helmstetter & Pierucci, 1976).

Also, many of the experiments designed to investigate the coupling between DNA replication and cell division were carried out in *E. coli* B/r strains which are now believed to carry two mutations affecting division control via the SOS response (W. Donachie, pers. comm.). Moreover, the *E. coli* B strain was shown to carry a mutation in *ftsZ* (Belhumeur & Drapeau, 1984) and the *ftsZ* gene product has recently been shown to play a crucial role in initiation of septation (section 1:15). Finally, many of these experiments involved a perturbation of the normal DNA replication period based on the assumption that this would not specifically affect division. It is now known that the SOS response (section 1:13) to DNA damage includes one or more specific links between replication and division (section 1:14). It is therefore difficult to assess the importance of much of the data.

In conclusion, the various models presented should be selectively reviewed in order to provide a framework for current experiments.

II The role of peptidoglycan synthesis in the determination of cell shape and cell division

As described in section 1:1, the *E. coli* cell grows by doubling in length and divides into two cells of equal length (Donachie *et al.*, 1976), by the formation of a septum. The division process must be well-regulated to ensure the segregation of sister chromosomes to sister cells and this is achieved via the precise positioning and timing of the synthesis of the septum. Many investigators have therefore concentrated their efforts on the characterisation of septum components and the regulation of synthesis of these components during division. This section aims to present the main findings on the structure of the septum and discusses models describing its assembly.

1:4 The bacterial cell wall

The Gram-negative *E. coli* cell envelope is a complex structure and consists of three layers (reviewed by Tipper & Wright, 1979). Figure 1.4a, is a representation of the *E. coli* cell envelope, redrawn from Lugtenberg and Van Alphen (1983).

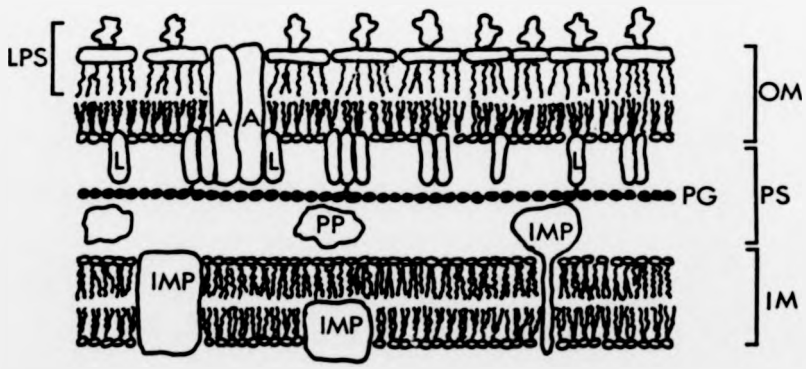
The inner, or cytoplasmic, membrane is a phospholipid bilayer with associated protein presumed to form a fluid mosaic (Singer & Nicholson, 1972), and is functionally equivalent to the eukaryotic plasma membrane It acts as the cells' main permeability barrier and is responsible for active transport.

The outer membrane is an asymmetric bilayer containing lipopoly-

Figure 1:4(a) Diagrammatic representation of the *E. coli* cell envelope.

The three main layers of the cell envelope are shown: outer membrane (OM), inner membrane (IM) and peptidoglycan layer (PG). Also shown are: OmpA (A); lipoprotein (L); inner membrane proteins (IMP); periplasmic protein (PP); periplasmic space (PS); lipopolysaccharide (LPS).

Redrawn from Lugtenberg & Van Alphen (1983).



saccharide and protein (reviewed by Wright & Tipper, 1979; Osborn & Wu, 1980). It provides a barrier to prevent loss of enzymes contained within the periplasmic space, but is permeable to nutrients and ions required for growth. Two of the outer membrane proteins, OmpA (protein II*) and lipoprotein (encoded by the *lpp* gene) are associated with the peptidoglycan layer, found between the inner and outer membranes.

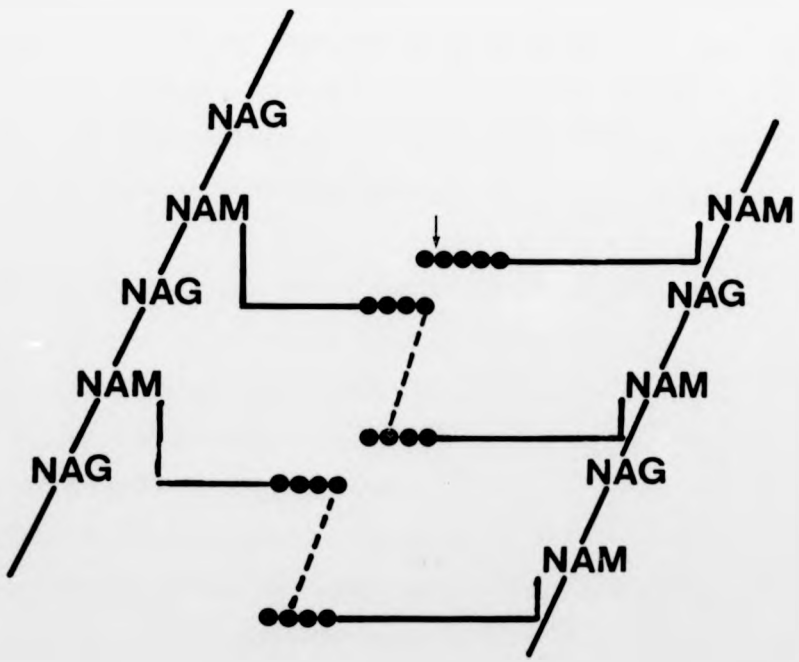
The peptidoglycan (PG) layer or sacculus consists of alternating residues of N-acetyl muramic acid pentapeptide (NAM) and N-acetyl glucosamine (NAG). Covalent bonds between side chains help form a net-like structure (figure 1:4b). PG precursors are incorporated into the mono-molecular sacculus and it is this extensive cross-linking which give the PG layer its strength. Thus, the strength and rigidity of the bacterial cell can largely be attributed to the PG layer. Many of the enzymes involved in the synthesis of PG are encoded by genes located in morphogene clusters (section 1:7) and these are shown in figures 1:7 and 1:12.

The role of PG in bacterial cell shape is unclear and the determination of cell shape is also likely to involve other components. For example, Wensink *et al.* (1982) showed that 10% of the peptide side chains of PG are covalently bound to lipoprotein in the outer membrane. This may suggest a role for lipoprotein in determining the strength and shape of the PG layer. Although mutant strains of *E. coli* which lack OmpA protein can grow and divide normally (Henning & Haller, 1975), the OmpA protein has been suggested to play a role in cell morphogenesis because cells lacking both OmpA and lipoprotein (*ompA,lpp* double mutants) lose their normal rod shape and grow as osmotically stable spheres (Sonntag *et al.*, 1978). Begg and Donachie (1984) examined the distribution of

Figure 1:4(b) Diagram to indicate the net-like structure of the sacculus.

Chains of alternating residues of NAM and NAG are shown. The strength and rigidity of the PG sacculus is derived from the covalent cross-linking of the NAM pentapeptide side-chains as indicated.

The terminal D-alanine residue is removed from the pentapeptide side chain by D-alanine carboxypeptidase enzymes as indicated by the arrow.



OmpA in the cell envelopes and found it to be predominantly located at the cell poles of the cell envelope.

The characterisation of cell shape mutants has led to a greater understanding of the structural and enzymatic processes involved in the maintenance of cell shape. This type of approach is described in detail in Part III.

1:5 Growth and assembly of the cell wall

The exact mechanism of biosynthesis and assembly of PG in *E. coli* remains unclear. In *Streptococcus faecalis*, a system of growth zones responsible for elongation and crosswall formation, result in the incorporation of polymers into the PG layer (Daneo-Moore & Shockman, 1977). These zones provide the basis for successive cycles of cell wall synthesis and septum formation. Attempts to identify similar zones in *E. coli* have produced confusing results.

Ryter *et al.* (1973, 1975), investigated the growth pattern of the murein sacculus and found well-defined growth zones at the centre of each daughter cell but they also showed that there was a rapid redistribution and mixing of murein within the sacculus. This rapid mixing was often a source of confusion. Begg and Donachie (1973, 1977) also investigated the growth of the cell envelope by following the distribution of attached phage particles and again conflicting results were obtained. Nanninga *et al.* (1982), reviewed much of this work and concluded that it was not possible to distinguish clearly between diffuse growth and zonal growth.

These technically difficult experiments were probably complicated by the lack of information on lateral diffusion in the outer membrane, of markers used to distinguish between old and new surface. Davison and Garland (1983) overcame this complication, by using an approach in which they crosslinked surface-bound antibodies and then followed growth using immunochemical labelling. In this way, they were able to classify the outer membrane antigens of *E. coli* into those which were laterally mobile and those which were not. The latter type was presumed to be immobile due to anchorage in PG. Thus, zones of old (labelled) surface growth and new (unlabelled) surface growth could be distinguished, and it was concluded that growth of PG was indeed zonal. However this does not imply that growth of the outer membrane is also zonal, but it does provide a basis for further experiments and supports the unipolar growth model of Begg and Donachie (1977).

1:6 Peptidoglycan assembly and cell division

1:6:1 Penicillin-binding proteins

The identification of the targets of action of β-lactam antibiotics, has provided much information about the role of PG assembly in cell division. These antibiotics (penicillins and cephalosporins) kill bacteria by inhibiting the terminal stages of PG synthesis. The penicillin binding proteins (PBPs) have been identified as the enzymes inhibited by β-lactams and the mechanisms by which this inhibition occurs have been reviewed (Spratt, 1977a, 1983; Waxman & Strominger, 1983).

Spratt (1977b) described a method whereby PBPs, located in the inner

(cytoplasmic) membrane, can be identified after binding with [^{14}C]-benzylpenicillin and anaylsis on SDS-polyacrylamide gels. Table 1:6:1 lists the properties of several PBPs. PBPs 4, 5 and 6 are non-essential for growth of *E. coli* under laboratory conditions and these PBPs are therefore not of major importance in the killing reaction of β-lactam antibiotics (Matsuhashi *et al.*, 1977; Spratt, 1980; Broome-Smith and Spratt, 1982). Inactivation of PBP 1A/1B, PBP2, or PBP3 is sufficient for death of the cells and β-lactam antibiotics exist which kill by each of these routes (Spratt, 1983). Characterisation of the mechanism of action of these antibiotics and subsequent observations of cell morphology, has suggested a role for some of these proteins in division and the maintenance of cell shape. For example, PBP2 (encoded by *pbpA*) is inhibited by the penicillin mecillinam which causes the production of spherical cells (Spratt, 1977a). The role of PBP2 in the regulation of cell shape was further strengthened by the isolation of two classes of mutant altered in PBP2. One class of mutants was resistant to the action of mecillinam and grew as stable spheres (Spratt, 1975). The other class of PBP2 mutants carried temperature-sensitive mutations in the *pbpA* gene and at the restrictive temperature, strains carrying such mutations also grew as spheres (Spratt, 1977a). PBP2 has therefore been assigned a role in the elongation of the cell. In agreement with this, Buchanan (1981) showed that *E. coli* minicells have reduced levels of PBP2, when the distribution of PBPs in minicell and normal rod-shaped cells was compared.

A different set of antibiotics (for example, cephalexin and furazlocillin) bind to PBP3 encoded by *pbp3/ftsI/sep* (see also section 1:9). Spratt (1977c), investigated the thermostability of the PBPs in various cell division mutants. Two independent, temperature-sensitive

Table 1:6:1 Characteristics of the penicillin-binding proteins

PBP	Mr	Gene	Location	Enzymic Activity	Function
1A	92000	*ponA*	73.5	TPase/TGase	Elongation
1B	90000	*ponB*	3.3	TPase/TGase	Elongation
2	66000	*pbpA*	14.4	TPase	Cell shape
3	60000	*ftsI/sep/pbpB*	1.8	TPase/TGase	Cell division
4	49000	*dacB*	68.0	CPase	?
5	42000	*dacA*	13.7	CPase	?
6	40000	*dacC*	?	CPase	?

TPase - transpeptidase
TGase - transglycosylase
CPase - D-carboxypeptidase

division mutants were found, which caused filamentation and the failure to bind [^{14}C]-benzylpenicillin at the restrictive temperature. PBP3 was therefore ascribed a role in PG synthesis specifically associated with septum formation (Botta & Park, 1981). The enzymatic nature of these proteins has also been investigated. Ishino *et al.* (1982) showed that PBP2 has transpeptidase activity, whereas PBP3 has both transpeptidase and transglycosylase activity *in vitro* (Ishino & Matsuhashi, 1981).

Recently the active site of these enzymes for the binding of penicillin has been determined (Broome-Smith & Spratt, 1985; Nicholas *et al.*, 1985). The active site was found to contain a unique residue which forms an acyl enzyme intermediate with penicillin G. This active site was also identified for certain β-lactamases and D-alanine carboxypeptidases. The nucleotide sequence of the genes encoding PBPs 1A and 1B of *E. coli* (Broome-Smith *et al.*, 1985a), and the sequence of the gene encoding PBP3 (Nakumura *et al.*, 1983) were both shown to contain a short identical sequence, corresponding to the active site of these enzymes (Broome-Smith *et al.*, 1985b). This active site contained the serine residue (Ser-307) known to be acylated by penicillin. Broome-Smith *et al.* (1985b), described the conversion of this serine residue of PBP3 in *E. coli*, to a cysteine residue, using site directed mutagenesis to produce a mutant PBP3 (thiol-penicillin-binding protein 3). Constitutive expression of the mutant PBP3 caused extensive filamentation of *E. coli* cells. These workers suggested that this division inhibition was probably due to interference of wild type PBP3 activity in division, by incorporation of mutant PBP3 into some active complex *in vivo*. Incorporation of mutant PBP3 into this complex would inactivate it, resulting in inhibition of division and filamentation (see also section 1:15).

1:6:2 An enzymatic model for cell division

If PBP2 plays a role in elongation and PBP3 is required for septation, it would be important to discover how these two enzymes and others like them, are co-ordinated during growth and division to produce normal rod-shaped cells.

Satta *et al.* (1979, 1980), proposed a model for shape regulation in Gram negative bacteria, suggesting that two sites exist for PG assembly, one responsible for septum formation and one for lateral wall formation, and that these two sites compete with each other for available PG precursors. Thus cell shape would be determined by a balance between these two competing sites. An enzymatic basis for this model was suggested by Mirelman *et al.* (1976, 1977) who investigated PG synthesis during cell division and noted that filamentation was associated with a decrease in the level of D-alanine carboxypeptidase activity. This enzyme removes the terminal D-alanine from the pentapeptide sidechains of murein thus preventing their action in cross-links and could act to 'loosen' pre-existing murein ready for septation. In filamenting cultures of the temperature-sensitive division mutant, PAT84, (induced by temperature-shift or inhibition of DNA replication), cells contained lower levels of D-alanine carboxypeptidase compared to the levels found in normally dividing cells. Transpeptidase enzymes catalyse the incorporation and cross-linking of newly-synthesised murein into the cell wall. Mirelman *et al.*, (1977) suggested that the inability to form septa was due to an imbalance between transpeptidase enzymes required for elongation and the D-alanine carboxypeptidase enzymes. Markiewicz *et al.* (1982) provided further support for this proposal when they showed that an increase in the level of PBP5 (a D-alanine carboxypeptidase) was involved in switching PG synthesis from elongation

to division.

Canepari *et al*. (1984) analysed these models and predicted that in cell division mutants, the inability to divide was due to a block in PG incorporation at the division site. Thus PG was assembled at the site for lateral wall elongation only, resulting in filamentation of the cells. This block could be due to the destruction of the septation site or simply the inhibition of PG incorporation at the site. Canepari *et al*. (1984) found a *pbpB*(Ts) mutation of the latter type (*sep2158*Ts) and showed that inhibition of elongation (by addition of mecillinam) allowed cell division to occur at the restrictive temperature. Thus, the balance had been redressed to alleviate the block at the septal site. These data support the idea that division involves a change in the balance between the incorporation of PG into the septum and cell wall elongation.

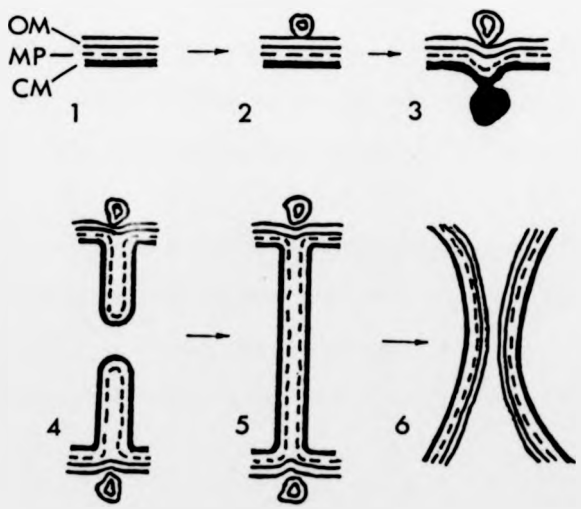
Recently, Begg and Donachie (1985) investigated the cell morphology of strains carrying mutations which blocked cell elongation and those which blocked septation. Their results suggested that neither of these processes was required for overall growth of the cell (as measured by general PG synthesis) and therefore, they postulated that the cell has a generalised system for cell growth in addition to a system for determination of cell shape.

The sequence of morphological events at septation was observed using electron microscopy of synchronised cultures *E. coli* B and B/r strains (Burdett & Murray, 1974b). A diagram representing this sequence is presented in figure 1:6:2. Burdett and Murray divided the septation process into three phases, including the initial modification of the

Figure 1:6:2 Diagrammatic representation of the morphological events observed during septation.

Stages 1-6 in the septation process were observed using electron microscopy of synchronous cultures of *E. coli* B and B/r strains (Burdett & Murray, 1974b). Stage 1 = cell envelope prior to septation; 2 = formation of a 'bleb' from the outer membrane (OM); 4 and 5 = invagination of the cytoplasmic membrane (CM) and mucopeptide (MP) layer; 6 = the OM 'bleb' is incorporated into the new septum.

Redrawn from Burdett & Murray (1974b).



cell wall, the assembly of septum 'precursors' and, finally, the organisation of these components into a septum.

The initial modification of the cell wall probably involves the action of murein hydrolases (autolysins) which have already been shown for *Streptococcus faecalis* (Higgins & Shockman, 1971). In *E. coli* the evidence of autolysin action is less direct and has been demonstrated using low concentrations of antibiotics (Schwarz *et al.*, 1969). When division is blocked by low doses of penicillin, a 'bulge' is formed in the middle of the cell at presumptive septum sites. Elongation of the cell can continue but ceases on addition of increased concentrations of penicillin which totally inhibit murein synthesis. The formation of the bulge is presumed to be due to the action of autolysins on the murein after inhibition of the transpeptidase reaction. These observations also support the proposal of a defined membrane growth site suggested in section 1:5. Burdett and Murray (1974a) studied the thermo-sensitive cell division mutant PAT84 and showed that 'bulge formation' did not occur at the restrictive temperature. This supported the idea that autolysins were involved in modifying murein at an early stage in septum formation - a stage in which the mutant PAT84 was defective.

The proposed stages in the septation process deduced from morphological observations are now being correlated with findings from the analysis of cell division mutants. The characterisation and subsequent classification of such mutants are described in Part III.

III The genetic analysis of cell division

1:7 The genetic approach

The genetic approach to studying cell division involves the isolation and characterisation of mutants defective in different stages of division. These mutant strains carry mutations in specific genes, the products of which are essential for division and cell viability. Thus, these mutations must be conditional so that under 'permissive' conditions the cells can grow normally whereas under restrictive conditions the mutant phenotype is expressed. These conditions are usually those of growth temperature, so that division can only occur at the permissive temperature.

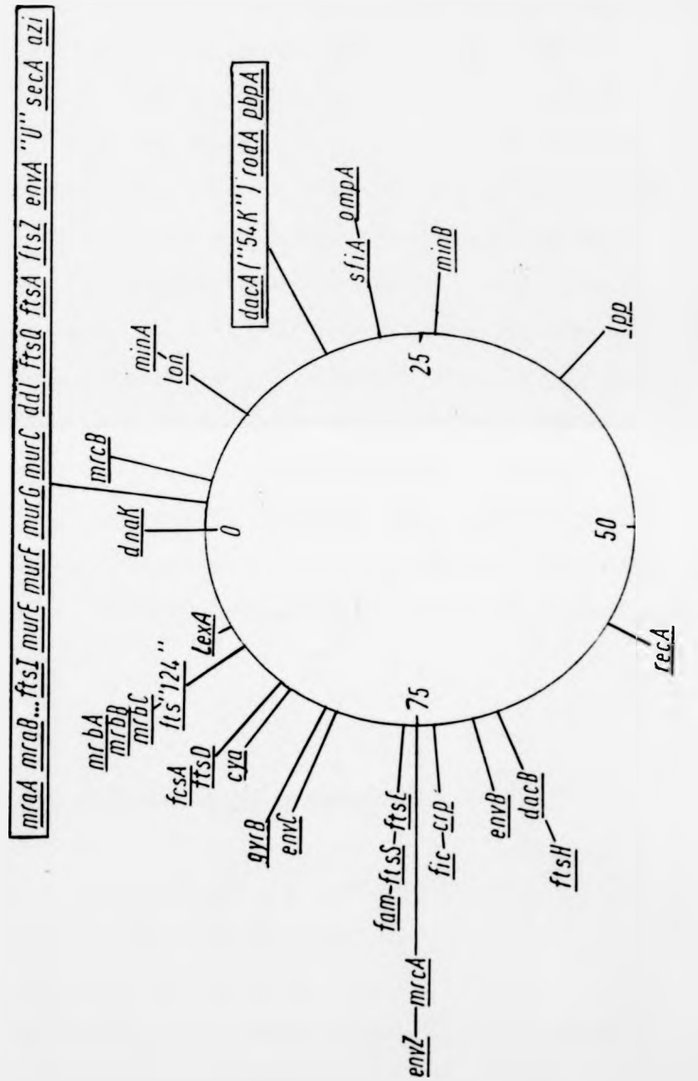
Once the mutants have been isolated their position on the *E. coli* linkage map (Bachmann, 1983) can be determined and the relevant gene cloned and analysed. Ultimately the aim of such an investigation is to ascribe a function to the gene product during division. Genes which have a primary function in the growth and shaping of the cell, have been termed 'morphogenes' (Donachie *et al.*, 1984), although it is impossible to rigorously classify such genes because many other genes involved in other cellular processes are also likely to have an effect on morphogenesis.

Figure 1.7 represents the *E. coli* chromosome showing the distribution of morphogenes. It can be seen that the distribution is not random but that some of the genes form 'clusters'. A cluster is a group of two or more genes of related function which constitute more than one

Figure 1:7 The distribution of morphogenes on the *E. coli* linkage map.

The *E. coli* linkage map is represented as a circle divided into 100-minutes. All the genes indicated are presumed to play a primary role in morphogenesis except for three SOS-inducible genes (see section 1:13) namely, *lexA*, *recA* and *sfiA*. The genes *crp* and *cya* may also play a secondary role (section 1:10). The gene clusters at 2-minutes and 15-minutes should be noted.

Redrawn from Donachie *et al.*, (1984).



independent operon. A large cluster of 14 genes located in the 2-minute region has been extensively studied (sections 1:11 and 1:12). The function, if any, of such clustering is unknown and has been the subject of much speculation (Donachie *et al.*, 1984; Vicente, 1984).

Examination of the cell morphology of strains carrying mutations in morphogenes, shows that these mutants can be divided into two broad categories, namely those which result in long aseptate filaments and those which result in rounded (spherical) cells. This would be expected if as suggested in section 1:6, cell shape is determined by competition betweeen the two enzymatically controlled processes of elongation and septation. A more detailed analysis of these mutants however, has revealed that they are blocked in different stages of division. A comprehensive review of these analyses has been presented by Donachie *et al.* (1984). The following sections aim to present an overview of the division process as deduced from information obtained from the analysis of division mutants.

1:8 Mutations which result in rounded cells

In section 1:4 it was noted that the double mutant *lpp ompA* showed a rounded or spherical morphology (Sonntag *et al.*, 1978). Certain other mutations also cause *E. coli* cells to adopt a round cell-shape, and these include mutations in *pbpA* (Henning *et al.*, 1972; Spratt, 1977c; Iwaya *et al.*, 1978) and *rodA* (Matsuzawa *et al.*, 1973; Spratt, 1977c; Stoker *et al.*, 1983a).

The genes *pbpA* and *rodA* are located in a small cluster of genes which

also include the gene *dacA* at approximately 14.5-minutes on the *E. coli* linkage map (Spratt *et al.*, 1980). As discussed previously (section 1:6), the addition of low concentrations of the β-lactam antibiotic mecillinam to an *E. coli* culture, produced rounded cells as a result of binding to PBP2. Strains carrying a *pbpA*(Ts) mutation also produce rounded cells at the restrictive temperature. Strains carrying *rodA* mutations are also rounded but can be distinguished from *pbpA* mutants because they are not defective in PBP2 (Spratt *et al.*, 1980; Tamaki *et al.*, 1980). The *rodA* gene product is required for growth of rod-shaped cells and presumably plays an essential role in the synthesis of the cell wall during elongation (Stoker *et al.*, 1983a).

Mutations in *rodA* and *pbpA* are distinct from another class of mutants which result in rounded cells. The *envB* mutation (Westling-Haggstrom & Normark, 1975) results in spherical cells with asymmetric constrictions, which appear to be abnormal septa. Also, cells defective in adenyl cyclase (*cya*) or in the *crp* gene encoding the adenosine 3',5'-monophosphate (cAMP) receptor protein (CAP) have been described as round although the rounded cells were actually found to be shortened rods (Scott & Harwood, 1981). The involvement of the cAMP-CAP complex in determining cell morphology may be indirect. Septation may be controlled by some metabolic process which is regulated by intracellular levels of cAMP. Utsumi *et al.* (1982), isolated mutants which filamented when treated with exogenous cAMP and designated the relevant mutation *fic* (filamentation induced by cAMP). This mutation mapped close to, and was possibly allelic with, *crp*. Donachie *et al.* (1984), suggested that the cAMP-CAP complex exerts negative regulation on PG synthesis at the site of septation and it would be interesting to analyse the data supporting this claim.

Unlike mutations in *rodA* or *pbpA*, mutants carrying mutations in *envB*, *cya* and *crp* retain the ability to filament when division is blocked by the addition of nalidixic acid (Donachie *et al.*, 1984). It is therefore presumed that these mutants are not completely blocked in cell wall elongation.

1:9 Mutations which result in filamentation

A large number of the mutants defective in cell division form long, aseptate filaments at the restrictive temperature, although the synthesis of DNA and RNA, and increase in cell mass are unaffected. If after only a few hours such mutants are returned to the permissive temperature, then the cells are usually still viable. The majority of these mutations have been designated *fts* (*f*ilamentation *t*emperature-*s*ensitive) and many of these are listed in table 1:9.

The physiology of these mutants has been summarised by Helmstetter *et al.*, (1979) and the studies have mainly concentrated on describing only a few characteristics.

Firstly, the conditional phenotype of some of these mutations is osmotically-reversible. The basis for this finding is unclear, but it is anticipated that it is due to an alteration in the cell envelope. Osmo-reversibility was shown to occur for strains carrying mutations in *ftsF*, *fts'ASH124'*, *ftsZ* and some alleles of *ftsE*, (Helmstetter *et al.*, 1979).

These mutants have also been studied with regard to the effect of

Table 1:9 Cell division mutations which result in filamentation

Gene	Location	Reference
ftsA	2′	Van de Putte, 1964
ftsD	86′	Ricard & Hirota, 1973
ftsE	76′	Ricard & Hirota, 1973
ftsF	82′	Ricard & Hirota, 1973
ftsH	61′	Santos & de Almeida, 1975
ftsI	2′	Walker *et al.*, 1975
ftsM	2′	Drapeau *et al.*, 1983
ftsS	76′	Salmond & Plakidou, 1984
ftsQ	2′	Begg *et al.*, 1980
ftsZ	2′	Lutkenhaus *et al.*, 1980
fts′ASH124′	90′	Holland & Darby, 1976*

This list does not include certain mutations which, although designated *fts*, have not been shown to be specifically blocked in septation. Such mutations have been discussed elsewhere (Donachie *et al.*, 1984).

*The *fts*′ASH124′ mutation is sometimes called *ftsH*

filamentation on peptidoglycan metabolism (Pages *et al.*, 1975), and the necessity of protein synthesis for the recovery of cell division after the release of the temperature-induced division block (Ricard & Hirota, 1979). Unfortunately, much of the information reviewed by Helmstetter *et al.*, is now obsolete, because in recent years, the detailed analysis of these mutants has revealed that either the mutants are not specifically blocked in septation, or they carry different alleles of the same gene. A review of this information and probably a repeat of many of the experiments in the light of current knowledge is now required.

Although the majority of these mutants are simply unable to form septa at the restrictive temperature, a more detailed analysis of the mutant phenotypes has allowed their further sub-classification. Mutants defective in the early stages of septation are probably blocked in the 'signal' for septum initiation whilst those blocked at later stages could be defective in septum assembly. Donachie *et al.* (1984) suggest that such a septum-initiation mutation is *ftsH* (Santos & de Almeida, 1975) located at 61-minutes on the *E. coli* linkage map. This mutation is similar to other *fts* mutations except that once shifted to the restrictive temperature, strains carrying this mutation, do not cease division immediately. Donachie *et al.* (1984), have suggested that FtsH is required for septum initiation and thus when strains defective in this gene are shifted to 42^{o}C, some of the cells which have already been initiated will go on to complete septum formation. However, this effect may also be due to slow inactivation of the mutant protein.

Other *fts* mutations which block septum formation are listed in table 1:9. With the exception of *ftsA*, all the genes listed have mutations

which block early septum formation. The *ftsA* mutation however (Van de Putte, 1964), results in filaments with visible constrictions presumed to be partially completed septa. These *ftsA* mutants are therefore blocked in the later stages of septation. Mutations in genes required for septum separation have also been identified. Strains carrying mutations in the *envA* gene (Normark, 1969, 1970), appear to form septa but cannot separate the daughter cells.

Four of the genes listed in table 1:9 are located in the 2-minute region and these are the most extensively studied *fts* genes. A summary of the information known about each of these genes is given in section 1:11.

1:10 Mutations affecting septum location

One type of mutant is not defective in initiation or in the formation of septation, but is defective in the correct positioning of the septum. Strains carrying the minicell mutation (*minA*, *minB*) constitutively produce minicells, which are small DNA-less cells (Adler *et al.*, 1967). The minicells are the result of a septum forming very close to one end of the bacterial rod-shaped cell. Davie *et al.* (1984), has recently cast doubt on the existence of the *minA* mutation, and suggested that only the *minB* mutation located at 26-minutes on the linkage map (Schaumberg & Kuempel, 1983) is required for the minicell phenotype. Although the positioning of the septa in this mutant is incorrect, it is not completely random. Septation occurs only at 'normal' division sites, in the centre and at the poles of the cell (Donachie & Begg, 1970). The number of septa formed per unit cell length is always the same as normal-sized cells, and thus the minicell mutation always

results in cells of multiple unit-lengths as well as minicells.

Donachie *et al.*, (1984) presented an interesting hypothesis to explain these observations. They suggested that the potential division site laid down at regular intervals during cell elongation can be used more than once unless it is inactivated. Thus, when division occurs in normal-sized cells, the potential division site, which usually ends up at the pole of the newborn cell, has been inactivated. In minicells, the division site can undergo septation again to produce small DNA-less cells.

Another mutant which is defective in the correct positioning of septa carries a *gyrB*(Ts) mutation (Orr *et al.*, 1979). This gene codes for one of the two subunits of the enzyme DNA gyrase which is a type II topoisomerase. This enzyme can introduce negative supercoils into a relaxed closed circular DNA molecule. The two subunits encoded by *gyrA(nalA)* and *gyrB(cou)* are inhibited by two types of antibiotics, nalidixic acid and coumermycin respectively. These antibiotics inhibit DNA replication suggesting that gyrase is necessary for DNA replication to proceed. Strains carrying a *gyrB*(Ts) mutation (located at 83-minutes) are unable to segregate nucleoids or form septa at the restrictive temperature (Orr *et al.*, 1979). Septation can occur however at apparently random locations along the length of the cell resulting in cells of many different sizes. Another mutant has also been isolated which results in a similar phenotype and this has been shown to map in the *ass* gene (*a*berrant *s*eptation and *s*egregation), a novel gene located at 85.5-minutes (K. Hussain & G. Salmond, unpublished results). In both mutants the chromosome is replicated but because the cell is unable to segregate, a large nucleoid forms in the middle of the cell. It is

possible that the gyrase mutant cannot segregate its chromosome at the restrictive temperature due to topological constraints imposed by the mutant GyrB activity. However, a function for the Ass protein is unknown (K. Hussain, pers. comm.) and the study of such mutants should help determine the mechanism of nucleoid segregation and septation. Particularly interesting is the apparent randomness of the positioning of septa. The control for the location of a septum must be relaxed in some way, perhaps by interfering with the 'signal' which couples DNA replication and division.

In 1980, Martinez-Salas and Vicente, identified the conditional *wee*(Am) mutation. Strains carrying this mutation do not affect the positioning of septa but they do allow septation to occur earlier than usual. Thus, when shifted to the restrictive temperature the mean cell length of the cell population was decreased. It was suggested that the *wee* gene product could be defective in the co-ordination between elongation of the cell and division (Martinez-Salas & Vicente, 1980) (see also 1:15).

In conclusion, the mutants described here are potentially useful in providing information about the way in which the number and location of septa are controlled.

1:11 The distribution of morphogenes in the *E. coli* chromosome

As shown in figure 1.7, the morphogenes of *E. coli* are scattered around the chromosome except for several clusters. A relatively small cluster of genes involved in cell-shape is located at approximately 14.5-minutes, as described in section 1:8. Another morphogene cluster is

emerging in the 76-minute region and the essential genes found in this new cluster are described in sections 1:19 and 1:20.

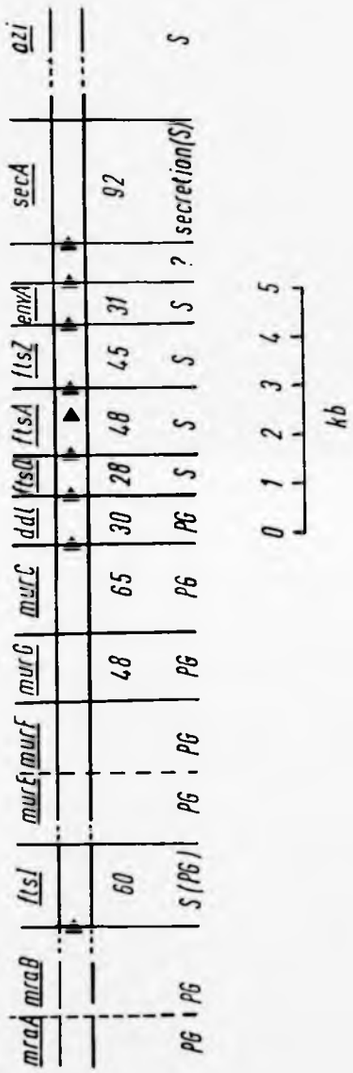
The largest cluster is located in the 2-minute region and has been extensively studied. An historical perspective of the work will not be presented here, but is reviewed by Sullivan (1984). Figure 1.11 summarises the current view of the arrangement of genes in this cluster. The close association of the genes and the fact that they are all transcribed in the same direction is of interest. The suggestion by Lutkenhaus and Wu (1980), that the *envA* gene was transcribed in the opposite direction has now been shown to be incorrect (Sullivan, 1984). These genes however, do not apparently represent a single transcriptional unit, because each gene has its own promoter (Lutkenhaus & Donachie, 1979; Lutkenhaus *et al.*, 1980; Lutkenhaus & Wu, 1980; Oliver & Beckwith, 1982; Sullivan, 1984). In fact, it has also been shown that several of these genes have more than one promoter, required for optimal expression of these genes. For example, Jones and Holland (1984), used transposon insertions to identify an additional *ftsZ* promoter present in the coding region of the adjacent *ftsA* gene. The level of transcription of each of these two promoters (designated PZ_1 and PZ_2 was measured using cloning vectors carrying each of the promoter sequences fused to the *E. coli galK* gene for β-galactokinase (Sullivan & Donachie, 1984a). It was found that the level of transcription obtained when both PZ_1 and PZ_2 were present, was greater than the separate activities of the promoters. This suggested the existence of a cooperative enhancement of transcription between these two promoters.

Donachie *et al.* (1983), used gene fusion technique to measure transcription from the *ftsA* promoter. The level of transcription was

Figure 1:11 The arrangement of morphogenes in the 2-minute cluster.

The approximate limit of each gene is represented by a vertical line. The arrow heads indicate the approximate locations and directions of known promoters. Each of the *mur* genes also has at least one promoter although the positions of these are not yet known. The main function of each gene is indicated as follows: PG = peptidoglycan synthesis; S = septation. The approximate relative molecular mass ($x10^{-3}$) of identified polypeptides is also shown.

Reproduced from Donachie *et al.*, (1984).



found to decrease in the presence of chromosomal mutations in the division genes *ftsI*, *ftsQ* and *ftsZ*, whereas it was unaffected by the presence of mutations outside the 2-minute region such as *ftsE*. Therefore, although the *ftsA* promoters allow transcription of the gene in isolation from the rest of the cluster, there appears to be a strong *cis*-acting enhancement effect observed, similar perhaps to that found for the two *ftsZ* promoters (Sullivan & Donachie, 1984a). The further investigation into the interaction of these genes is obviously required. Section 1:12 describes the information known about the proteins encoded by these genes.

1:12 Gene products encoded in the 2-minute region

Out of all the morphogenes isolated, most information is available about the products of the *envA*, *ftsI*, *ftsA* and *ftsZ* genes. The *envA* mutation causes *E. coli* to form chains of cells during fast growth in rich media, presumably because it requires longer than the wild type to complete a separation-specific step (Normark, 1969). The *envA* gene product must therefore be required for one of the final stages in septation. Wolf-Watz and Normark (1976) presented evidence that the *envA* mutation was associated with low levels of N- acteylmuramyl-L-alanine amidase, an enzyme involved in splitting PG molecules between NAM (see section 1:4) and the pentapeptide side chain. Although much of the evidence is indirect, the failure of *envA* mutants to sufficiently separate cells could be due to abnormally low levels of this enzyme. Recently, Sullivan and Donachie (1984b) showed that a DNA fragment coding for the *envA* gene was not stably maintained in multiple copies and that the *envA* gene product itself was responsible for this effect. The authors

tentatively suggested that the overproduction of this protein resulted in an increase in *envA*- associated activity, which affected the structural integrity of the sacculus in some way, but the precise activity of the *envA* product is unknown.

A function has been identified for the *ftsI* gene product. The FtsI protein plays an essential role during cell division because inactivation of this protein via a temperature-sensitive mutation or by selective inhibition with β-lactam antibiotics, results in filamentation (Spratt, 1977c). The *ftsI* gene (also *sep* or *pbpB*) encodes PBP3 and the enzymatic role of this protein was discussed in sections 1:6:1 and 1:6:2. Recently, evidence has been presented for an interaction of FtsI with another septation protein, FtsA (Tormo *et al.*, 1986).

Much attention has been focused on the role of FtsA in cell division, especially as a candidate for the 'termination protein' proposed by Jones and Donachie (1973). The termination protein would be synthesised at the end of DNA replication and would act as a signal to coordinate DNA replication and division (see section 1:6). Evidence for FtsA being a termination protein was presented by Donachie *et al.*, (1979) and by Tormo *et al.*, (1980), who showed that the synthesis of FtsA was required during the later stages of septation, specifically during the last 10 minutes preceding division. The findings of Tormo *et al.* (1980, 1985), provided further evidence in favour of determining FtsA as a termination protein, when it was shown that DNA replication was required for FtsA synthesis. A structural role for FtsA in the septum has also been suggested (Tormo & Vicente, 1984). Vicente (1984), reviewed the information known about FtsA and suggested that synthesis of FtsA would be controlled by temporal expression from its promoter. Evidence for

the interaction of FtsA and FtsI was provided by Tormo *et al.*, (1986) who showed that mutations in *ftsA* conferred a higher resistance to lysis by certain β-lactam antibiotics. For example, the presence of a mutant form of FtsA prevented the binding of ampicillin to PBP3 at the restrictive temperature whereas it showed no major effect on the binding of antibiotic to other PBPs. The authors pointed out that the interaction of FtsI and FtsA suggests a way in which the enzymatic activity of FtsI can be combined with the timing properties of FtsA to allow septation to occur at a precise time during the division cycle.

Many of the cell division mutants isolated carry mutations which are found to map in the *ftsA* gene and approximately 23 temperature-sensitive missense and two nonsense mutations have now been mapped to *ftsA* (Donachie *et al.*, 1984). The cell division mutation in strain PAT84, used in many early studies on cell division, was found to be linked to the *leu* gene in the 2-minute region and was also designated *ftsA* (Hirota *et al.*, 1968). However, Lutkenhaus *et al.*, (1980), demonstrated that PAT84 carried a temperature sensitive mutation in a previously unidentified gene and designated this gene, *ftsZ*.

Early work using PAT84 included that by Burdett and Murray (1974a) who investigated the effect of antibiotics on septation (see section 1:6:2). These workers showed that the materials necessary for septation were synthesised at the restrictive temperature, but could not be assembled. This suggested that PAT84 was blocked in the early stages of septation when the presence of autolysins at the septum is required for the insertion of new material.

In 1985, Begg and Donachie brought together the information known about

the septation proteins and proposed a model to show where the action of each of these proteins was required in septation. They investigated the effects of double mutations on cell shape. Figure 1.12 is a diagram of the model, showing that FtsZ is required very early in septation and that FtsA is required in the later stages. The FtsI and FtsQ proteins are required at intermediate stages.

In conclusion, the isolation of cell division mutants has allowed the preliminary dissection of the septation processes. The investigation into the role of each protein, its possible interaction with other division proteins and the overall regulation of these proteins must now be undertaken. Such studies have so far provided data which indicates a crucial role for the FtsZ protein in the regulation of division and in the organisation of septum assembly. Preliminary evidence for this central role for FtsZ was obtained from analysis of division inhibition during the SOS response and this is discussed in section 1:13.

1:13 The SOS response

To combat damage to DNA, *E. coli* has evolved an inducible repair system. This has been called the 'SOS response' (Radman, 1975), and this system operates after DNA has been damaged (by UV light or various mutagens) or when DNA replication has been inhibited. The induced response includes inhibition of cell division leading to filamentation and an enhanced capacity for DNA repair and mutagenesis. These responses allow the cell time to repair damaged DNA and when repair is completed, the SOS response is relieved allowing nucleoid segregation and division to take place.

Figure 1:12 Proposed steps in the morphogenesis of *E. coli* cells.

Three morphogenetic systems are represented:

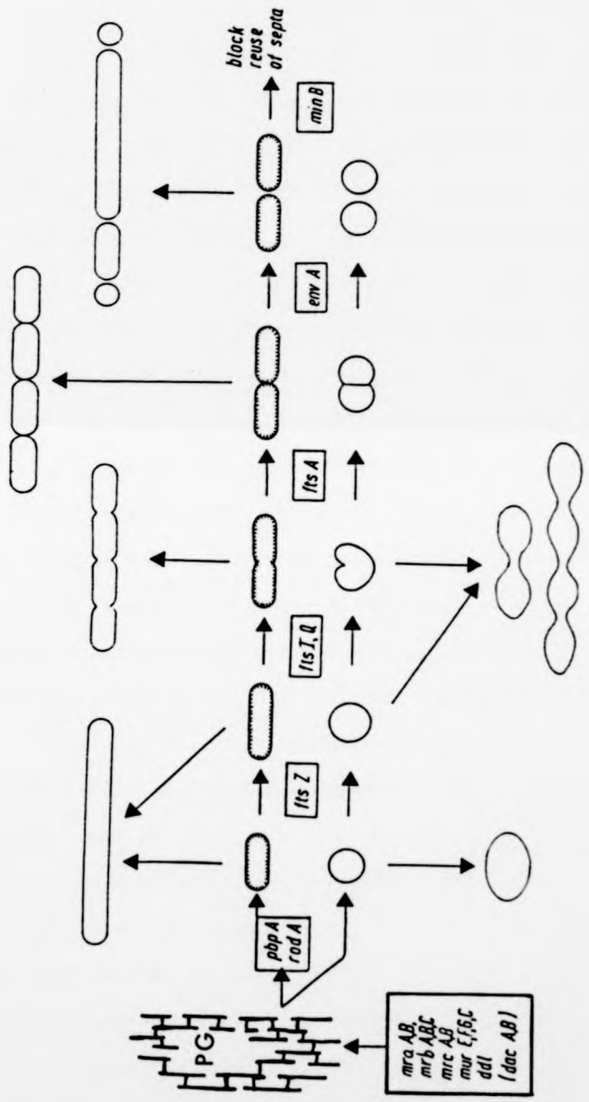
(i) The enzymes involved in the synthesis of PG are encoded by *mraA*, *mraB*, *mrbA*, *mrbB*, *mrbC*, *mrcA*, *mrcB*, *murE*, *murF*, *murG*, *murC*, *ddl* and possibly *dacA* and *dacB*.

(ii) The products of two genes *rodA* and *pbpA* are involved in the maintenance of the rod-shape of *E. coli*. Cells defective in these proteins grow as spheres.

(iii) At least six gene products (those of the *ftsZ*, *ftsI*, *ftsQ*, *ftsA*, *envA* and *minB* genes) are required for the initiation, formation, completion, separation and inactivation of potential division sites.

The model of Begg and Donachie (1985) predicts the stages at which each of these gene products is required. The phenotypes of mutants carrying mutations in each of these genes is indicated. The various mutants have been discussed in detail in previous sections.

Reproduced from Begg & Donachie, (1985).



The SOS response is now well characterised and the genetical and biochemical data which led to this understanding have been extensively reviewed (Witkin, 1976; Gottesman, 1981; Little & Mount, 1982; Witkin, 1982; Kenyon, 1983; Gottesman & Neidhardt, 1983; Walker, 1984). This discussion is an attempt to present the salient features of the SOS response in its connection with the inhibition of cell division. Figure 1.13 shows the main events leading to cell division inhibition during induction of SOS. Two genes primarily involved in SOS regulation are *recA* and *lexA*. In the current model, the *lexA* gene product is a repressor of several unlinked genes involved in SOS responses (including *recA* and the *lexA* gene itself). Parts (i) and (ii) of figure 1.13 show the activation of the SOS response. A signal generated by DNA damage activates RecA to RecA*, resulting in the activation of the protease activity of this protein (Roberts *et al.*, 1978; Craig & Roberts, 1980). The nature of the inducing signal has been extensively studied (reviewed by Little & Mount, 1982; Walker 1984). Data has been presented for the role of both specific oligonucleotides (Irbe *et al.*, 1981) and single stranded DNA (Craig & Roberts, 1980, 1981) in the induction of RecA* protease activity. RecA* can cleave both cellular and prophage repressors causing induction of functions normally repressed in the absence of DNA damage. One of the repressors cleaved by RecA* is the LexA protein itself (Little *et al.*, 1980). Many of the genes which are derepressed during SOS have been identified and are listed in table 1:13. A major approach used to do this, was the isolation of gene fusions using the bacteriophage Mud(Ap*lac*) in genes which were DNA-*d*amage *in*ducible, (*din* genes) (Kenyon & Walker, 1980). This approach however, was only used to identify non-essential genes and perhaps other genes remain to be identified.

Figure 1:13 Diagrammatic representation of the SOS response in *E. coli*.

Part i: Uninduced state.

A balance is maintained between production of LexA and the repression of *lexA*, *recA* and SOS-inducible genes.

Part ii: SOS response.

The inducing signal activates RecA to RecA*, which cleaves LexA. De-repression of the SOS-inducible genes allows synthesis of the division inhibitor, SfiA and proteins required to repair DNA damage. A basal level of these proteins may be present during the uninduced state.

Part iii: Recovery.

Once DNA damage is repaired the inducing signal is removed and LexA can re-establish repression. Remaining SfiA division inhibitor is degraded by Lon protease.

Symbols are as follows:

- ● – LexA
- ◔ – inactivated LexA
- ○ – RecA
- ◎ – RecA* (activated)
- ◇ – Lon protease
- ◆ – SfiA
- ⧫ – inactivated SfiA
- ✡ – inducing signal

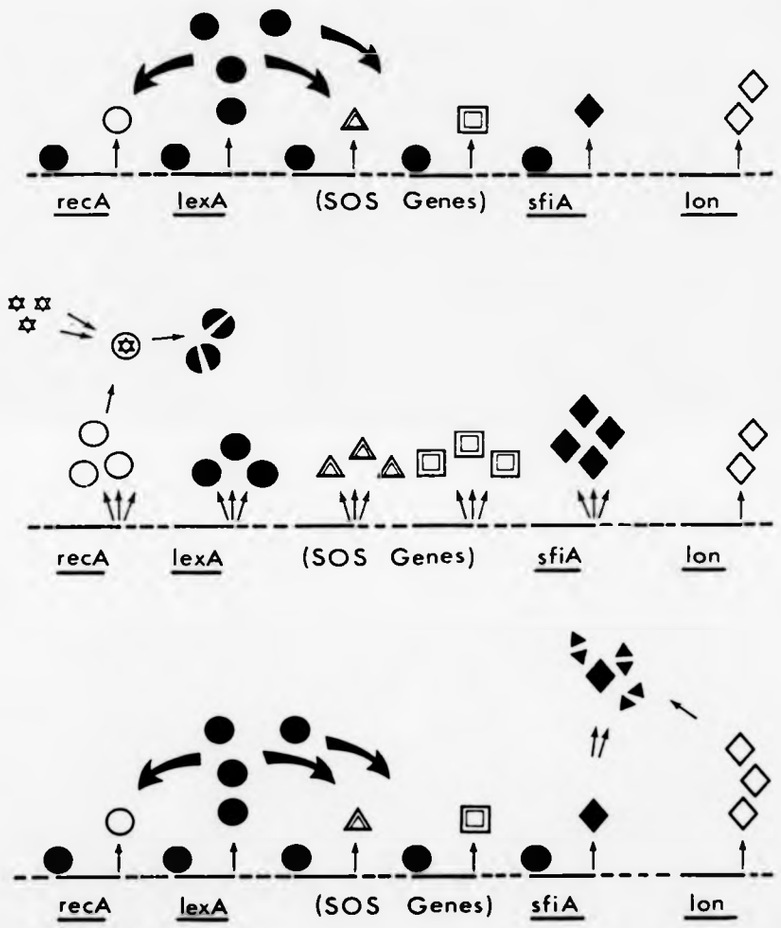


Table 1:13 SOS-induced genes

Gene	Location	Function
lexA	91	SOS repressor
recA	58	General recombination SOS protease
umuC	25′	Mutagenesis
himA	38′	Site-specific recombination
uvrA	92′	Excision repair
uvrB	17′	Excision repair
sfiA	22′	Division inhibitor
dinA	2′	Unknown, Kenyon & Walker, 1980
dinB	8′	
dinD	80-85′	
dinF	91′	

Once DNA damage is repaired and the inducing signal removed (figure 1.13 part iii), the level of RecA* activity is reduced and LexA can re-establish repression of the SOS genes. Rapid recovery from the SOS response is mediated via Lon (product of the *lon* gene) which is one of a set of proteases found in *E. coli* (Zehnbauer *et al.*, 1981). Lon protease has the ability to degrade both abnormal proteins (Shineberg & Zipser, 1973; Gottesman & Zipser, 1978) and normal proteins (Gottesman *et al.*, 1981; Mizusawa & Gottesman, 1983). The action of Lon in SOS-induced division inhibition is described in section 1:14.

1:14 SOS-induced division inhibition

The analysis of mutations in the *recA* and *lexA* genes (Kirby *et al.*, 1967; Mount *et al.*, 1972; Gudas, 1976) and the characterisation of suppressors of these mutations, has led to an understanding of the mechanism of LexA repression. Similarly, the gene encoding the SOS division inhibitor (*sfiA*) was identified in this way. Darby and Holland (1979) showed that SOS-induced filamentation was allowed to continue in *lon* mutants, even after DNA damage had been repaired. Presumably this was due to the mutant Lon protease being unable to degrade the division inhibitor. George *et al.*, (1975) isolated two mutants which carried suppressor mutations of a *lon* mutation and found them to map in two genes, *sfiA(sulA)* at 22- minutes, and *sfiB(sulB)* at 2-minutes on the *E. coli* linkage map (*sfi*: *s*uppressor of *f*ilamentation). Thus, whereas *lon* continued to filament after SOS induction, *lon sfi* double mutants did not.

Work on the *sfiA* mutation has allowed the identification of SfiA as the

division inhibitor. Huisman and D'Ari (1981) isolated mutants with a Mu d(Ap*lac*) insertion in the *sfiA* gene and showed that *sfiA* expression was SOS-inducible. Cole (1983), characterised the *sfiA* promoter sequence and compared this with similar sequences from other SOS-induced genes to reveal an 'SOS box'. This sequence constitutes the site at which LexA protein binds to repress the expression of SOS-regulated genes. Table 1:14 shows the consensus sequence for the SOS box or LexA-binding site and some examples from LexA-regulated genes (Little & Mount, 1982; Walter, 1984). Kenyon and Walker (1980) showed that different damage-inducible genes had slightly different SOS boxes. They also showed that the different binding sites bind LexA with characteristic affinities, which might provide a way of coordinating different SOS functions. Thus, genes, the products of which are required early in the SOS response, would have LexA-binding sites with lower affinities for LexA, than those required at a later stage. The variation in affinity for LexA may be related to the extent of the dyad symmetry in the SOS box (Kitagawa *et al.*, 1985).

The role of Lon protease in the regulation of SOS-mediated division inhibition was elucidated by Mizusawa and Gottesman (1983) who measured the stability of SfiA protein in *lon* and *lon*$^+$ strains. They found that the presence of the *lon* mutation increased the half-life of SfiA from 1.2 minutes to 19 minutes. It was thus proposed that one function of Lon protease was to degrade the SfiA division inhibitor during recovery from the SOS response.

The question remained however, as to how the *sfiB* gene was involved in this division inhibition. Lutkenhaus (1983) showed that the *sfiB* mutation was an allele of the division gene *ftsZ*, and proposed that the

Table 1:14 LexA-binding sites of some SOS inducible genes

Gene	Sequence
recA	TACTGTATGAGCATACAGTA
uvrA	TACTGTATATTCATTCAGGT
uvrB	AACTGTTTTTTTATTCAGTA
uvrD	ATCTGTATATATACCCAGCT
sfiA	TACTGTACATCCATACAGTA
lexA-1	TCCTGTATATACTCACAGGA
lexA-2	AACTGTATAACAACCCAGGG
Consensus	taCTGTatata-a-aCAGta

Adapted from Walker, 1984.

sfiA product somehow inactivates or inhibits the action of FtsZ in initiating septation during normal cell division. Section 1.15 describes the role of FtsZ in division.

1:15 The role of FtsZ during cell division

In section 1:12, work was described which showed that the FtsZ protein was required during the very first stages of septum formation (Burdett & Murray, 1974a; Walker *et al.*, 1975; Begg & Donachie, 1985). As discussed in section 1:14, the *sfiB* mutation was shown to be an allele of FtsZ (Lutkenhaus, 1983). Several possible models were proposed to explain how the *sfiB* mutation was able to suppress filamentation in strains carrying the *lon* mutation. It was suggested that the division inhibitor SfiA, functioned by inactivating the essential FtsZ function required for division.

In 1983, D'Ari and Huisman investigated the effects of SfiA, as distinct from those of the other SOS functions. An operon fusion was constructed in which the *sfiA* gene was placed under the control of the *lacZ* promoter. The addition of the *lac* operon inducer β-D-thiogalactopyranoside (IPTG) was shown to be sufficient to cause inhibition of division. It was also shown that mutations in the *sfiB* gene could suppress the IPTG-induced, *sfiA*-mediated division inhibition.

This clearly supported the hypothesis that the FtsZ protein was required for septation and was the target of action of SfiA. Despite all this data, it was not known how SfiA inhibited division via FtsZ, nor was it known how the *sfiB* mutation affected this.

Insight into the mechanism of FtsZ action came from several groups of workers. Jones and Holland (1985) investigated the way in which the FtsZ protein, essential for division, is inhibited by SfiA. They showed that SfiA did not decrease the synthesis of FtsZ protein, nor did it decrease its stability. One way in which division inhibition could occur however, was via the interaction of SfiA and FtsZ proteins. Jones and Holland, (1984) showed that in the presence of FtsZ, the half-life of SfiA synthesised in maxicells was approximately 12 minutes, as opposed to 3 minutes in the absence of FtsZ (or in the presence of the mutant FtsZ protein, SfiB). These results suggested that a SfiA-FtsZ interaction *in vivo* would increase the stability of SfiA (perhaps by blocking access of Lon protease to SfiA cleavage sites) and thus increase the duration of division inhibition. Simultaneously, the action of FtsZ would be inhibited by the SfiA protein binding to it. This model can also explain the effect of the *sfiB* mutation, if it is proposed that the mutant SfiB protein is no longer capable of binding to SfiA. Under these conditions, it can be said that SfiB is resistant to division inhibition via SfiA. The measurements of the half-life of SfiA estimated by Jones and Holland (1985) compared favourably with the measurements made by Mizusawa and Gottesman (1983).

Work by Ward and Lutkenhaus (1984), showed that overproduction of a hybrid protein 'FtsZZ' consisting of part of LacZ fused to the FtsZ protein, caused inhibition of division as expected, but could be suppressed in two ways. Suppression was achieved firstly, by increasing the *ftsZ* copy number, and secondly by the presence of a *sfiB* mutation in the chromosome. This finding suggested that FtsZ could act as an activator/promoter of cell division in one of two ways. Either FtsZ functioned in a multimer and assembly of FtsZZ (or SfiB) into this

multimer inhibits division, or FtsZ has some specific site of interaction and the molecular competition between FtsZZ (or SfiB) inhibits division.

Thus, a model began to emerge in which FtsZ was a key protein in the initiation of septation and inhibition of division during the SOS response was achieved by the binding of SfiA to FtsZ. Holland and Jones (1985) proposed that FtsZ and other proteins required for division formed a complex or 'septalsome' which was activated at the precise time of septation, by some cell cycle-dependent signal, or by the attainment of a critical concentration of FtsZ. The activation of the septalsome via FtsZ may then serve to organise the other essential division proteins for septum formation. Some evidence has already accumulated for the interaction of certain division proteins. An interaction of essential division components FtsA and FtsI was discussed in section 1.12. Jones and Holland (1985) showed that both FtsZ and SfiA have a high affinity for the inner (cytoplasmic) membrane, suggesting that the septalsome is probably associated with the membrane during septum formation.

Ward and Lutkenhaus (1985) proceeded to test this model, which predicted that increased levels of FtsZ should lead to hyper-division activity and the production of small-sized cells. These workers showed that a 2 to 7-fold increase in the level of FtsZ protein resulted in the initiation of cell division earlier in the cycle, leading to additional division events and the production of very small cells. These results indicated that the level or activity of FtsZ controls the frequency of cell division, and thus support the hypothesis that FtsZ is an activator of division.

Recent results (Maguin *et al.*, 1986), showed that the action of SfiA to inhibit division was completely reversible in the absence of *de novo* protein synthesis, suggesting that active FtsZ protein was eventually released from the FtsZ-SfiA complex. SfiA protein released from this complex rapidly decays as predicted by Jones and Holland (1985). Therefore, during recovery from the SOS response, all SfiA in the cell is degraded by Lon protease (unless it is bound to FtsZ) and once LexA repression is re-established, no more SfiA protein is present to inhibit division. This was confirmed by Lutkenhaus *et al.* (1986), who showed that increasing the level of FtsZ in the cell also suppressed DNA damage-induced filamentation in *lon* mutants. Presumably, the increased stability of SfiA observed in *lon* mutants could be overcome by an excess of FtsZ, thus allowing division to occur. These studies also showed that whereas excess FtsZ was able to overcome SfiA-mediated division inhibition, it could not override the division requirement for termination of chromosome replication.

Maguin *et al.* (1986) suggested that the termination protein (Jones & Donachie, 1973) required for septation (should such a protein exist) must act earlier than FtsZ. Such a protein would be synthesised at a specific point in the cell cycle and would not accumulate, and it would act earlier than FtsZ. A candidate for this protein could be the *wee* gene product discussed in section 1:10. The *wee* product could control the accumulation of FtsZ protein and thus, in strains carrying the *wee* mutation this control would be relaxed resulting in the increased concentration of FtsZ. Overproduction of FtsZ would then lead to earlier initiation of division in a similar way to that described by Ward and Lutkenhaus (1985) and by Martinez-Salas and Vicente (1980) for *wee* mutants.

To summarise, the essential division component FtsZ is postulated to be an activator of division. It could function by initiating the assembly of the 'septalsome' which would consist of all the proteins required for formation of the septum. The FtsZ protein would require activation in some way and this activation could be regulated by some cell cycle-dependent signal, such as the attainment of a crucial concentration. Septation could then take place. If the cell needed to arrest division perhaps in order to repair damaged DNA, then the SOS response is switched on. The SOS response induces the synthesis of the division inhibitor protein, SfiA. This protein then acts to block division by binding to FtsZ, thereby inactivating its essential function. Once the SOS response is switched off, the LexA protein can re-establish repression of the *sfiA* gene and any SfiA protein remaining in the cell is degraded by the Lon protease. FtsZ protein then proceeds to activate division once again. A diagrammatic representation of these events is presented in figure 1.15.

This model predicts that FtsZ is the crucial protein for control of cell division and therefore a simple mechanism for inhibiting division for any reason, is via this protein. The data obtained for the action of SfiA and the mutant SfiB protein can also be explained by this model, providing a pathway for SOS-mediated division inhibition. It might also be expected that other pathways of division inhibition also operate via the FtsZ protein and the data for this is discussed in section 1:16.

1:16 Other pathways of division inhibition

The coupling of DNA replication and cell division during the SOS

Figure 1:15 Diagram to represent the proposed action of FtsZ in division.

(i) Cell elongation: The cell wall continues to elongate. Essential division components are not yet assembled due to the relatively low concentration of FtsZ in the cell.

(ii) Initiation of septation: The level of FtsZ in the cell reaches a critical concentration and the septalsome can be activated.

(iii) Division: The activated septalsome can now form a septum and the cell goes on to divide.

(iv) Division inhibition: During inhibition the SfiA protein (or other inhibitor protein) binds to FtsZ. The essential FtsZ division function is inactivated. SfiA protein which is not bound to FtsZ is rapidly degraded by Lon protease. Septum formation occurs only when the level of SfiA protein decreases and FtsZ is free to activate the septalsome.

Symbols are as follows:

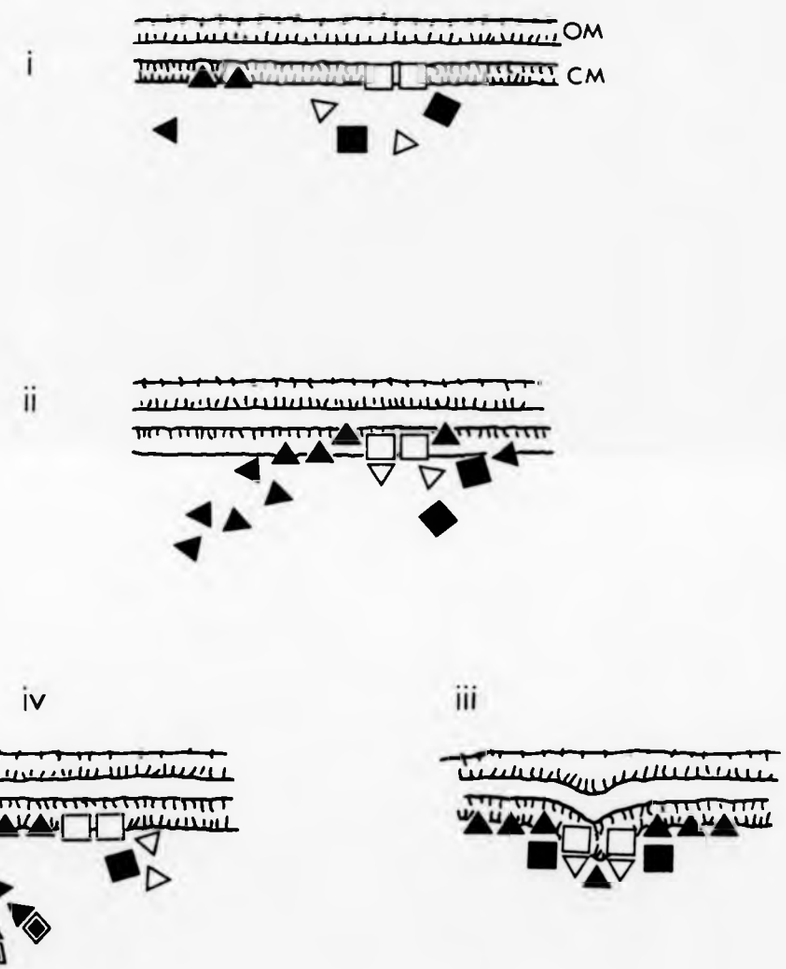
▲ - FtsZ

△ - FtsA

□ - FtsI

◈ - SfiA

■ - EnvA



response was shown to be achieved by the division inhibitor SfiA (sections 1:14 and 1:15). Huisman *et al.* (1980) showed that the action of SfiA was specific to the SOS response. SfiA inhibits division in order to prevent the production of DNA-less cells since nucleoid segregation must only occur after DNA repair is completed. Obviously, this co-ordination is very important to the cell and therefore, it is perhaps not surprising that other pathways of division inhibition have also been found.

D'Ari and Huisman (1983) demonstrated the existence of another pathway when they discovered that certain strains carrying *recA*(*tif*),*sfiA* mutations were observed to filament at the restrictive temperature. The *tif* mutation is a temperature-sensitive *recA* mutation so that, at the restrictive temperature there is constitutive expression of the SOS response (Kirby *et al.*, 1967). This filamentation was due to the *sfiC* locus, although the nature and activity of the *sfiC* gene product is unknown. Both *sfiA*- and *sfiC*-dependent division inhibition are suppressed by *sfiB* mutations suggesting that both pathways have FtsZ as their target molecule. Recently, the SfiC pathway has been further characterised (Maguin *et al.*, 1986). Table 1:16 presents a comparison of both pathways of division inhibition. Due to the irreversibility of the SfiC-mediated division inhibition, the SfiC pathway is dominant over the SfiA pathway. Further investigation is required to assess the possible role (if any) of SfiC in normal division control.

A third mechanism of division inhibition is *sfi*-independent and is only revealed when there are mutations present to inactivate the first two pathways (Gottesman *et al.*, 1981; Burton & Holland, 1983). This pathway does not require protein synthesis and only operates in cells active in

Table 1:16 A comparison of *sfiA*- and *sfiC*-mediated division inhibition

SfiA pathway	SfiC pathway
Reversible	Irreversible
Positively regulated by RecA	Positively regulated by RecA
Negatively regulated by LexA	Not regulated by LexA
Suppressed by excess FtsZ	Not suppressed by excess FtsZ
Unstable protein	Relatively stable protein

DNA replication. Burton and Holland (1983) have suggested that the need for DNA replication may reflect the normal cell cycle requirement for termination of chromosome replication.

Division inhibition also can occur when the replication of certain plasmids resident in the cell is blocked, even though replication of the host chromosome is unimpaired (see also section 1:2). Analysis of F plasmid replication revealed the presence of the *ccd* locus (*c*oupled *c*ell *d*ivision) composed of two genes *ccdA* and *ccdB* (Ogura & Hiraga, 1983; Miki *et al.*, 1984a,b). The model proposes that CcdB acts to inhibit division unless repressed by CcdA. Once division is completed, CcdA is either inactivated or diluted out by an increase in cell mass. The balance between these two proteins is determined by completion of replication and CcdA may therefore be a 'termination protein' (Jones & Donachie, 1973). Genetic analysis has shown that *ccd* functions can induce the SOS response but that division inhibition is independent of both *sfiA* and *sfiC* genes (Jaffé *et al.*, 1985).

Recently, Noel and Drapeau (1986) have proposed yet another pathway of division inhibition via the cell division gene *ftsM* which maps in the 2-minute region (Drapeau *et al.*, 1983), although apparently distinct from the morphogene cluster. The *fstM* mutation was distinguished from other *fts* mutations by its sensitivity to UV-irradiation, a phenotype also shared by *lon* mutants. The *ftsM* gene was also shown to be repressed by LexA (Drapeau *et al.*, 1984). These properties suggested that *ftsM* was a division inhibitor or an effector of division inhibition mediated in a similar way to *sfiA*. This was indeed shown to be the case when Belhumeur and Drapeau (1984) isolated *ftsZ* mutants which were altered for the interaction of FtsZ with both SfiA and FtsM proteins. The

action of these proteins could represent alternative mechanisms within the same pathway. The new *ftsZ* alleles are unusual because they encode mutant FtsZ proteins which are *more* responsive to SfiA (rather than less responsive as in the case of SfiB). In subsequent work, Noel and Drapeau (1986) isolated extragenic suppressors of the *ftsM* mutation which should be useful in future attempts at characterising division inhibition. One suggestion is that FtsM is an activator of FtsZ and that, in strains carrying *ftsM* mutations, the mutant FtsM protein is less efficient in its activation, resulting in filamentation.

In conclusion, although these pathways appear separate, genetic analysis should indicate whether one or more of them are linked. It is difficult to imagine how completely separate mechanisms could work efficiently in the same cell without some fundamental regulation. Gottesman and Neidhardt (1983), postulated the existence of 'global control systems' responsible for co-ordinating cell division and other essential processes in response to stress. Such a mechanism would ultimately control induction of division inhibition, the SOS response and the heat shock response. This latter phenomenon is discussed in section 1:17.

1:17 The heat shock response

The heat shock response of *E. coli* is similar to that of other organisms (Schlesinger *et al.*, 1982; Schlesinger, 1986). It is induced by a shift to high temperature and also by other conditions of cell stress, such as the presence of ethanol (Marx, 1983; Gottesman & Neidhardt, 1983). Following such stress the rate of synthesis of a class of proteins (the heat shock proteins) is dramatically increased.

Genetic analysis has provided much information about the molecular mechanism of heat shock induction. In 1975, Cooper and Ruettinger isolated a mutant in which the heat shock response was abolished. Neidhardt and Van Bogelen (1981) showed that this was the result of a mutation in the *htpR* gene (*h*igh-*t*emperature *p*roduction *R*egulation). The same conclusions were drawn from observations by Yamamori and Yura (1982) who called the gene *hin* (*h*eat *in*ducible). The *htpR* gene was cloned and its product identified (Neidhardt *et al.*, 1983a). The DNA sequence of *htpR* revealed that the product showed some homology with the sigma subunit (sigma-70) of RNA polymerase (Landick *et al.*, 1984), itself a heat shock protein (Gross *et al.*, 1982; 1984). Grossman *et al.*, (1984) showed that the *htpR* product was an alternative sigma subunit (sigma-32) which allowed RNA polymerase to initiate transcription from the promoters of heat shock genes. They proposed that *htpR* be re-named *rpoH*. Further work (Bloom *et al.*, 1986) has demonstrated that sigma-32 specifically stimulates expression of heat shock genes but not of the *rpoH* gene itself. Thus *rpoH* is a positive regulator of the heat shock response.

This molecular mechanism allows the increased synthesis of 17 proteins, seven of which have been identified as the products of known genes (table 1:17). The identification of two of these heat shock proteins has already revealed possible links between heat shock and the SOS response.

Phillips *et al.*, (1984) showed that the Lon protease was induced by the heat shock response. It was found that sigma-32 was degraded more slowly in cells carrying the *lon* mutation (Grossman *et al.*, 1983) suggesting that Lon may have a proteolytic function in heat shock

Table 1:17 Heat shock proteins which have been identified

Name	Gene	Possible Function	Essential
GroEl	*mopA/groEl*	Morphology of coliphage Some role in RNA and DNA synthesis	Yes
DnaK	*dnaK*	Phage DNA replication Modulation of heat shock response	Yes
Sigma	*rpoD*	Promoter recognition RNA polymerase subunit	Yes
GroES	*mopB/groES*	Morphology of coliphage Some role in RNA and DNA synthesis	?
lysyl-tRNA synthetase form II	*lysU*	Charging of tRNA	-
Lon	*lon*	ATP-dependent protease	No
DnaJ	*dnaJ*	Role in RNA and DNA synthesis	Yes

These proteins appear to be involved in the major macromolecular processes the cell: DNA replication (DnaK and DnaJ), RNA synthesis (sigma factor), protein synthesis (LysU), protein processing or assembly (GroES and GroEL) protein degradation (Lon), taken from Neidhardt *et al.*, (1984).

recovery just as it does during SOS recovery (see section 1:14). The elucidation of the role of Lon in the regulation of RpoH stability should prove very interesting. Gayda *et al.*, (1985) determined the DNA sequence of the *lon* gene. These workers showed that there was no consensus sequence for an SOS-box in the DNA sequence 5' to the start of the gene, but provided some evidence for the presence of a heat shock (sigma-32) promoter sequence (Neidhardt *et al.*, 1984).

A further link between heat shock and SOS was found with the identification of another heat shock protein, DnaK. The *dnaK* gene was originally identified because mutations in this gene blocked bacteriophage λ DNA replication, and the *dnaK* product was subsequently shown to be essential for *E. coli* viability at 42^{o}C (Georgopoulos, 1977). This protein has also been ascribed both ATPase and autophosphorylase activity (Zylicz *et al.*, 1983) and is a highly conserved protein in biology, showing strong amino acid sequence homology with the Hsp70 heat shock protein of Drosophila (Bardwell & Craig, 1984).

The determination of the fundamental role of DnaK under both normal and stressed conditions in the cell should prove highly informative. Mutants carrying mutations in the *dnaK* gene fail to switch off the heat shock response and continue to synthesise heat shock proteins, whereas overproduction of DnaK protein in bacteria, results in a diminished heat shock response (Tilley *et al.*, 1983). These data suggested a role for DnaK in the negative regulation of the heat shock response, directly or indirectly affecting the activity of RpoH. Tilley *et al.*, (1983) suggested that DnaK modulates the intracellular levels of RpoH activity perhaps by utilising the autophosphorylation activity of DnaK (Zylicz *et*

al., 1983). An alternative model involves an indirect effect of DnaK on RpoH activity. The DnaK protein could positively regulate some other protein which would subsequently negatively regulate RpoH.

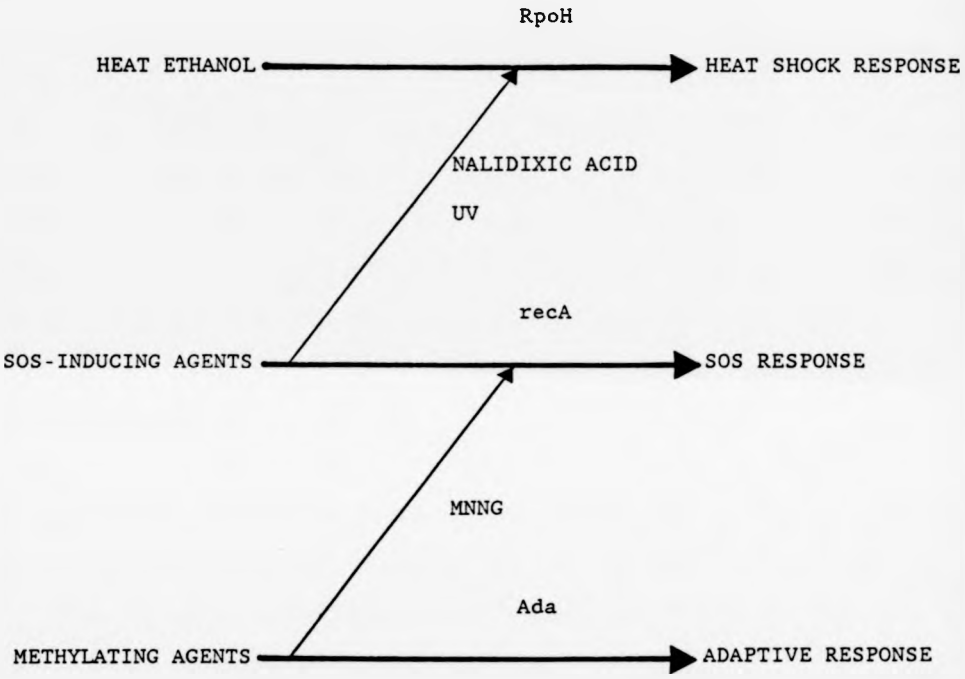
In 1984, Kruegar and Walker showed that DnaK and another heat shock protein, GroEL, were both induced by UV-irradiation and nalidixic acid along with the SOS response. Induction of DnaK and GroEL however, was independent of the *recA* and *lexA* gene products, but was dependent on the *rpoH* gene product. Although Kruegar and Walker offered no explanation for the physiological significance of these findings, they suggested that the control of the SOS and heat shock responses was more complex than was originally thought. Section 1:18 describes the current ideas on the possible interactions between these responses within the cell.

1:18 The interaction of stress-induced responses in *E. coli*

Gottesman and Neidhardt (1983) discussed the idea of 'global regulatory systems' such as the SOS and heat shock responses. These systems were presumed to be so fundamental to the survival of the cell that they had been conserved throughout prokaryotic and eukaryotic evolution. Walker (1984) expanded this theory to include all stress-induced responses and proposed that these systems interact to enable the cell to cope with a variety of stressful conditions.

The SOS and heat shock responses were discussed in sections 1:13 and 1:17 respectively. Figure 1:18 is a simple model to explain known interactions between these systems and a third system - the adaptive response.

Figure 1:18 The interaction of three stress responses in *E. coli*



The three responses can be induced by agents that damage DNA. A subset of the SOS-inducing agents also induce the heat shock response and evidence has been presented indicating that a subset of agents which induce the adaptive response also induce SOS (see text for details).

Redrawn from Walker, (1984).

This latter response is another independent regulatory network known to be induced by DNA-damaging agents and was first discovered by Samson and Cairns (1977). It was observed that if *E. coli* was first exposed to low concentrations of methylating or ethylating agents such as N-methyl-N-nitro-N'-nitrosoguanidine (MNNG), the cells became more resistant to the mutagenic effects of increasing concentrations of such agents. *De novo* protein synthesis was required and these physiological responses were collectively called the adaptive response (Jeggo *et al.*, 1977). Analysis of the regulation of this response (reviewed by Walker, 1984), revealed the presence of a positively activing regulator, Ada (Sedgwick, 1982). Evidence has also been presented indicating that a subset of agents which induce the adaptive response also induces SOS (Bagg *et al.*, 1981; Quillardet *et al.*, 1982).

Perhaps another stress-induced system should also be included in this list, namely the stringent response. In 1985, Grossman *et al.*, showed that this response induces expression of heat shock proteins in *E. coli*. The stringent response is induced when the availability of an aminoacyl-tRNA species becomes limiting for synthesis (reviewed by Gallant, 1979). This results in the accumulation of two unusual guanine nucleotides, guanosine-5'-diphosphate-3'-diphosphate (ppGpp) and guanosine-5'-triphosphate-3'-diphosphate (pppGpp). A variety of adjustments in gene expression is also observed, including a decrease in the rate of synthesis of stable RNA and ribosomal proteins, and an increase in the rate of synthesis of other proteins. One of the proteins for which the rate of synthesis is increased is the heat shock protein RpoD (sigma -70)(Grossman *et al.*, 1985). In addition, the synthesis of all heat shock proteins measured so far have also been shown to be induced during the stringent response although the requirements for this induction are differ -ent from those induced by high-temperature during the heat shock response.

Grossman *et al.*, described how the product of the *rpoD* gene (the third gene in 3-gene operon) was induced. Induction was achieved via the activation of an internal promoter whereas expression from the major operon promoter is reduced. The details of this mechanism are not yet understood.

Thus, the stringent response joins the growing list of 'stress' conditions known to induce the synthesis of heat shock proteins. In order to control the complex interaction of all of these stress-induced responses, the cell probably requires a fundamental regulatory network which, although sensitive to stress is also part of the normal cellular processes. The molecular approach used to investigate these processes has obviously helped to identify the components involved, but a more phenomenological approach is now required to assimilate the known information into a working model.

1:19 The characterisation of morphogenes in the 76-minute region

As discussed in section 1:17, the *rpoH* (*htpR*) gene for control of the heat shock proteins was cloned and the gene product identified (Neidhardt *et al.*, 1983a). The gene was sub-cloned from a plasmid (pLC31-16) which was also shown to complement the *rpoH* nonsense mutation *htpR*. Characterisation of this plasmid revealed that it also complemented the cell division mutation *ftsE* (see table 1:9), suggesting that these two essential genes must be closely linked. The *ftsE* mutation carried by strain MFT1181 (Ricard & Hirota, 1973) was originally mapped to 73-minutes on the *E. coli* linkage map (Bachmann & Low, 1980) but subsequent revisions placed it at 76- minutes close to the

rpoH gene (Bachmann, 1983).

The location of these two genes in this region was interesting because several other essential genes were also found at this position. The mutation *fam715* is in an essential gene, identified as causing defects in lipoprotein synthesis (Torti & Park, 1976), although probably via some indirect mechanism rather than by alteration of the structural gene (Torti & Park, 1980). Another mutation which maps in the 76-minute region is *dnaM*, which does not affect DNA synthesis as its name suggests (Sevastopoulos *et al.*, 1977; Glassberg *et al.*, 1979), but maps in an essential gene involved in cell envelope integrity (G. Salmond, pers. comm.). Finally, a psychrosensitive filamentation mutant JS10 (Sturgeon & Ingram, 1978) was isolated which was presumed to carry an *ftsE* mutation, due to the phenotypic similarities of JS10 and MFT1181.

Thus, several essential genes including one cell division gene were located in the 76-minute region and could represent a novel morphogene cluster. Previously, the intense study of the 2-minute morphogene cluster had led to identification of new genes (section 1:13) and it was possible that other unidentified genes were also contained in the 76-minute cluster. This has in fact proved to be the case, with the subsequent identification of a new cell division gene, *ftsS* (Salmond & Plakidou, 1984).

1:20 The isolation of a new cell division gene *ftsS*

Table 1:20 lists all the mutations mapping in the 76-minute region. Among them, are two cell division mutants OV22 and OV32 derived from OV2

Table 1:20 Mutants mapping in the 76-minute region

Strain	Mutation	Function	Reference
TsnK165	*htpR*(Am)	heat shock control	Neidhardt & Van Bogelen, 1981
MFT1181	*ftsE*	cell division	Ricard & Hirota, 1973
ST715	*fam715*(Am)	?	Torti & Park, 1976
JGC127	*dnaM*	?	Sevastopoulos *et al.*, 1977
JS10	*ftsE2000*	cell division	Sturgeon & Ingram, 1978
OV22	*ftsS*(Am)	cell division	Salmond & Plakidou, 1984
OV32	**fts-32*(Am)	cell division	Unpublished
TOE22	**fts-22*	cell division	Salmond & Plakidou, 1984
TC35	**fts-35*	cell division	Salmond & Plakidou, 1984

*These cell division mutations were all designated alleles of *ftsE* (Plakidou, 1983).

(a strain with a well-characterised cell cycle (Donachie *et al.*, 1976)), by MNNG mutagenesis (Lutkenhaus & Donachie, 1979). These mutants were later found to carry amber mutations in cell division genes (Salmond & Plakidou, 1984). Strains TOE22 and TC35 were isolated using a temperature-oscillation-enrichment procedure (Begg *et al.*, 1980) from a parent strain not known to carry a temperature-sensitive suppressor. These strains therefore carried temperature-sensitive missense mutations.

All the mutations listed in table 1:20 were mapped by Plakidou (1983) and Salmond and Plakidou (1984). The first step in the characterisation of these mutants, was to map each mutation with respect to a closely-linked (40-60%) Tn*10* insertion (*zhf-1*::Tn*10*). Figure 1.20 shows the results of mapping studies in this region. The cell division mutations found in MFT1181, TOE22, TC35, JS10, OV22 and OV32 were each transduced via the Tn*10* insertion to clean genetic backgrounds using bacteriophage P1 and it was found that filamentation and temperature-sensitivity were phenotypic effects of single mutations.

Salmond and Plakidou (1984), isolated a λ transducing phage, λpGS22, which complemented the cell division mutation carried by OV22, and went on to isolate derivatives of this phage carrying the different cell division mutations. These were λSP22 (from TOE22), λSP35 (from TC35) and λSP22(Am) (from OV22). *Cis-trans* tests using these λ phages showed that the mutations found in MFT1181, TC35, TOE22 and JS10 were alleles of *ftsE* whereas OV22 possessed a mutation in a previously unidentified locus designated *ftsS*. The mutation carried by OV32 was only tentatively assigned to *ftsE* due to equivocal data.

1:21 Aims of this work

Because two cell division genes, *ftsE* and *ftsS*, and several other essential genes, including *rpoH*, *fam* and *dnaM* were located at 76-minutes, it was possible that this region could represent a morphogene cluster. The phage λpGS22 was shown to complement the five potential *ftsE* mutations and the *ftsS* mutation of OV22, but not mutations in the other essential genes (Salmond & Plakidou, 1984). The aim of this work was to further characterise *ftsE* and *ftsS* as follows:

1. To subclone DNA fragments of λpGS22 to locate the position of the *ftsE* and *ftsS* genes.
2. To further characterise the *ftsE* mutations and to map the mutation carried by OV32.
3. To use transposon mutagenesis to analyse the direction of transcription of the genes.
4. To identify the *fts* gene products.

CHAPTER 2

Materials and Methods

2:1 Bacterial strains

The bacterial strains used in this study were *E. coli* K-12 derivatives and are listed in table 2:1. Derivatives of strains carrying various plasmids were constructed by transformation and are defined in the text.

2:2 Bacteriophage strains

The bacteriophage strains used in this study are listed in table 2:2, and were stored at 4°C in phage buffer over a few drops of chloroform.

2:3 Plasmids

All plasmids used in this study are listed in table 2:3.

2:4 Chemicals

Table 2:4 lists all the chemicals used in this study.

2:5 Media

All media and solutions used in this study are described in table 2:5(a) and were prepared in double-distilled water, and sterilised by autoclaving at 121°C for 20 min unless otherwise indicated. Nutrient broth (NB) and Double Difco (DD) were solidified with 1.5% (w/v) Bacto

Table 2:1 Bacterial strains

Strain	Characteristics	Source	Reference
QD*SupF*	F^-, *pro*, *supF*	G. Salmond	Wilson *et al.*, 1979
DH1	F^-, *recA1*, *endA1*, *gyrA96*, *thi-1*, *hsdR17*, ($r_k^- m_k^+$), *supE44*, *relA1*	J. Hinton	Hanahan, 1983
CSH26ΔF6	F^-, *ara*, Δ(*lac-pro*), *thi*, *rpsL*, Δ(recA-srl)F6, sup^o	C. Jones	Jones & Holland, 1984
RB308	F^+, *deoC*, *lacY*, *thyA*	C. Jones	Jones & Holland, 1984
JC10240	Hfr PO45, *srl300*::Tn*10*, *recA56*, *thr300*, *ilv318*, *rpsE*	G. Salmond	Csonka *et al.*, 1980
TsnK165	F^-, *trp*(Am), *pho*(Am), *mal*(Am) *htpR*(Am), *supC*(Ts), λ^R(Ts)	G. Salmond	Neidhardt & Van Bogelen, 1981
OV2	F^-, *trp*(Am), *leu*, *ilv*, *his* *thyA*, *deo*, *ara*(Am), *lac-125*(Am), *galU42*(Am), *galE*, *tsx*(Am), *supF*(Ts), Nal^r	G. Salmond	Donachie *et al.*, 1976
OV22	F^- as OV2, but *fts22*(Am)	G. Salmond	Salmond & Plakidou, 1984

Table 2:1 (continued)

Strain	Characteristics	Source	Reference
OV32	F⁻ as OV2, but *fts32*(Am)	G. Salmond	Plakidou, 1983
JS10	F⁻,*leuB6*,*proA180*,*purE42*, *trpE38*,*lysA23*,*metE70*, *ftsE2000*,*thi-1*,*ara-14*, *xyl-5*,*mtl-1*,*azi-6*,*rpsL109*, *tonA23*,*tsx-67*,*supE44*	G. Salmond	Sturgeon & Ingram, 1978
MFT1181	F⁻,*leu-6*,*thr-1*,*argH1*, *his-1*,*trp-1*,*ftsE*,*thyA*, *lacY1*,*malA1*,*mtl-2*, *xyl-7*,*tonA2*,*supE44*,*Str*^{r}	G. Salmond	Ricard & Hirota, 1973
TOE22	F⁻,*thr*,*leu*,*proA*,*his*,*argE*, *fts-22*,*lac*,*gal*,*ara*,*xyl*, *mtl*,*thi*,*tsx*,*thyA*,*deo*,*Str*^{r}	G. Salmond	Salmond & Plakidou, 1984
TC35	F⁻, as TOE22 but *fts-35*	G. Salmond	Salmond & Plakidou, 1984
DW36	F⁻, as OV32 but *srl300*:: Tn*10*,*recA56*		This study
DW40	F⁻, as TOE22, but *srl300* ::Tn*10*,*recA56*		This study
DW52	F⁻, as MFT1181, but *srl300*::Tn*10*,*recA56*		This study

Table 2:1 (continued)

Strain	Characteristics	Source	Reference
DW53	F^-, as TC35, but *srl300* ::Tn*10*,*recA56*		This study
DW55	F^-, as OV22 but *srl300* ::Tn*10*,*recA56*		This study
DW159	F^-, as JS10, but *srl300* ::Tn*10*,*recA56*		This study

Table 2:2 Bacteriophages

Phage	Characteristics	Source	Reference
λ^+	wild type	G. Salmond	
λvir	virulent	G. Salmond	
λ_{540}	*Hin*dIII cloning vector	G. Salmond	Borck *et al.*, (1976)
$\lambda supF$	λ_{540}, *supF*	K. Hussain	Borck *et al.*, (1976)
T4GT7	generalised transducing derivative of T4	J. Hinton	Wilson *et al.*, (1979)

Table 2:3 Plasmids used in this study

Plasmid	Parent	Characteristics	Reference
pBR322		Multi-copy, Ap^rTc^r	Bolivar *et al.*, 1977
pBR325		Multi-copy, $Ap^rTc^rCm^r$	Bolivar, 1978
pDB1	pBR322	4.5 kb *Hin*dIII fragment from λpGS22	This study
pDB2	pBR322	pDB1 but opposite orientation	This study
pDG1	pBR325	4.5 kb *Hin*dIII fragment from λpGS22	This study
pDB14	pBR322	1.1 kb *Hin*dIII fragment from λpGS22	This study
pDB113	pBR322	0.9 kb *Hin*dIII fragment from λpGS22	This study
pDB1Δ5	pDB1	0.39 kb *Eco*RV fragment deleted	This study
pDB1Δ7	pDB1	1.75 kb *Sph*I fragment deleted	This study
pDB38	pDB1	Tn*1000* insertion 38 in 5'-end of ORF2 (2.9 kb)	This study
pDB63	pDB1	Tn*1000* insertion 63 in 3'-end of ORF2 (3.2 kb)	This study
pDB65	pDB1	Tn*1000* insertion 65 in 3'-end of ORF1 (2.6 kb)	This study
pDB92	pDB1	Tn*1000* insertion 92 in 3'-end of ORF1 (2.5 kb)	This study
pDB95	pDB1	Tn*1000* insertion 95 in 3'-end of ORF3 (4.0 kb)	This study

Table 2:3 (continued)

Plasmid	Parent	Characteristics	Reference
pDB184	pDB1	Tn*1000* insertion 184 in 5'-end of ORF1 (1.4 kb)	This study
pDG1Ω4	pDG1	Insertion of Ω at *Stu*I site in ORF1	This study
pDB232	pBR322	4.5 kb *Hin*dIII *fragment from OV32 (ftsX*$^-$)	*This study*
pDB233	pDB232 & pDB2	4.8 kb *Ava*I fragment from pDB232 3.9 kb *Ava*I fragment from pDB2	This study
pDB1181	pBR322	4.5 kb *Hin*dIII fragment from MFT1181 (*ftsE*$^-$)	This study
pDB1182	pDB1181 & pDB1	3.2 kb *Ava*I fragment from pDB1181 5.5 kb *Ava*I fragment from pDB1	This study
pHP45Ω	pBR322	2.0 kb *Eco*RI Ω fragment, Ap^r	Prentki & Krisch, 1984
pHCP2	pBR322	3.0 kb *Eco*RI-*Bgl*II fragment carrying *lamB*, Ap^r	Clement *et al.*, 1982
pTU101	pSC101	4.0 kb *Eco*RI-*Sma*I fragment carrying *ompA*, Tc^r	Bremer *et al.*, 1982
pLC31-16	ColE1	reported to complement *ftsE* mutations	Nishimura *et al.*, 1977
pLC31-32	ColE1	identical to pLC31-16	Nishimura *et al.*, 1977
pLC19-48	ColE1	reported to complement *ftsE* mutations	Nishimura *et al.*, 1977

Table 2:4 Chemicals used in this study

Chemical	Supplier
restriction enzymes	Amersham International
pBR322 DNA	Amersham International
^{35}S-methionine	Amersham International
T4 DNA ligase	Amersham International
ethidium bromide	BDH
dimethyldichlorosilane	BDH
2,5-diphenyloxazole (PPO)	BDH
liquid paraffin	BDH
phenol	BDH
N:N'-methylene-bis-acrylamide	Biorad
N,N,N'-N'-tetramethylene-ethylenediamine (TEMED)	Biorad
ammonium persulphate	Biorad
sodium dodecylsulphate	Biorad
glycine	Biorad
β-mercaptoethanol	Biorad
sodium N-lauroylsarcosinate (Sarcosyl)	Ciba Geigy
casamino acids	Difco
tryptone	Difco
yeast extract	Difco
D19 developer	Eastman Kodak
Unifix	Eastman Kodak
acrylamide	Eastman Kodak
caesium chloride	Fisons

Table 2:4 (continued)

Chemical	Supplier
glycerol	Fisons
butan-1-ol	May & Baker
chloroform	May & Baker
iso-amyl alcohol	May & Baker
propan-2-ol	May & Baker
glacial acetic acid	May & Baker
gelatin	Oxoid
ethanol	reagent grade
methanol	reagent grade

Table 2:5(a) Media

Medium	Constituents per litre[(a)]
Nutrient broth (NB)	13 g Oxoid nutrient broth
Luria broth (LB)	10 g Bacto tryptone 5 g Bacto yeast extract 5 g NaCl [pH 7.2]
SOB	20 g Bacto tryptone 5 g Bacto yeast extract 10 ml 1M NaCl 2.5 ml 1M KCl (10 ml 1M $MgSO_4$, 1M $MgCl_2$, filter sterile [pH 6.8]
Stab agar medium	as NB + 7 g Bacto agar
Double Difco medium (DD)	20 g Bacto tryptone 8 g NaCl (10 ml 20% (w/v) maltose + 10 ml 1M $MgSO_4$ for λ work)
M9 salts (10x) (M9)	60 g Na_2HPO_4 anhydrous 30 g KH_2PO_4 anhydrous 10 g NH_4Cl 5 g NaCl [pH 7.4]

Table 2:5(a) (continued)

Medium	Constituents per litre[a]
M9 minimal medium (M9 MM)	(100 ml 10x M9 salts)
	(10 ml 10 mM $CaCl_2$, 0.1 M $MgSO_4$)
Hershey salts	3.0 g KCl
	5.4 g NaCl
	1.1 g NH_4Cl
	15 mg $CaCl_2.2H_2O$
	0.2 g $MgCl_2.6H_2O$
	0.2 mg $FeCl_3.6H_2O$
	87 mg KH_2PO_4 anhydrous
	12.1 g Tris-HCl
	[pH 7.4]
Hershey medium	Hershey salts
	(20 ml 20% (w/v) glucose)
	(10 ml 2% (w/v) proline)
	(1 ml 0.1% (w/v) thiamine)
K medium	M9 MM
	(50 ml 20% (w/v) Bacto casamino acids)
	(0.1 ml 0.1% (w/v) thiamine)
	(10 ml 20% (w/v) glucose)

Table 2:5(a) (continued)

Medium	Constituents per litre[a]
Phage buffer	7 g Na_2HPO_4 anhydrous
	3 g KH_2PO_4 anhydrous
	5 g NaCl
	2.5 g $MgSO_4.7H_2O$
	1 ml 1% (w/v) gelatin
Freezing solution (2x)	126 g K_2HPO_4 anhydrous
	0.9 g sodium citrate
	0.18 g $MgSO_4.7H_2O$
	1.8 g $(NH_4)_2$ SO_4
	3.6 g KH_2PO_4 anhydrous
	88 g glycerol

[a]Items in brackets were added after autoclaving from sterile stocks.

agar where necessary. Minimal medium was supplemented with amino acids (20 μg ml^{-1} final concentration) and sugars (0.2% (w/v) final concentration) when required. Antibiotics were prepared as 100x stocks and used at the final concentration shown in table 2:5(b). Tetracycline (Tc) and chloramphenicol (Cm) were dissolved in 50% (v/v) ethanol and stored at -20°C. All other antibiotics were dissolved in sterile double-distilled water and stored at 4°C. TE buffer was used in all DNA manipulations, and contained 10 mM Tris-HCl, 1 mM EDTA, pH 8.0.

2:6 Growth and maintenance of bacterial cultures

All *E. coli* strains were grown at 37°C, or 30°C in the case of temperature-sensitive strains. The one exception was the cold-sensitive strain JS10 (table 2:1) which was always grown at 37°C and maintained at 4°C.

Bacterial liquid cultures were grown in 25 ml universal bottles in a Gallenkamp Orbital Shaker (150 rpm). Cultures required for transformation (section 2:21) were grown in 250 ml flasks in a New Brunswick Gyrotory waterbath (275 rpm). Culture density was monitored at 450 nm or 600 nm with an LKB Ultrospec 4050.

Bacterial strains were maintained on agar plates at 4°C for 1-2 months and stored at room temperature in stabs. Liquid overnight cultures were mixed with an equal volume of 2x freezing solution and stored at -20°C.

Cells were harvested by centrifugation in an MSE Chilspin (5000 rpm, 10 min) or, when using Eppendorf tubes, in an MSE Microcentaur (high speed,

Table 2:5(b) Antibiotic concentrations

Antibiotic	Abbreviation	Final concentration ($\mu g\ ml^{-1}$) in media
Sodium ampicillin	Ap	50
Chloramphenicol	Cm	50
Kanamycin sulphate	Km	50
Spectinomycin	Sp	50
Streptomycin sulphate	Sm	100
Tetracycline	Tc	10

2 min).

2:7 Use of bacteriophage λ

2:7:1 λ transfection

An overnight culture of the recipient strain was diluted 1 ml in 20 ml of fresh NB and grown to A_{600} = 0.5. The culture was incubated on ice (20 min) and harvested (5000 rpm, 10 min). The pellet was resuspended in 10 ml ice-cold 0.1 M $MgCl_2$ and re-pelleted immediately. The pellet was resuspended in 2 ml ice-cold 0.1 M $CaCl_2$ and incubated on ice for a further 20 min. Aliquots (200 μl) of cells were taken and the DNA sample added and mixed by vortexing. The samples were incubated on ice for 30 min, heatshocked (42°C, 2 min), and returned to ice. Serial dilutions, plus 500 μl of the overnight culture and 3 ml soft DDA (supplemented with 10 mM $MgSO_4$) were mixed then poured over fresh, wet DDA plates and incubated overnight to generate plaques.

2:7:2 Preparation of high-titre λ lysates

A single fresh plaque was picked and resuspended, by vortexing, in 1 ml phage buffer. An overnight culture of the host strain was grown in NB + 0.2% (w/v) maltose, 10 mM $MgSO_4$. The culture (300 μl) and the lysate (200 μl or 10^5 pfu) were mixed and left at room temperature (30 min) for the phages to adsorb to the cells. Following the addition of 3 ml of soft DDA, the mixture was poured onto fresh, wet, thick DDA plates, and, when set, these were incubated until confluent lysis was observed (by comparison with a phage-free control lawn). The top agar was then removed using a glass spreader, and the plate was washed with 3 ml of phage buffer. A few drops of chloroform were added and the mixture was

vortexed for at least 5 min, before centrifuging (5000 rpm, 20 min) to pellet the agar. The supernatant was decanted and stored over chloroform at 4°C.

2:7:3 Isolation of λ lysogens

Phage lysate (10 μl) was spotted onto a seeded bacterial lawn and allowed to dry. After incubation overnight, cells were taken from the turbid zone of lysis and streaked to single colonies on agar plates. Single colonies were tested for phage release by stabbing into a λ-sensitive bacterial lawn. Lysogens gave zones of lysis when compared with lysogenic and non-lysogenic controls. Putatative lysogens were then tested for immunity to a homoimmune λ phage and sensitivity to λ*vir*, according to Silhavy *et al.* (1984).

2:8 Use of bacteriophage T4GT7

2:8:1 Preparation of T4GT7 high titre lysate

An overnight bacterial culture (300 μl) was mixed with 10^5-10^6 pfu of phage, and used to inoculate 3.0 ml soft DDA. This was poured onto fresh, wet, thick DDA plates and incubated. These were then treated as for the preparation of high titre λ lysates (section 2:7:2).

2:8:2 T4GT7 transduction

Recipient cells were grown overnight in NB, harvested (5000 rpm, 10 min) and resuspended in 1 ml cold phage buffer plus L-tryptophan (25 μg ml^{-1} final concentration). Phage was added to give an m.o.i. of 0.1-0.25, and allowed to adsorb to the cells for 15 min at room temperature. The mixture was then incubated in 1 ml NB, for 1 h if gene expression time

was required, before spreading aliquots onto selective plates. Transductants usually appeared after 2-3 days.

When screening transductants for UV-sensitivity, a Hanovia bacteriocidal lamp was used. Individual colonies were streaked on agar plates alongside appropriate controls (when screening for inheritance of *recA* alleles, known *recA* and *recA*$^+$ strains were used). The plates were irradiated for a range of time intervals (1-20 sec, depending on the *recA* allele) and then wrapped in aluminium foil prior to incubation overnight. The growth of the colonies and thus the degree of UV-sensitivity was then compared.

2:9 Tn*1000* ($\gamma\delta$) mutagenesis

The F$^+$ strain RB308*recA* Sm^s was transformed with the plasmid to be mutagenised, and used as the donor strain. The donor and Sm^r recipient strain were grown in NB (with appropriate antibiotics) with very gentle agitation, to A_{450} = 0.2. The two cultures were then mixed in the ratio 1:10, donor to recipient, the mating culture was left incubating for 2-3 h and was then harvested (5000 rpm, 10 min). The pellet was resuspended in a small volume of phage buffer and serial dilutions were plated onto selective media.

2:10 Temperature-sensitivity test

Temperature-sensitivity was tested by streaking a single colony onto agar plates in duplicate, and incubating one at the permissive and one

at the restrictive temperature overnight. Growth at both temperatures was examined using light microscopy. Quantitative results were obtained by growing cultures to A_{450} = 0.5 in NB, and spotting dilutions onto duplicate agar plates. Viable counts were then made after incubation at the permissive and restrictive temperature. Colony formation at the restrictive temperature was calculated as a percentage of colony formation at the permissive temperature. When measuring plasmid complementation, a result >50%, was considered positive (+).

2:11 Bacterial conjugation

This was performed on NB agar plates, by mixing a loopful of donor and recipient cells, incubating for several hours at 37°C (or 30°C overnight) and streaking onto selective media.

2:12 Large-scale preparation of plasmid DNA

The basic procedure was that of Clewell and Helinski (1970). A 5 ml overnight culture was added to 500 ml NB containing appropriate antibiotics for selection, and grown to 2-4 x 10^8 cells ml^{-1} (A_{600} = 0.5). To amplify the plasmid, spectinomycin (Sp) was added to a final concentration of 300 μg ml^{-1}, and incubated overnight. Cells were harvested (10000 rpm, 10 min), resuspended in 16.5 ml Tris-sucrose solution (0.05 M Tris-HCl, pH 8.0, 25% (w/v) sucrose) and transferred to 50 ml polycarbonate "Oakridge" tubes. After adding 5 ml lysozyme mix (0.25 M Tris-HCl, pH 8.0, 5 mg ml^{-1} lysozyme), followed by incubation on ice for 5 min, 4.5 ml EDTA (0.25 M, pH 8.0) was added and the solution

was kept on ice for a further 5 min. Cells were lysed by the addition of 18 ml of lysis mix (0.05 M Tris-HCl, 0.0625 M EDTA, 2% (w/v) Brij 58, 0.4% (w/v) sodium deoxycholate, pH 8.0) and the mixture alternately inverted and warmed to 42°C, until it cleared. The mixture was centrifuged (18000 rpm, 20 min, 4°C) to pellet unlysed cells, cell debris and chromosomal DNA. The supernatant ("cleared lysate") was carefully decanted and was used to make three 10 ml CsCl gradients, or stored at 4°C until required.

For a 10 ml gradient, 7.76 ml cleared lysate, 7.38 g CsCl and 270 µl ethidium bromide (2.5%, w/v) were gently mixed until the CsCl had dissolved. The R_I of the resulting solution was *ca*. 1.393, and this was poured into 13.5 ml Beckman polyallomer tubes and the remaining space was filled with liquid paraffin. Gradients were centrifuged in a Beckman L8 ultracentrifuge using a 70 Ti rotor (36000 rpm, 60 h, 15°C). The plasmid DNA, visualised by long-wave UV light, was removed through the side of the tube with a size 21 needle and a 1 ml syringe. The DNA was extracted three times by vortexing with an equal volume of propan-2-ol equilibrated with CsCl and TE (80 ml propan-2-ol, 20 g CsCl, 20 ml TE) followed by centrifugation in an MSE Microcentaur (high speed, 1 min). The upper layer containing ethidium bromide was discarded. The DNA solution was dialysed against 2L of TE overnight.

2:13 Rapid small scale preparation of plasmid DNA

This method was that of Maniatis *et al*. (1982) except it was necessary to extract the cleared lysate once with phenol mix, and once with chloroform mix. DNA pellets were dried under vacuum and resuspended in

100 μl TE, by incubation at 65^{o}C for 5 min. For each restriction digest 10 μl of the DNA solution was used.

2:14 Preparation of chromosomal DNA

The procedure for the large scale preparation of plasmid DNA (section 2:12) was followed exactly, to prepare chromosomal DNA from a plasmid-free strain (except that amplification was no longer necessary). All volumes and conditions of incubation were the same, but instead of centrifuging the resulting mixture at 18000 rpm for 20 min, the mixture was phenol extracted twice and then extracted with chloroform mix. This mixture was essentially treated as a 'cleared lysate' and loaded onto CsCl gradients as described in section 2:12.

2:15 Extraction of DNA with phenol/chloroform

Phenol/chloroform mix was prepared by dissolving 100 g phenol crystals and 100 mg 8-hydroxyquinoline in 100 ml chloroform and 4 ml iso-amyl alcohol. This was equilibrated by shaking the mixture with two changes of 0.2 volume 1 M Tris pH 8.0, and two changes of 0.2 volume TE, before storage in foil-covered bottles. For the extraction procedure, DNA samples were mixed with an equal volume of phenol mix to form an emulsion and the phases were separated by a 1 min spin in an MSE microcentaur. The upper aqueous phase was recovered, taking care not to disturb the interface. A further extraction with chloroform/iso-amyl alcohol (24:1) was carried out to remove any remaining phenol. DNA was recovered by ethanol precipitation.

2:16 Ethanol precipitation

To a DNA solution, 0.5 volumes of ammonium acetate (7.5 M, pH 7.5) and three volumes of ethanol were added, mixed, and chilled at -20°C (2 h or overnight) or in an ethanol/dry ice bath (10 min). The DNA was recovered by centrifugation (high speed, 10 min, room temperature). The supernatant was discarded and the DNA pellet was dried under vacuum, and resuspended in TE buffer.

2:17 Agarose gel electrophoresis

Horizontal slab gels were prepared by boiling agarose in TAE electrophoresis buffer (0.04 M Tris-acetate, 0.001 M EDTA) and 0.6% (w/v) gels were used routinely. Ethidium bromide was added (0.5 μg ml^{-1} final concentration) and the agarose cooled slightly before pouring. DNA samples were prepared by adding 0.1 volume of loading buffer (0.25% (w/v) bromophenol blue, 15% (w/v) Ficoll-type 400) and they were then loaded into the gel slots. Electrophoresis was carried out with the gels completely submerged in buffer at 100-125 volts, or 25 volts overnight. As DNA molecular weight markers, λ^{+} bacteriophage DNA digested with the restriction endonuclease *Hin*dIII was used. This generated fragments of sizes: 23.131, 9.418, 4.361, 2.322, 2.028, 0.564 and 0.125 kb.

Restriction fragment sizes were determined with the DNASIZE programme (G. Russell, pers. comm.) adapted from Schaffer & Sederoff (1981), and run on a BBC Model B microcomputer.

2:18 Preparation of DNA fragments from agarose gels

2:18:1 DE81 method

An adaptation of the method published by Dretzen *et al.*, (1981) was used. DNA fragments were electrophoresed on 0.6% (w/v) agarose gels until acceptable band separation had been achieved. DNA bands were visualised using longwave UV light. The gel was rotated through 90°; slits were made at one end of the bands required, a piece of DEAE-cellulose (Whatman DE81 paper; pre-washed in 2.5 M NaCl overnight, washed several times in water and stored in 1 mM EDTA at 4°C) was placed into each split, and the gel was squeezed carefully against the papers to close the incision. Electrophoresis was resumed until the DNA had entered the DEAE-cellulose paper strips. The strips of paper were blotted dry, placed in 1.5 ml Eppendorf tubes, and 400 µl of high-salt buffer (20 mM Tris-HCl (pH 7.5), 1 mM EDTA, 1.5 M NaCl) was added. The paper was shredded by vortexing and then incubated (37°C, 2 h) in the dark. The mixture was then transferred to Eppendorf tubes plugged with glass wool (to trap the paper fibres). A small hole was made in the bottom of the tube, which was placed on the lip of another Eppendorf tube which in turn was placed in a universal bottle. After centrifugation (4000 rpm, 1 min), the eluate was extracted with water-saturated butan-1-ol, and the DNA was ethanol precipitated (section 2:16).

2:18:2 Freeze-squeeze method

This procedure was adapted from Tautz and Renz, (1983) and was used for size fractionation of total digests of chromosomal DNA. The DNA fragments of the required size were cut out of the gel using a razor. The gel slice was equilibrated for 30 min in 0.3 M NaAc, 1 mM EDTA, in

the dark and then transferred to a 0.6 ml Eppendorf tube (previously pierced with a small hole and plugged with glass wool). This tube was frozen in an ethanol/dry ice bath and centrifuged (still frozen) for 10 min inside a 1.5 ml Eppendorf tube. To the eluate, which collected in the bottom of the tube, was added 0.01 volumes of 1 M $MgCl_2$ and 0.1 volumes of acetic acid before ethanol precipitation of the DNA. The isolated DNA was washed in 70% (v/v) ethanol before drying and resuspension in TE buffer.

2:19 Restriction endonuclease digestion

The low, medium and high salt buffers described by Maniatis *et al.*, (1982) were prepared (at 10x) and used accordingly. Digestions were carried out for at least 90 min at 37°C and for up to 4 h when digesting chromosomal DNA. RNaseA (100 $\mu g\ ml^{-1}$) was added to restriction digests of plasmid DNA prepared by the rapid small-scale method.

2:20 DNA ligation

DNA samples were mixed in appropriate volumes of TE buffer. To subclone fragments, a 4:1 fragment to vector ratio was used with a DNA concentration < 50 $\mu g\ ml^{-1}$. For construction of gene libraries and subcloning using 'blunt-end' restriction endonucleases, a ratio of fragment to vector of 6:1 was used and the DNA concentration was increased to 100 $\mu g\ ml^{-1}$. To allow recircularisation only, the DNA concentration was reduced to 10 $\mu g\ ml^{-1}$. The DNA sample mix was then heated to 65°C for 5 min and incubated on ice for 60 min to allow DNA

fragments to reanneal slowly. After adding the appropriate amount of 10x concentration ligation buffer (4 mM ATP, 66 mM $MgCl_2$, 0.1 M DTT, 0.66 M Tris-HCl, pH 7.6) and T4 DNA ligase, the mixture was incubated at 15°C overnight. Aliquots of ligation buffer were stored at -20°C and were discarded after thawing once.

2:21 Transformaton

2:21:1 Routine transformation

Recipient cells were grown (by dilution of an overnight culture 1 ml in 20 ml of fresh NB) for 3 h with shaking. Cells were chilled on ice for 20 min, harvested (5000 rpm, 10 min), and the pellet resuspended in 10 ml ice-cold 0.1 M $CaCl_2$ (Sigma Grade I no. C-3881). The cells were incubated on ice for a further 20 min, harvested, and resuspended in 1 ml 0.1 M $CaCl_2$. The cells were then 'competent' and could be kept for 2 days. Aliquots of competent cells (100 μl) were mixed with DNA samples by vortexing, and placed on ice for 30'. The cells were heat shocked (42°C, 2 min) and if gene expression time was required, 1 ml of NB was added and incubated for 1 hr, before plating out on selective media.

2:21:2 Efficient transformation

For the efficient transformation of gene libraries and other cloning work, a few minor modifications were made to the method outlined in section 2:21:1. All cells were grown in SOB instead of NB and the cells were grown in a 250 ml flask (275 rpm) to A_{600} = 0.35. The expression time was increased to 2 h and all centrifugation steps were performed at 2000 rpm for 15 min.

2:22 Gene product identification using the maxicell system

This procedure was an adaptation of the method proposed by Sancar *et al.*, (1979) to label plasmid-coded proteins *in vivo* with ^{35}S-methionione. A *recA* strain carrying the plasmid of interest was grown in 15 ml K-medium (Rupp *et al.*, 1979. See also table 2:5:(a)) to a cell density of A_{600} = 0.5 (2 x 10^8 cells ml^{-1}), and placed on ice. Cells (10 ml) were UV-irradiated (using gentle agitation) with a dosage of *ca.* 50 Jm^{-2}, in a Petri dish. The cells were then transferred to a sterile universal bottle and freshly prepared cycloserine was added to a final concentration of 200 μg ml^{-1}. The culture was incubated with shaking for 14-16 h (150 rpm).

Cells were harvested (5000 rpm, 5 min) and washed twice in an equal volume of Hershey salts (Worcel & Burgi, 1974) and finally resuspended in 5 ml Hershey medium (table 2:5(a)). Following incubation for a further hour, 30 μCi ^{35}S-methionine was added and incubation continued for another hour. Cells were then harvested (5000 rpm, 5 min), and washed once in 10 mM Tris-HCl, pH 8.0. SDS-sample buffer (50 μl of 2x concentration) was added to the pellet and samples boiled for 5 min to solubilise cell proteins. The samples were stored at -20^{o}C and re-boiled (3 min) before electrophoresis (15 μl per slot).

2:23 SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

Proteins were analysed on 10-30% (w/v) exponential gradients or 11% (w/v) linear, SDS-polyacrylamide gels, (Kelly & Dow, 1985; Laemmli, 1970). The constitution of buffer and acrylamide solutions are given in

tables 2:23a,b, and c. Ammonium persulphate was always freshly prepared and the gel, once poured, was overlayed with water-saturated butan-1-ol. Electrophoresis was carried out using a Bio-Rad Protean gel system, at a constant current of 16 mA for 12 h or 25 mA for 4 h. ^{14}C-methylated proteins (Amersham) were used as molecular weight markers, and consisted of lysozyme (Mr 14300), carbonic anhydrase (Mr 30000), ovalbumin (Mr 46000), bovine serum albumin (Mr 69000), phosphorylase b (Mr 92500) and myosin (Mr 200000).

2:24 Treatment of SDS-polyacrylamide gels

2:24:1 Autoradiography

For autoradiography, gels were fixed for 30 min in 50% methanol (v/v) and dried onto a sheet of Whatman 3MM paper using a Bio-Rad model 1125B gel drier (80°C). The dried gels were placed in a Harmer film cassette with a sheet of Kodak X-omat S X-ray film, and were left at room temperature overnight or for a few days. The exposed X-ray film was developed using Kodak LX-24 developer (4 min), a 15 sec wash in water and 2 min in Kodak FX-40 X-ray fixer.

2:24:2 Fluorography

For fluorography, the method of Skinner & Griswold, (1983) was used. Gels were fixed in 200 ml of glacial acetic acid (5 min) and then immersed in 200 ml of 20% (w/v) 2,5-diphenyloxazole (PPO) in glacial acetic acid for 90 min. The gels were then washed in distilled water and left to soak for 30 min. The gels were then dried as section 2:24:1 (except temperature was 60°C), exposed to X-ray film, and left at -70°C overnight.

Table 2:23(a) Stock solutions for SDS-PAGE

Stock solution	Composition
Separating gel buffer	3 M Tris-HCl pH 8.8
Stacking gel buffer	0.05 M Tris-HCl pH 6.8
Low-bis acrylamide (60%)	60% (w/v) acrylamide, 0.3% (w/v) N,N'-methylene-bis-acrylamide
High-bis acrylamide (60%)	60% (w/v) acrylamide 1.6% (w/v) N,N'-methylene-bis-acrylamide
Stacking gel acrylamide	10% (w/v) acrylamide 0.5% (w/v) N,N'-methylene-bis-acrylamide
Electrophoresis buffer	0.025 M Tris HCl, 0.192 M glycine, 0.1% (w/v) SDS
Sample buffer x 2	0.05 M Tris-HCl, pH 6.8 10% (v/v) glycerol 2% (w/v) SDS 5% (v/v) β-mercaptoethanol

Table 2:23(b) Composition of polyacrylamide separating gels

Stock solution	Gradient Gel		Linear Gel
	10 ml 30%	25 ml 10%	11%
Separating gel buffer	1.25 ml	3.1 ml	3.76 ml
Low bis-acrylamide	5.0 ml	-	-
High bis-acrylamide	-	4.2 ml	5.45 ml
SDS, 10% (w/v)	0.1 ml	0.25 ml	0.3 ml
Glycerol, 75% (v/v)	3.65 ml	-	20.35 ml
Water	-	17.45 ml	
Ammonium persulphate, 10% (w/v)	20.0 µl	50.0 µl	60.0 µl
TEMED	2.0 µl	5.0 µl	6.0 µl

Table 2:23(c) Composition of polyacrylamide stacking gel

Stock solution	Volume in stacking gel
Stacking gel buffer	2.4 ml
Stacking gel acrylamide	3.0 ml
SDS, 10% (w/v)	0.1 ml
Water	4.4 ml
Ammonium persulphate 10% (w/v)	100.0 μl
TEMED	5.0 μl

2:24:3 Coomassie blue staining

Gels were stained for at least an hour in 300 ml 10% (v/v) glacial acetic acid, 30% (v/v) propan-2-ol, 0.05% (w/v) Coomassie brilliant blue. De-staining was carried out in the above solution, without stain, for 30 min and then in 300 ml 10% (v/v) glacial acetic acid for *ca*. 2 h.

2:25 Isolation of bacterial cell envelopes

The basic procedure was that described by Churchward and Holland, (1976). Exponentially growing cells at A_{450} = 0.5 or ^{35}S-methionine labelled maxi cells were resuspended in ice cold 10 mM sodium phosphate buffer, pH 7.2 (+ or - 10 mM $MgSO_4$). The total volume was *ca*. 8 ml and contained *ca*. 20 units of cells at A_{450}. Samples were transferred to a 25 ml beaker, and sonicated for 4x 30 sec, with 30 sec cooling on ice at amplitude 6 μm, using the 3/4 inch end diameter probe in a 150 Watt MSE ultrasonic disintegrator. The sonicated samples were centrifuged (7000 rpm, 5 min) in an MSE Hi-Spin 21 centrifuge using an 8 x 50 rotor, to remove unlysed cells. The samples were transferred to 10 ml polycarbonate tubes and the membrane fragments pelleted using an MSE Super-Speed 65 centrifuge and 10 x 10 Ti rotor (30000 rpm, 30 min). The supernatant was discarded or retained to prepare cytoplasmic proteins (see section 2:26). The membrane pellet was carefully resuspended using a sealed off, bent Pasteur pipette in 1 ml 10 mM sodium phosphate buffer (+ or - $MgSO_4$), and re-pelleted (30000 rpm 30 min, 4^{o}C). This was repeated with one or more 5 ml washes as required. The final pellet was carefully drained and resuspended in 50 μl sodium phosphate buffer transferred to an Eppendorf tube and solubilised by the addition of 50 μl of 2x SDS-sample buffer. Samples were boiled for 5 min and stored at

-20°C until required.

2:26 Preparation of cytoplasmic proteins from sonic-lysates

Cytoplasmic proteins were prepared from the supernatant after removal of envelope material from sonic lysates (section 2:25). Aliquots of supernatant (2 ml) were added to 8 ml ice-cold acetone in 15 ml Corex tubes and incubated at -20°C for 2 h or overnight. The precipitated proteins were pelleted (10000 rpm, 30 min, 4°C) in an MSE Hi-Spin 21 centrifuge using an 8 x 50 rotor. The precipitate was dried under vacuum and resuspended in 100 µl of 10 mM sodium phosphate buffer pH 7.2 and transferred to Eppendorf tubes. After the addition of 100 µl 2x SDS-sample buffer, the samples were boiled for 5 min and stored at -20°C until required.

2:27 Separation of outer and cytoplasmic membranes

The washed membrane pellets (setion 2:25) still in the 10 ml polycarbonate tubes were resuspended in 200 µl of 0.5% (w/v) sarkosyl NL97. If required, 50 µl of the resuspension was removed for a total envelope fraction. The remainder was incubated at room temperature for 30 min before centrifugation in the 10 x 10 Ti rotor (section 2:25) for 1 h (35000 rpm, 15°C). The supernatant represented the solubilised cytoplasmic membrane proteins and the pellet contained the sarkosyl-insoluble outer membrane proteins. The pellet was washed by resuspending in 1 ml 0.5% (w/v) sarkosyl NL97, incubating for 30 min at room temperature and centrifuging for 1 h at 35000 rpm, 15°C as before.

The washed pellet was then finally resuspended in 100 μl of 10 mM sodium phosphate buffer, pH 7.2 and 100 μl of 2x sample buffer added before boiling for 5 min and storing at -20^{o}C until required.

2:28 Isolation of periplasmic proteins

Periplasmic proteins were released in response to cold osmotic shock treatment of cultures, according to the method of Neu and Heppel (1965). Maxicell cultures were labelled with ^{35}S-methionine (section 2:22), and buffered by the addition of 0.5 ml of a solution of 0.5 M Tris.HCl, pH 7.8. The culture was incubated (10 min) at room temperature and then pelleted in an MSE Chilspin (5000 rpm, 5 min, 4^{o}C). The pellet was resuspended in 0.8 ml of sucrose solution (30 mM Tris.HCl, pH 7.8; 40% (w/v) sucrose, 2 mM EDTA) and after incubation at 30^{o}C for 10 min, the cells were re-pelleted by centrifugation (1 min) in an MSE Microcentaur. The supernatant was carefully removed and the cells rapidly resuspended in 0.5 ml of ice-cold distilled water. The suspension was then left on ice for 10 min and the 'shocked' cells removed by a 5 min centrifugation in an MSE Microcentaur.

The resulting shock fluid was then added to 4 ml of ice-cold acetone and precipitated at -20^{o}C for 2 hours in 15 ml Corex tubes. After centrifugation in an MSE Hi-Spin 21 (10000 rpm, 30 min, 4^{o}C) using an 8 x 50 rotor, the precipitate was air-dried and resuspended in 30 μl of distilled water and 30 μl of 2x SDS-sample buffer. Aliquots (15 μl) were routinely used for analysis using SDS-PAGE (section 2:23).

2:29 Zubay *in vitro* transcription-translation system

Plasmid DNA or linearised DNA fragments were used to programme the synthesis of polypeptides *in vitro*. The *E. coli* extracts (S30) and low molecular weight mix (LMM) prepared as described by Pratt *et al.*, (1984), were a generous gift from C. Jones, and were stored at -20°C until required. Extracts were thawed once only and then discarded. Transcription-translation incubations were carried out at 37°C and contained:

7.5 µl	LMM
2.0 µl	^{35}S-methionine (≈25 µCi)
3.5 µl	0.1 M magnesium acetate
5.0 µl	S30 extract
2.5 µg	DNA in 10 mM Tris.HCl; 1 mM EDTA, pH 7.0

The reaction mix was made up to 30 µl with TE buffer. After 30 min incubation, 5 µl of prewarmed 44 mg ml^{-1} unlabelled methionine was added and the incubation continued for a further 5 min. SDS-sample buffer (30 µl) was added and the sample frozen at -20°C until required for analysis using SDS-PAGE.

CHAPTER 3

Subcloning and analysis of the λ transducing phage λpGS22

3:1 Introduction

The λ transducing phage λpGS22, complemented the cell division genes *ftsE* and *ftsS*, but not nearby genetic markers (Salmond & Plakidou, 1984). The essential gene *rpoH/htpR/hin*, (Cooper & Ruettinger, 1975; Yamamori & Yura, 1982; Grossman *et al.*, 1984) mapped close to the cell division genes, but not close enough to lie on the bacterial fragment cloned into λpGS22. To determine the locations of the cell division genes on λpGS22, fragments of the 6.5 kb partial *Hin*dIII insert were subcloned both for restriction analysis and complementation assays.

3:2 Results

3:2:1 Subcloning of λpGS22

DNA from the λ transducing phage, λpGS22 was digested with the restriction endonuclease *Hin*dIII and the digest analysed by electrophoresis (figure 3:2:1). Together with the λ 'arms', three *Hin*dIII fragments of sizes 4.5, 1.1, and 0.9 kb were seen. Each of these *Hin*dIII fragments were subcloned into the unique *Hin*dIII site of the multicopy plasmid vector pBR325 (Bolivar, 1978), to make plasmids pDG1, pDG14 and pDG113 respectively. These plasmids were used to transform *recA* and *recA*$^+$ derivatives of the cell division mutants already shown to be complemented by λpGS22. The *recA* derivatives of all the strains (except for TOE22 and MFT1181) were constructed using the generalised transducing phage T4GT7 according to the method of Plakidou *et al.*, (1984). Strains TOE22 and MFT1181 were made *recA*, by conjugation with JC10240, (Csonka & Clarke, 1980).

Figure 3:2:1 Agarose gel electrophoresis of λpGS22 DNA digested with *Hin*dIII.

Samples were analysed on a 0.8% (w/v) agarose gel. Track 1 = λ DNA digested with *Hin*dIII; track 2 = λpGS22 DNA digested with *Hin*dIII; track 3 = λpGS22 DNA uncut.

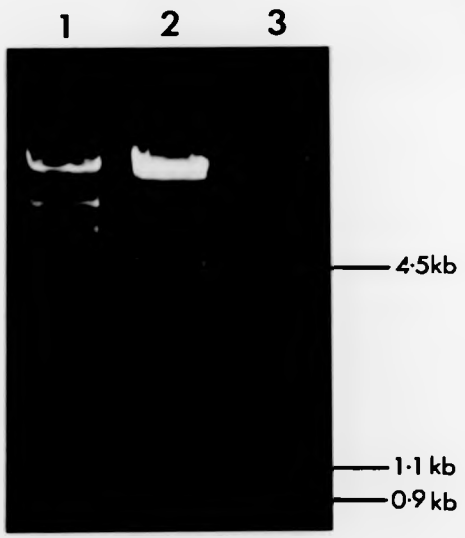


Figure 3:2:1 Agarose gel electrophoresis of λpGS22 DNA digested with *Hin*dIII.

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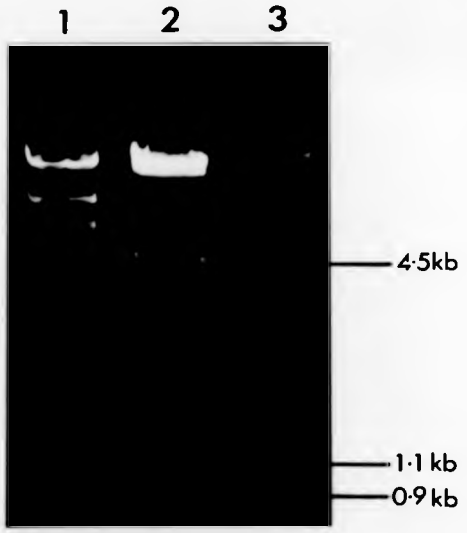


3:2:2 Complementation tests using λpGS22 subclones

Data presented in table 3:2:2 show that plasmid pDG1 complemented all but OV22*recA*. Plasmids pDG14 (this study) and pDG113 (N. Crickmore, pers. comm.) did not complement any of the mutants. Strain OV22 was previously shown to carry a mutation in a novel cell-division gene, *ftsS*, whereas the other strains were presumed to carry alleles of *ftsE* (Salmond & Plakidou, 1984). On the basis of these results, it was decided to further analyse the 4.5 kb fragment in an attempt to localise the *ftsE* gene.

3:2:3 Restriction mapping the 4.5 kb fragment

Plasmid pDG1 was digested with the restriction endonucleases listed in figure 3:2:3, and a preliminary restriction map of the 4.5 kb fragment constructed, by comparison of the products of single and multiple digests (Maniatis *et al.*, 1982). The 4.5 kb *Hin*dIII fragment was also purified using the DE81-method (section 2:18:1), for sequence analysis by the random, shotgun method (Bankier & Barrell, 1983). The complete nucleotide sequence of the fragment is presented in Appendix I (Gill *et al.*, 1986). For the final DNA sequence compilation the complete 4.5 kb fragment was sequenced at least twice on both strands and up to six times in certain regions of the fragment (G. Salmond, pers. comm.). Salient features of the sequence are represented and discussed in the text as appropriate. The DNA sequence was used, both to confirm the preliminary restriction map, and to provide additional well-defined enzyme sites for subsequent manipulations.

3:2:4 Position of the 4.5 kb fragment in the chromosome

In order to determine the position of the *ftsE* and *ftsS* genes relative to the *rpoH* gene at 76 minutes of the *E. coli* chromosome (Bachmann,

Table 3:2:2 Complementation data for λpGS22 and pDG1 containing strains

STRAIN	λpGS22	pDG1
OV22*recA*	?	-
OV32*recA*	+	+
JS10*recA***	+	+
TOE22*recA*	+	+
TC35*recA*	+	+
MFT1181*recA*	+	+

** the complementation tests for cold sensitive strain JS10 were done at 37°C and 25°C.

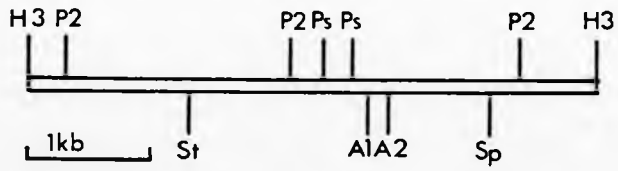
\+ indicates complementation

\- indicates no complementation

? indicates some complementation observed

Figure 3:2:3 Restriction map of the 4.5 kb insert of pDG1.

Abbreviations for restriction enzymes have been listed earlier. Restriction enzymes which cut the 4.5 kb *Hind*III fragment are indicated in the diagram. The enzymes which did not cut the fragment include: Bm, Bg, El, Kp, Sa, Sm and Ss.



1983), four plasmids reputed to complement the *ftsE* mutant MFT1181 (Nishimura *et al.*, 1977), were investigated. The plasmids were isolated from a ColE1 hybrid plasmid bank (Clarke & Carbon, 1976). One of the plasmids, pLC19-48, was shown to have no homology to the 4.5 kb fragment, when probed in a Southern blot experiment (data not shown). Plasmids pLC31-16 and pLC31-32, were shown to be identical and complemented the mutations *ftsE*, *htpR*, *livJ* and *livK*, (Neidhardt *et al.*, 1983b). Plasmid pLC31-16 was investigated further. The published restriction map of this plasmid (Neidhardt *et al.*, 1983a), was verified for several restriction endonuclease sites and it was conclusively shown that the plasmid contained only one *Hin*dIII site (figure 3:2:4). This proved that pLC31-16 could not contain the complete 4.5 kb fragment, and when the two restriction maps were compared it was possible to align them as shown in figure 3:2:4. In 1984, Landick *et al.* published the DNA sequence of a 1.125 kb fragment, derived from pLC31-16, which carried the *rpoH* gene and this shows an overlap (66 identical base pairs) with the 4.5 kb sequence. From these data the two smaller *Hin*dIII fragments carried by λpGS22 must lie at the *rpoH*-distal end of the 4.5 kb fragment. This has now been confirmed (N. Crickmore, pers. comm.).

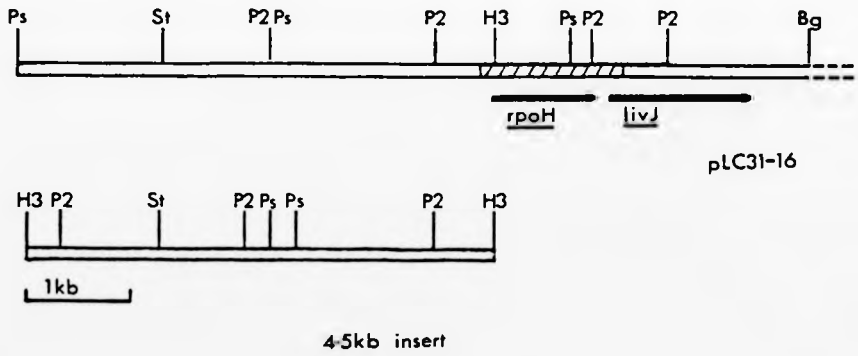
3.3 Discussion

3:3:1 The relative position of the *rpoH* and *ftsE* genes

The data presented in this chapter bring together the information known about the arrangement of genes in the 76-minute region of the *E. coli* chromosome. Salmond and Plakidou (1984) mapped many of the genes located in this region using generalised transduction and showed that

Figure 3:2:4 Comparison of the restriction maps for the 4.5 kb insert of pDG1 and plasmid pLC32-16.

Arrows indicate the direction of transcription of the *rpoH* and *livJ* genes. The hatched area of pLC31-16 ▨ represents the extent of the DNA sequence reported by Landick *et al.*, (1984). There is an overlap of 66 bp with the DNA sequence of the 4.5 kb fragment (see text).



the λ transducing phage λpGS22 complemented mutations in the cell division genes *ftsE* and *ftsS*, but not the closely mapped *htpR*, *fam715* and *dnaM* mutations. Plasmid pDG1 carried a 4.5 kb *Hin*dIII fragment subcloned from λpGS22, which was shown to complement *ftsE*-carrying mutants, but not the *ftsS*-carrying mutant, OV22. The position of the *ftsS* cell division gene is currently being investigated in this laboratory.

The position of the very tightly linked *rpoH* gene was investigated using a previously characterised plasmid pLC31-16. The rather preliminary restriction mapping of the chromosomal fragment carried by this plasmid (Neidhardt *et al.*, 1983a) did not allow the conclusive positioning of the 4.5 kb fragment carrying the *ftsE* gene. However, the publication of the *rpoH* DNA sequence (Landick *et al.*, 1984) and further restriction analysis of pLC31-16 clarified this as shown in figure 3:2:4. Initial confusion arose due to the existence of the *PstI* restriction site present on the chromosomal fragment of pLC31-16 which was not present on the 4.5 kb fragment. Chromosomal fragments from this region cloned by other workers (Oxender *et al.*, 1980) also lacked this *PstI* site. A possible explanation for this observation is that the *PstI* site was generated during the construction of pLC31-16 at the junction between chromosomal and ColE1-vector DNA. The hybrid plasmids were constructed by random shearing of chromosomal DNA and annealing to ColE1 DNA by the poly(dA.dT) "connector" method outlined by Clarke and Carbon (1975, 1976).

The location of the *rpoH* gene very close to the *ftsE* and *ftsS* genes may have some exciting implications for the regulation of cell division, (Crickmore & Salmond, 1986; see also Chapter 7), further emphasising the

importance of the "morphogene cluster" at 76-minutes (Donachie *et al.*, 1984). Even if the close proximity of *rpoH* is found to be unrelated to cell division control, this work has clarified the position and orientation of the 4.5 kb fragment relative to genes in this region of the *E. coli* linkage map (Bachmann, 1983).

3:3:2 What is complementation? - A cautionary note

In *recA*$^+$ strains, complementation results can be complicated by the phenomenon of "marker rescue". An example of this was described by Neidhardt *et al.*, (1983a) where the *htpR* mutation was "rescued" by plasmids carrying DNA for only part of the *htpR/rpoH* gene, after prolonged growth at the permissive temperature. It was assumed that *recA*$^+$-dependent recombination between the chromosome and one copy of the plasmid, had generated a functional chromosomal *rpoH* gene. To eliminate such a source of confusion in this study, all complementation tests were carried out in *recA* derivatives as described in section 3:2:1.

For cell division mutants, there are additional problems encountered when trying to ascertain the degree of complementation. Ideally, a temperature-sensitive cell division mutant would produce healthy rods at the permissive temperature, but would fail to form colonies at the restrictive temperature. In practice, this is not usually the case, the test being complicated firstly by the "leakiness" of some of these mutants (Salmond & Plakidou, 1984) and secondly by the variable degree of filamentation observed. For example, some strains can produce micro-colonies at the restrictive temperature which may affect the accuracy of viable count data. These small colonies are difficult to re-streak and are seen to be filamentous when examined under the microscope. The formation of such micro-colonies may be medium-dependent (G. Salmond,

pers. comm.). Also, phenotypic differences in the cell division mutants often make it difficult to compare complementation. The cold-sensitive cell division mutant JS10, (figure 3:3:2) shows a variable degree of filamentation at both permissive and restrictive temperatures.

Finally, one must also be wary when interpreting data from complementation tests involving multicopy plasmids, because the overproduction of a gene product may interfere with phenotypic complementation. An extreme example of this, is the gene for the outer membrane protein OmpA, which is lethal to the cell when cloned into multicopy plasmid vectors (Bremer *et al.*, 1980).

Once aware of these potential complications, the degree of complementation can be assessed in each case. The strains MFT1181, TC35, TOE22, OV32 and JS10 were all complemented satisfactorily by plasmid pDG1, and therefore the 4.5 kb fragment was further analysed to locate the *ftsE* gene.

Figure 3:3:2 Photomicrographs showing the variable degree of filamentation observed for the cold-sensitive mutant JS10.

i = Growth at the permissive temperature of 37°C;

ii = Growth at the restrictive temperature of 25°C.

i

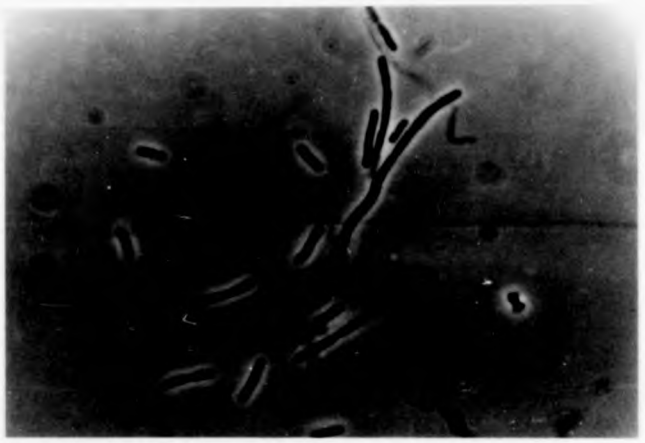
ii

Figure 3:3:2 Photomicrographs showing the variable degree of filamentation observed for the cold-sensitive mutant JS10.

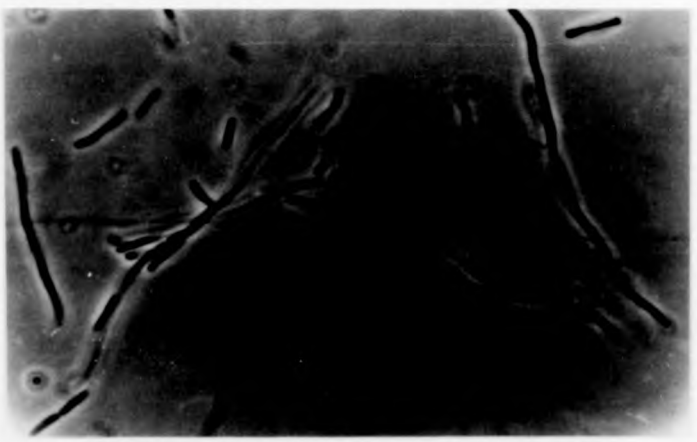
i = Growth at the permissive temperature of 37°C;

ii = Growth at the restrictive temperature of 25°C.

i



ii



CHAPTER 4

The location of the *ftsE* gene on the 4.5 kb fragment

4:1 Introduction

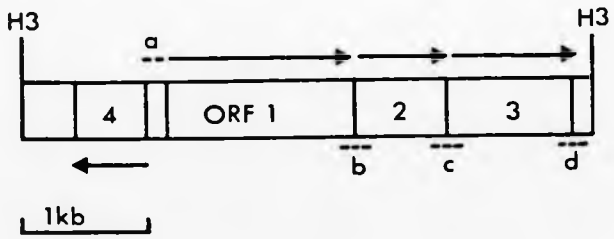
4:1:1 Predictions from the 4.5 kb nucleotide sequence

Once the complete nucleotide sequence of the 4.5 kb *Hin*dIII fragment had been compiled, the sequence was analysed using the programme ANALYSEQ (Staden, 1984). The preliminary restriction map shown in figure 3:2:3, was confirmed and the salient features of the sequence are presented in figure 4:1:1. There are four major open reading frames (ORFs), three of which have the same predicted direction of transcription (ORFs 1, 2 and 3), and one, which is transcribed in the opposite direction and from the other DNA strand (ORF 4). The close arrangements of ORFs 1, 2 and 3 is suggestive of an operon with an interval of two base pairs, between the end of ORF1 and the beginning of ORF2, and an overlap of seven base pairs between the end of ORF2 and the beginning of ORF3. This overlap could be indicative of translational coupling (Oppenheim & Yanofsky, 1980; Aksoy *et al.*, 1984). The putative operon is translationally terminated by tandem stop codons as indicated in figure 4:1:1.

The sequence 5' to the start of ORF1 looks particularly interesting since there is sequence homology in this region with the tryptophan (*trp*) operon promoter/operator region (Appendix II). This may be the regulatory region of the putative operon. Various aspects of the sequence are discussed in the text as appropriate. From the results presented in chapter 3, the *ftsE* gene was shown to be on the 4.5 kb fragment and therefore insertion mutants were used to determine in which ORF the *ftsE* mutation mapped.

Figure 4:1:1 Diagram to show the salient features of the operon.

The major open reading frames (ORFs) are shown (1-4) and arrows indicate the predicted direction of transcription. Other salient features include: a = putative regulatory region (see Appendix II); b = interval of 2 bp between ORF1 and ORF2; c = overlap of 7 bp between ORF2 and ORF3; d = tandem stop codons.



4:1:2 The use of transposons to investigate transcriptional organisation

Transposons are mobile genetic elements which are able to insert independent of the host *recA* function into DNA sequences, and can be used in the genetic manipulation of bacteria. The properties and uses of transposons have been extensively discussed (Kleckner *et al.*, 1977; de Bruijn & Lupski, 1984). Two properties of transposons which have been successfully exploited in order to mutate and locate genes are, firstly, the almost random manner of insertion and, secondly, the ability of the insertion to have polar effects on expression of downstream genes. Once inserted into a gene, the translational and transcriptional stop signals present in the transposon's flanking insertion sequences, effectively prevent expression of the gene, and will normally result in the complete loss of function of that gene (Kleckner *et al.*, 1977).

The widely-used transposon Tn*5* (D. Berg & C. Berg, 1983: review) was used in this way to investigate the transcriptional organisation of the isoleucine-valine (*ilv*), biosynthetic genes in *E. coli* (C. Berg *et al.*, 1979). By using the property of Tn*5* to cause polarity on expression of downstream genes, Berg *et al.*, were able to identify two internal promoters within the *ilv* operon. Incomplete polarity due to transposons is rare (de Bruijn & Lupski, 1984).

In order to use transposons to analyse the genes on the 4.5 kb fragment, it was necessary to obtain insertions throughout the DNA, to screen the effect of insertion on the complementation of the cell division mutants, and then to locate the point of insertion. The latter would help determine which ORF was the *ftsE* gene.

4:1:3 The choice of transposon mutagenesis system

A relatively simple method of generating transposon insertions into pDG1 was required, and the Tn*1000* or $\gamma\delta$ mutagenesis procedure was chosen. The transposition of Tn*1000* from the F plasmid, to the multicopy plasmid pBR322 and its derivatives during a simple bacterial conjugation, was first described by Guyer (1978). This phenomenon has since been successfully exploited by several groups of workers (Buxton & Drury, 1983; Delencastre *et al.*, 1983; Jones & Holland, 1984) in order to identify genes cloned into multicopy plasmid vectors.

Although little seems to be known about Tn*1000* (Grindley, 1983; Guyer, 1983) this transposon was chosen instead of the better-characterised Tn*5*, because it was reported to encode only two polypeptides (Buxton & Drury, 1983). Transposon Tn*5* encodes five polypeptides (Rothstein & Reznikoff, 1981) and there was a possibility that these proteins would obscure the *ftsE* gene product in subsequent gene product-identification experiments.

4:2 Results

4:2:1 Tn*1000* mutagenesis of the 4.5 kb fragment

Tn*1000* mutagenesis was carried out as described in section 2:9. For convenience, the 4.5 kb *Hin*dIII fragment was sub-cloned into the multicopy vector pBR322 (Bolivar *et al.*, 1977), which is smaller than pBR325, and lacks the gene for chloramphenicol (Cm)-resistance. The sub-cloning was achieved by preparation of the 4.5 kb *Hin*dIII fragment and ligation into the *Hin*dIII site of pBR322 (position 29). Maniatis *et al.* (1982), have compiled a detailed restriction map of pBR322 and this

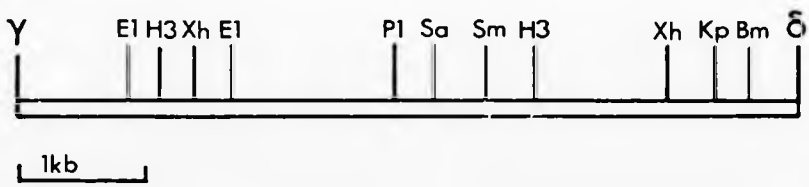
is referred to throughout. Ampicillin-resistant transformants were then screened for sensitivity to tetracycline (Tc) which indicated that the Tc-resistance gene on pBR322 had been inactivated due to insertion of the 4.5 kb fragment at the *Hin*dIII site. One such plasmid, pDB1, was used to transform the F^+ donor strain RB308 to Ap^r. The resultant strain subsequently referred to as DW214 was then mated with the recipient TOE22*recA* (DW40), and Ap^r,Tc^r transconjugants selected. The transconjugant colonies were purified and then tested for complementation of the *ftsE* mutation carried by TOE22. Colonies which carried the plasmid pDB1 but which were temperature-sensitive were presumed to be the result of a Tn*1000* insertion in the *ftsE* gene. Of 100 colonies tested, 10 colonies appeared to be temperature-sensitive, and these colonies were retained for further study. Several colonies which had remained temperature-sensitive, were also kept for analysis.

4:2:2 Restriction mapping of the Tn*1000* insertion mutations

Plasmid DNA was prepared from both the temperature-sensitive and temperature-insensitive colonies and used to re-transform the strain TOE22*recA* to Ap^r. The transformants were re-tested for complementation at the restrictive temperature to ensure that the plasmid alone was responsible for the phenotype. The plasmids were then restriction mapped using single and multiple restriction endonuclease digestions, as described by Maniatis *et al.* (1982). The digests were subjected to electrophoresis and DNA fragment sizes calculated (section 2:17). Rapid screening of the plasmid DNA was achieved by digestion with the restriction endonuclease *Sal*I. This enzyme cuts the vector pBR322 at a unique *Sal*I site (position 650 in the vector), and does not cut the 4.5 kb fragment. Figure 4:2:2(a) shows the restriction map of the Tn*1000* fragment (Guyer, 1978). It can be seen that Tn*1000* contains a single,

Figure 4:2:2(a) Restriction map of the Tn*1000* fragment.

Redrawn from Guyer (1978). All abbreviations for restriction enzymes have been listed earlier.



centrally located *Sal*I site, and therefore digestion of the pDB1::Tn1000 derivatives with the *Sal*I enzyme was used to ensure that the plasmids contained only one Tn*1000* insertion. The central location of the *Sal*I site also allowed an estimate to be made of the Tn*1000* insertion point. Of the 10 plasmids which no longer complemented TOE22*recA*, four of these (designated pDB38, pDB63, pDB65, and pDB92) were investigated further. Figure 4:2:2(b) shows the results of digesting pDB1 and the four pDB1::Tn*1000* derivatives, with the enzyme *Sal*I. The plasmid pDB1 contains only one *Sal*I site as described above, whereas the Tn*1000* derivatives contain two *Sal*I sites, therefore producing two *Sal*I fragments. The position of the Tn*1000* insertion, in each case, was determined more accurately by single digestion with the restriction endonucleases, *Eco*RI and *Xho*I, and by double digestion with either of these two enzymes and *Sal*I. Neither *Eco*RI nor *Xho*I cut the 4.5 kb fragment (figure 3:2:4) and thus using the restriction map of pBR322 (Maniatis *et al.*, 1982) and the restriction map of the Tn*1000* element, presented in figure 4:2:2(a), the position of insertion in each of the plasmids was determined. The orientation of the Tn*1000* in each plasmid was then ascertained, by digestion with the restriction endonuclease *Bam*HI, which does not cut the 4.5 kb *Hin*dIII fragment, but cuts the vector pBR322 once only at position 375, and cuts the Tn*1000* element asymmetrically. The position and orientation of each of the four insertions is shown diagrammatically in figure 4:2:2(c). They are as follows: pDB38, 2.9 kb; pDB63, 3.2 kb; pDB65, 2.6 kb and pDB92, 2.5 kb. All base pairs in the sequence of the 4.5 kb fragment have been numbered, left to right, zero to 4480, (4.48 kb) for convenience. The position of the Tn*1000* insertions in five plasmids which had retained the ability to complement TOE22*recA* was similarly determined. In all five cases the transposon was inserted in vector DNA and not in the

Figure 4:2:2(b) Agarose gel electrophoresis of plasmids pDB1, pDB92, pDB65, pDB38 and pD63 digested with SalI.

Samples were run on a 0.6% (w/v) agarose gel. Tracks 1 and 7 = λ DNA digested with *Hin*dIII; tracks 2-6 = plasmids pDB1, pDB92, pDB65, pDB38 and pDB63 respectively, digested with *Sal*I.

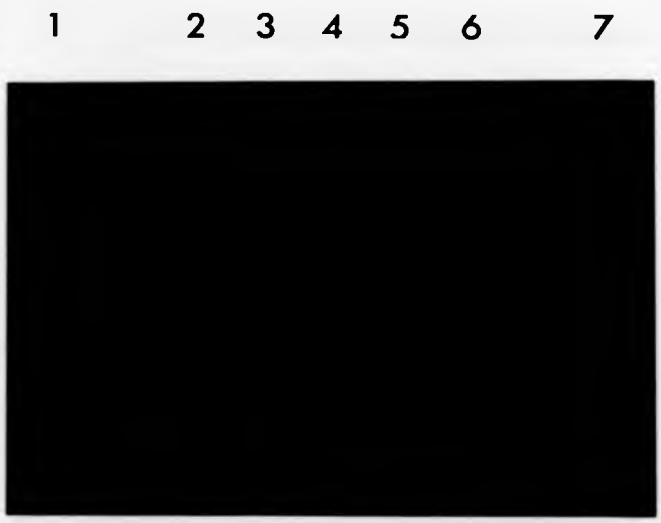


Figure 4:2:2(b) Agarose gel electrophoresis of plasmids pDB1, pDB92, pDB65, pDB38 and pD63 digested with SalI.

Samples were run on a 0.6% (w/v) agarose gel. Tracks 1 and 7 = λ DNA digested with *Hin*dIII; tracks 2-6 = plasmids pDB1, pDB92, pDB65, pDB38 and pDB63 respectively, digested with *Sal*I.

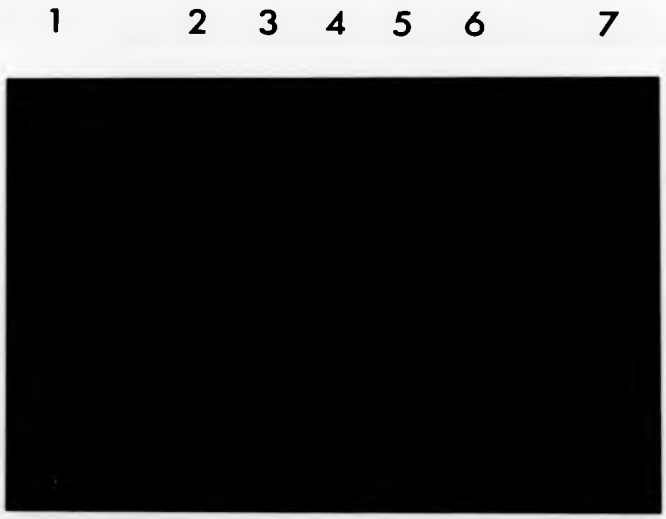
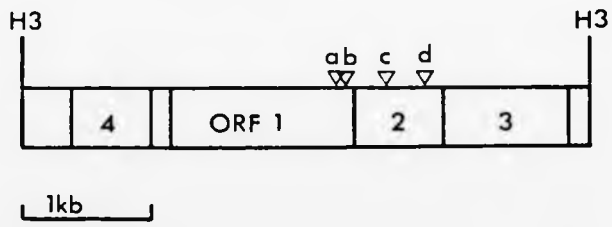


Figure 4:2:2(c) Diagram to show the relative position of the Tn*1000* insertions 92, 65, 38 and 63 in the 4.5 kb fragment.

Insertion a = 92; b = 65; c = 38 and d = 63. Insertions a and b are oriented γ to δ with respect to the 4.5 kb fragment (0 to 4.5 kb) and insertions c and d are located in the opposite orientation. See text for details.



4.5 kb sequence (data not shown).

4:2:3 Identification of the *ftsE* gene

Once the Tn*1000* insertions had been located, the four pDB1::Tn*1000* derivatives were used to transform all the *ftsE* mutants, and the Ap^r transformants screened for temperature-sensitivity. The results are presented in table 4:2:3. It was shown that whilst pDB1 was able to complement all the *ftsE* mutants, none of the four Tn*1000*-plasmids complemented any of the mutants.

4.3 Discussion

The results indicated that Tn*1000* insertions into ORF2 and the 3'-end of ORF1 inactivated the *ftsE* gene, since plasmids carrying these insertions no longer complemented the *ftsE* mutations. On the basis of these results, which ORF is *ftsE*?

As discussed in section 4:1:2, a transposon inactivates the gene into which it is inserted by interruption of the coding sequence and subsequent termination of transcription (Kleckner *et al.*, 1977). A transposon may also affect expression of genes downstream of the insertion point, due to polarity. Insertions 38 and 63 (found on plasmids pDB38 and pDB63 respectively) interrupted the continuity of ORF2, whilst insertions 92 and 65, (plasmids pDB92 and pDB65) interrupted ORF1. If ORF2 was *ftsE*, then expression of the gene would be affected by insertions 38 and 63 as predicted and by insertions 92 and 65 due to the polar nature of these insertions in ORF1. These latter insertions would interrupt expression of ORF2, if both ORF1 and

Table 4:2:3 Summary of complementation data of pDB1 and the Tn*1000*-insertion plasmids pDB38, pDB63, pDB65 and pDB92

	PLASMID				
STRAIN	pDB1	pDB38	pDB63	pDB65	pDB92
MFT1181*recA*	+	-	-	-	-
TOE22*recA*	+	-	-	-	-
TC35*recA*	+	-	-	-	-
OV32*recA*	+	-	-	-	-
JS10*recA*	+	-	-	-	-

\+ indicates complementation

\- indicates no complementation

ORF2 were co-transcribed.

If ORF3 was *ftsE*, then expression of this gene would be affected by insertions in ORFs 1 and 2 due to polarity, and thus it would be postulated that ORFs 1, 2 and 3 were co-transcribed. Consequently the data suggest that either ORF2 or ORF3 is *ftsE*.

It should be noted, however, that the ORF1 insertions are very 3' within the gene and it is possible that these insertions interrupt sequences required for optimal expression of ORF2 (and perhaps ORF3). Thus the possibility remains that ORF1 and ORF2 do not represent an operon in the 'classical' sense. An example of this is found in the morphogene cluster at 2-minutes on the *E. coli* linkage map (Bachmann, 1983). Jones and Holland (1984) showed, using Tn*1000* mutagenesis, that the 3'-end of the *ftsA* gene contained a regulatory sequence essential for maximal expression of the adjacent gene, *ftsZ*, although these two genes are not co-transcribed (see also section 1:11).

One question remains however, and that is, why were no ORF3 insertions isolated, if ORF3 is *ftsE*? It is possible that of the six discarded plasmids which no longer complemented the TOE22 *ftsE* mutation due to insertional inactivation, one or more could have contained an ORF3 insertion. These six plasmids were discarded, to avoid isolates which contained more than one Tn*1000* insertion, or appeared to have rearrangements of its DNA. Such plasmids would have caused confusion during interpretation of complementation data. Screening of colonies in this way revealed that approximately 60% of the plasmids isolated carried a single Tn*1000* insertion.

In conclusion, the ease of isolation of Tn*1000* insertions and their subsequent utility in gene-product identification, render Tn*1000* mutagenesis a powerful technique in the identification and analysis of genes and operons. This technique has been used to predict that either ORF2 or ORF3 is *ftsE* and this is resolved in the next chapter.

CHAPTER 5

Identification of a novel cell division gene *ftsX*

5:1 Introduction

Results presented in chapter 4 suggested that the cell division gene *ftsE* could be either ORF2 or ORF3 on the 4.5 kb fragment. In order to investigate these predictions, deletion derivatives of pDB1 were constructed in which part, or all, of ORF3 was removed. These plasmids were then used to transform the cell division mutants, and the effect on complementation was determined. A transposon insertion in ORF3 was also obtained using Tn*1000* mutagenesis of pDB1 in the strain JS10*recA*, and the effect of this insertion on complementation was also studied.

5:2 Results

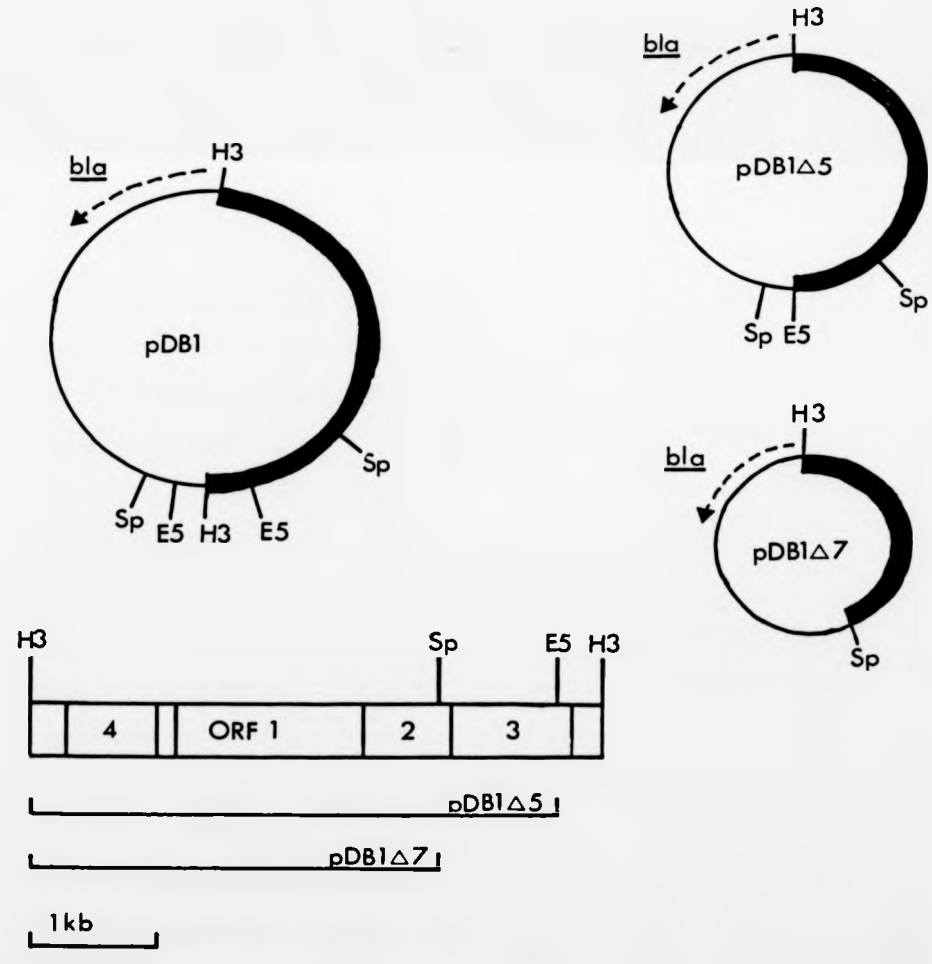
5:2:1 Construction of pDB1Δ5 and pDB1Δ7

As shown in figure 5:2:1, pDB1 contained two *Eco*RV restriction sites. These sites were used to construct a deletion derivative, in which the 0.39 kb *Eco*RV fragment of pDB1 was removed. In order to do this, pDB1 was digested with *Eco*RV, religated in a large volume (to promote intramolecular recircularisation) and the ligation mix was used to transform the *recA* strain DH1. The resultant Ap^r plasmid had lost the 0.39 kb *Eco*RV fragment, which removed 0.12 kb of the 3' end of ORF3. This was confirmed by restriction mapping and the plasmid was designated pDB1Δ5. Figure 5:2:1 also shows that pDB1 contains two *Sph*I sites and these were used to construct a plasmid in which the 1.75 kb *Sph*I fragment was removed from pDB1. After digestion with *Sph*I, and subsequent ligation and transformation as described above, Ap^r transformants were screened, and yielded plasmid pDB1Δ7. The

Figure 5:2:1 Diagram to show plasmid pDB1 and the construction of plasmids pDB1Δ5 and pDB1Δ7.

Abbreviations for restriction enzymes have been listed earlier.

Arrow----➤ indicates position of gene for Ap-resistance.



construction removed ORF3 and a small part of ORF2 as shown (figure 5:2:1).

5:2:2 Complementation data for pDB1Δ5 and pDB1Δ7

The plasmids pDB1Δ5 and pDB1Δ7 were used to transform *recA* derivatives of the cell division mutants, and Ap^r colonies were purified and tested for temperature-sensitivity. Table 5:2:2 summarises the results obtained from these tests. It was found that although pDB1 complemented all five mutants, the plasmid pDB1Δ7, in which ORF3 and part of ORF2 had been deleted, no longer complemented any of them. However, plasmid pDB1Δ5, in which only part of ORF3 had been removed, was able to complement three of the mutants, MFT1181, TOE22 and TC35. The significance of these data is discussed in section 5:3:1.

5:2:3 Tn*1000* mutagenesis in JS10*recA*

A second approach used to determine whether ORF2 or ORF3 was *ftsE*, was that of transposon mutagenesis. Several attempts were made to isolate an insertion in ORF3 using Tn*1000* mutagenesis as described in chapter 4. When the strain JS10*recA* (DW159) was used to screen for such insertions, a plasmid, designated pDB95, carrying a single insertion in ORF3 (insertion 95) was successfully isolated. This plasmid was restriction mapped as outlined in section 4:2:2, and the Tn*1000* element found to be inserted at position 4.0 kb within the 4.5 kb fragment. Figure 5:2:3 indicates the relative position of this insertion and the restriction fragments produced when pDB95 was digested with the restriction enzyme *SalI* (see also section 4:2:2).

5:2:4 Complementation data for pDB95

The cell division mutants were transformed with plasmid pDB95 and

Table 5:2:2 Complementation data for pDB1Δ5, pDB1Δ7 and pDB95

	PLASMID			
STRAIN	pDB1	pDB1Δ5	pDB1Δ7	pDB95
MFT1181	+	+	-	+
TOE22	+	+	-	+
TC35	+	+	-	+
JS10	+	-	-	-
OV32	+	-	-	-

\+ indicates complementation in *recA* background

\- indicates no complementation

Figure 5:2:3 Agarose gel electrophoresis of the Tn*1000*-insertion plasmid pDB95 and a diagram to indicate the relative position of insertion 95 in the 4.5 kb fragment.

Samples were run on a 0.6% (w/v) agarose gel. Tracks 1 and 8 = λ digested with *Hin*dIII; tracks 2-7 = plasmids pDB1, pDB92, pDB65, pDB38, pDB63 and pDB95 respectively, digested with *Sal*I. See section 4:2:2 for details of restriction enzyme mapping.

a = insertion 92; b = 65; c = 38; d = 63 and e = 95. The orientation of insertion 95 is γ to δ relative to the 4.5 kb *Hin*dIII fragment (0-4.5 kb).

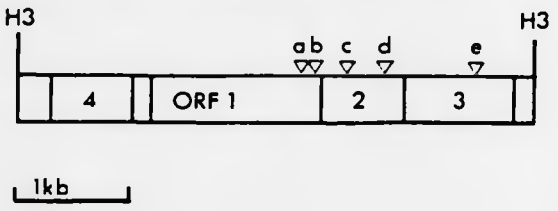
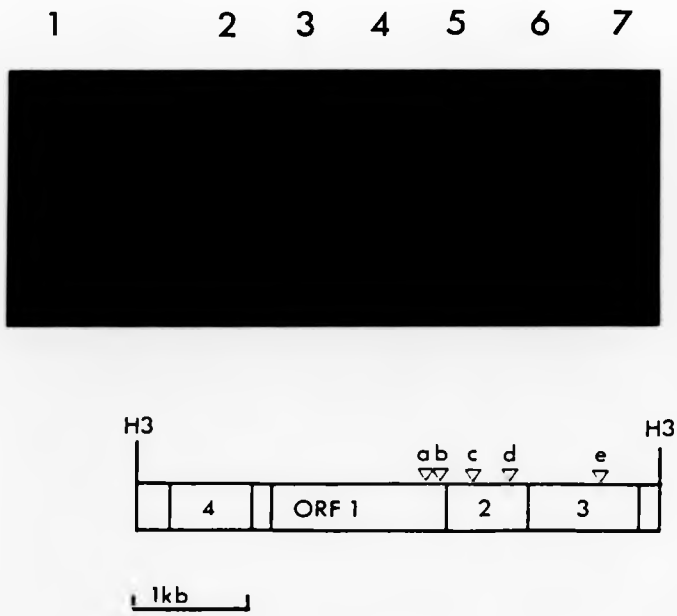


Figure 5:2:3 Agarose gel electrophoresis of the Tn*1000*-insertion plasmid pDB95 and a diagram to indicate the relative position of insertion 95 in the 4.5 kb fragment.

Samples were run on a 0.6% (w/v) agarose gel. Tracks 1 and 8 = λ digested with *Hin*dIII; tracks 2-7 = plasmids pDB1, pDB92, pDB65, pDB38, pDB63 and pDB95 respectively, digested with *Sal*I. See section 4:2:2 for details of restriction enzyme mapping.

a = insertion 92; b = 65; c = 38; d = 63 and e = 95. The orientation of insertion 95 is γ to δ relative to the 4.5 kb *Hin*dIII fragment (0-4.5 kb).



resultant Ap^r colonies tested for temperature-sensitivity. The results are summarised in table 5:2:2, and show that pDB95 only complemented the mutations carried by strains MFT1181, TOE22 and TC35. These results reflect those obtained for plasmid pDB1Δ5 and in both cases, the plasmid carries an inactivated ORF3 but leaves ORF2 intact.

5:3 Discussion

5:3:1 Deletion studies

Results presented in this chapter showed that inactivation of ORF3, by transposon insertion or by partial deletion of the gene, led to loss of complementation of only two of the cell division mutants, namely JS10 and OV32. Deletion of both ORF3 and ORF2, led to loss of complementation of all five mutants. Therefore it is postulated that strains MFT1181, TOE22 and TC35 are defective in ORF2, since they are no longer complemented by pDB1Δ7 (section 5:2:2). The results discussed in chapter 4 suggested that the cell division gene *ftsE*, as defined by MFT1181 (Ricard & Hirota, 1973), could be ORF2 or ORF3, but results presented here indicate that ORF2 is in fact *ftsE*.

Although the failure of plasmid pDB1Δ5 to complement JS10 and OV32 was surprising, these results can be explained if it is proposed that these strains carry mutations in ORF3. Consequently inactivation of ORF3 would affect complementation of only these two strains but not of the mutants defective in ORF2.

The strain JS10 was reported to carry an *ftsE* mutation, on the basis of Hfr interrupted-mating experiments (Sturgeon & Ingram, 1978), and the

observed phenotypic similarities with MFT1181. Salmond and Plakidou (1984), presented results of *cis-trans* tests involving the λpGS22 derivatives, λpSP22 and λpSP35, carrying the *ftsE* alleles from TOE22 and TC35 respectively. These results indicated that MFT1181, TOE22, TC35 and JS10 were all defective in the same gene, *ftsE*.

However, close inspection of the data presented by Plakidou (1984) revealed that the complementation result recorded by Salmond and Plakidou (1984), was based on the microscopic examination of the λpSP22 and λpSP35 lysogens. The microscopy revealed a degree of filamentation not observed when JS10 was lysogenised with λpGS22. Thus the absence of complementation of JS10 by ORF2 alleles could be explained by the "leaky" complementation and the requirement for an intermediate temperature which is restrictive for both low and high temperature-sensitive strains at which complementation tests can be performed (Sturgeon & Ingram, 1978). Since JS10 has a conditional cell division phenotype like the *ftsE* mutants, and is defective in ORF3, the gene is designated *ftsX*.

Unfortunately it is not possible to draw the same conclusion for OV32, since the mutation carried by this strain is a nonsense mutation. Although the data suggest that OV32 is also defective in *ftsX*, the same result could be obtained if the nonsense mutation was found in ORFs 1, 2 or 3, should the mutation exhibit polarity.

5:3:2 The strategy for isolation of pDB95

The isolation and subsequent characterisation of plasmid pDB95, further substantiated the hypothesis that JS10 carried a mutation in ORF3. If JS10 was defective in ORF3 then a transposon insertion in ORF3 would

only affect complementation of JS10, and not that of ORF2 mutants. Complementation tests using plasmid pDB95, which carries such an insertion, produced exactly this result (section 5:2:4).

The possibility that ORF3 was *ftsE* was discussed in chapter 4, and several explanations were presented as to why insertions in ORF3 were not obtained. However the finding that ORF2 was in fact *ftsE* clarifies the situation and explains why only insertions in ORF1 and ORF2 were obtained. The original experiment to isolate Tn*1000* insertions in the *ftsE* gene (section 4:2:1) was performed in strain TOE22. Since plasmids with insertions were screened for loss of complementation of the TOE22 mutation, only insertions which inactivated *ftsE* were obtained in this way. These insertions were located within ORF1 and 2. Insertions in ORF3 were not isolated since such an insertion does not appear to affect expression of the upstream *ftsE* (section 5:2:4).

Several attempts at the isolation of ORF3 insertions were made before pDB95 was successfully isolated using the JS10 complementation assay. Problems were encountered when screening for temperature-sensitivity due to transposon insertion in pDB1. This plasmid was known to complement the lesion carried by JS10, since the presence of this plasmid in the strain allowed colony formation at the restrictive temperature (section 3:2:2). However, during the mutagenesis experiment, it was found that the degree of filamentation observed in the potential insertion mutants, was very variable (see also figure 3:3:2). As a result, out of several plasmids isolated, only one, pDB95 was further characterised.

The problems discussed here could be the result of the psychrosensitive nature of the JS10 mutation and also, perhaps, the difficulty

experienced in handling the strain successfully. When manipulating this strain, care was taken to avoid over-exposure to room temperature (25°C) since the strain filaments at this temperature.

5:3:3 Conclusions

In summary, several conclusions can be drawn from these results. Firstly, strains MFT1181, TOE22 and TC35 are all defective in ORF2, which is therefore designated *ftsE*. Secondly, strain JS10 is defective in ORF3 and this is designated *ftsX* in keeping with current nomenclature. Finally, the observation that insertions in all three ORFs show polarity implies that all three genes are co-transcribed or at least contain sequences necessary for maximal expression of the third gene *ftsX*.

In order to investigate the relationships between these genes more accurately, it would be necessary to determine the messenger RNA transcripts from this region (Berk & Sharp, 1977), but these findings suggest a transcriptional unit of three genes. This supports the idea, initially discussed in chapter 3, that these cell division genes constitute an operon.

CHAPTER 6

The identification of the *ftsE* and *ftsX* gene products

6:1 Introduction

6:1:1 Choice of *in vivo* gene expression system

In order to identify the polypeptides encoded by the 4.5 kb fragment it was necessary to express the genes located on plasmids pDB1 and/or pDG1 in an *E. coli* gene expression system. The two *in vivo* gene expression systems applicable to recombinant plasmids, in common use, are minicells (Clarke-Curtiss & Curtiss, 1983) and maxicells (Sancar *et al.*, 1979). The uses and relative merits of these systems are discussed by Stoker *et al.*, (1984).

The maxicell system was chosen because it is versatile, amenable to use with large numbers of samples, and with careful handling can produce very good results. In this way the pDB1-derivatives described in chapters 4 and 5 could be used to unequivocally identify the gene products encoded by the operon.

The use of maxicells, relies on the deficiency in DNA repair observed in UV-sensitive strains, such as those which carry a *recA* mutation. The *recA* strain carrying the plasmid of interest, is UV-irradiated and incubated overnight to allow the breakdown of UV-damaged chromosomal DNA, whilst leaving undamaged plasmid DNA molecules intact. Plasmid-coded proteins can then be preferentially labelled with ^{35}S-methionine and samples analysed using SDS-PAGE. The *recA* strain used routinely throughout this study was CSH26ΔF6, but other *recA* strains were also used where the need arose, for example strains also carrying the suppressor *supF* (chapter 8).

6:1:2 Predicted proteins encoded by pDB1

The 4.5 kb sequence, analysed using the computer programme ANALYSEQ (Staden, 1984), gave the predicted molecular weights of the deduced proteins as 48117 (ORF1); 24439 (ORF2); 38543 (ORF3); and 21677 (ORF4). Thus, when plasmid pDB1/pDG1 was studied using the maxicell system, proteins of these relative molecular weights were anticipated, as well as the gene products encoded by the vector DNA. Sancar *et al.* (1979), identified the proteins produced by vector pBR322 and showed these to include the Mr 37000 protein of the Tc-resistance gene and the two Ap-resistance gene products, of Mr 28000 and Mr 30000. These two products correspond to the mature and precursor forms of β-lactamase respectively (Clement *et al.*, 1982), although usually only one polypeptide band is observed (Stoker *et al.*, 1984). Wherever plasmid pBR325 was used as vector, as in the case of pDG1, then a protein of Mr $\simeq$ 23000 was also anticipated, corresponding to the Cm-resistance gene product (Stoker *et al.*, 1983). Using this information, the radiolabelled protein products from maxicells containing the various plasmids were analysed.

6:2 Results

6:2:1 Maxicells of pDB1 and pDG1

The maxicell strain CSH26ΔF6 was transformed with plasmids pDB1 and pDG1, and using the maxicell protocol (section 2:22), radioactive protein samples were obtained for each. The preparations were then analysed using SDS-PAGE (section 2:23). Two gel systems were employed: the 10-30% (w/v) gradient polyacrylamide gel was used to obtain good resolution of polypeptides over a wide range of molecular weights; and the 11% (w/v) linear gel system was used to obtain optimum separation of

polypeptides in the range of Mr 20000 - Mr 40000.

Figures 6:2:1 (a) & (b) show autoradiograms of ^{35}S-methionine labelled samples of pDB1, pDG1 and pBR322 maxicells electrophoresed on both types of gel. Figure 6:2:1(a) indicates the vector-encoded proteins, as described in section 6:1:2, and the polypeptides encoded by the 4.5 kb fragment. Several of these correspond extremely well to the predicted molecular weights of the deduced proteins. The Mr 20000 polypeptide is a candidate for the ORF4 product; the Mr 25000 polypeptide for the ORF2 product; and the Mr 38000/36000 polypeptides are possible candidates for the ORF3 product. There appears to be no polypeptide band corresponding to the predicted molecular weight of the ORF1 protein, and an unassigned polypeptide of Mr ≃ 92000 is also observed.

Figure 6:2:1(b) shows an autoradiogram of pDG1 maxicell samples electrophoresed on an 11% (w/v) linear gel, and polypeptides of similar relative mobility are seen. Small differences observed are probably due to slight variations between the gel electrophoresis systems used. One obvious difference however, is that there is only one ORF3 candidate protein (Mr 38000) observed.

The Mr 37000 protein corresponding to the *tet* gene product (Sancar *et al.*, 1979) is observed using both gel systems, although the *tet* gene was inactivated during the construction of plasmids pDG1 and pDB1 (sections 3:2:1 and 4:2:1 respectively). The *Hin*dIII site of pBR322 (position 29) is found within the promoter region of the *tet* gene (Bolivar, 1977) and thus the *tet* gene product is sometimes observed, in spite of insertion at this site resulting in Tc-sensitivity (C. Jones, pers. comm.). The DNA sequence of the *tet* gene (Sutcliffe, 1979) has since been revised

Figure 6:2:1(a) Autoradiogram of ^{35}S-methionine labelled maxicell samples of pBR322 and pDB1.

Samples were analysed by SDS-PAGE using a 10-30% (w/v) gradient gel. Track 1 = pBR322; track 2 = pDB1.

Figure 6:2:1(a) Autoradiogram of ^{35}S-methionine labelled maxicell samples of pBR322 and pDB1.

Samples were analysed by SDS-PAGE using a 10-30% (w/v) gradient gel.
Track 1 = pBR322; track 2 = pDB1.

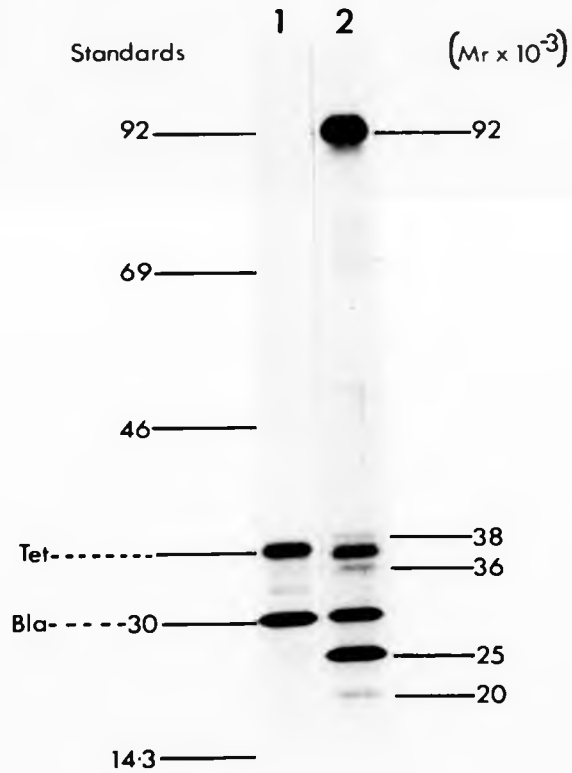


Figure 6:2:1(b) Autoradiogram of ^{35}S-methionine labelled maxicell sample of pDG1.

The sample was analysed by SDS-PAGE using an 11% (w/v) linear gel and subsequent fluorography.

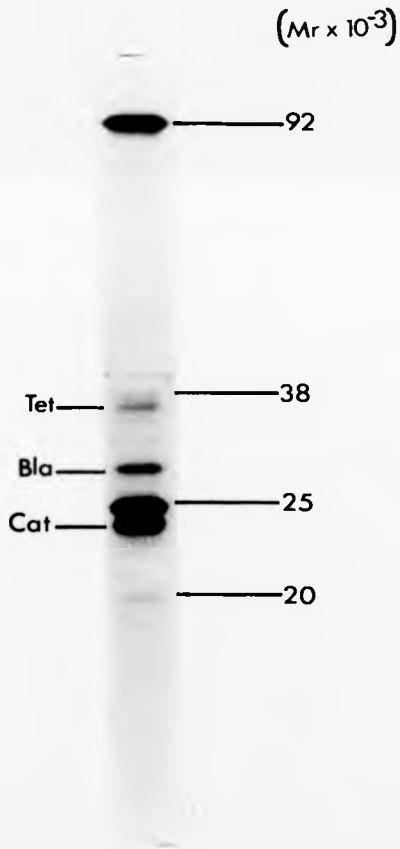
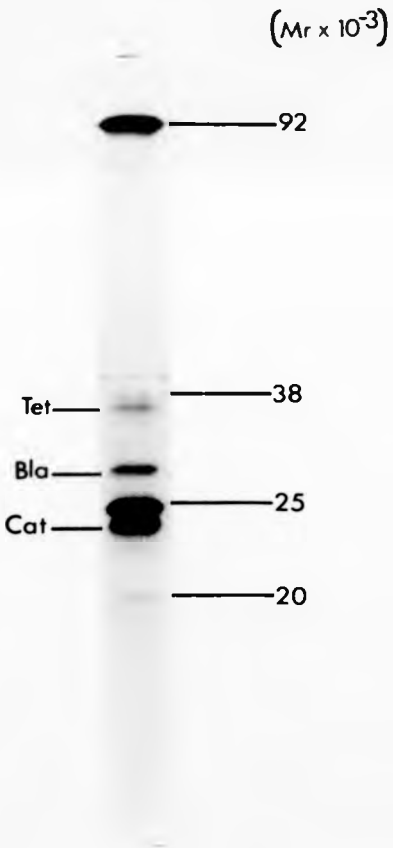


Figure 6:2:1(b) Autoradiogram of ^{35}S-methionine labelled maxicell sample of pDG1.

The sample was analysed by SDS-PAGE using an 11% (w/v) linear gel and subsequent fluorography.



(Peden, 1983), resulting in a change of reading frame within the gene, and predicts that the *tet* gene product should be of Mr 41000. These reports on the variable size of the *tet* gene product may be a consequence of the hydrophobicity of this protein affecting binding of SDS (Beyreuther *et al.*, 1980; Padan *et al.*, 1983).

6:2:2 Maxicells of Tn*1000*-insertion, and deletion-derivatives of pDB1

Proteins coded by the five Tn*1000* insertion plasmids: pDB38, pDB63, pDB65, pDB92 and pDB95, and the two deletion derivatives pDB1Δ5 and pDB1Δ7, were all labelled with ^{35}S-methionine using the maxicell procedure. Figure 6:2:2 shows the gene products which are observed after SDS-PAGE and subsequent autoradiography.

The position of the transposon insertions in the Tn*1000*-carrying plasmids can be correlated with the appearance and disappearance of the candidate proteins. It can be seen that the Mr 20000 candidate ORF4 protein remains unaffected, as would be predicted from the Tn*1000* insertion data. Insertions 92 and 65, which were mapped to the 3'-end of ORF1, prevent the synthesis of the candidate polypeptide for the ORF3 product (Mr 38000) and the candidate ORF2 product (Mr 25000), as does insertion 38 which was mapped to the 5' region of ORF2.

Insertion 63 however, also abolishes ORF2 and ORF3 candidate products but causes the synthesis of a novel polypeptide of Mr 23000. Considering the position of insertion 63 (located in the 3' region of ORF2), this novel polypeptide is presumed to be a truncated form of the Mr 25000, ORF2 product. Insertion 95, which was mapped to the 3'-end of ORF3, does not affect the synthesis of ORF2 but prevents the synthesis

Figure 6:2:2 Autoradiogram of ^{35}S-methionine labelled maxicell samples of pBR322, pDB1, pDB92, pDB65, pDB38, pDB95, pDB1Δ5 and pDB1Δ7.

Samples were analysed by SDS-PAGE using an 11% (w/v) linear gel and subsequent fluorography. Track 1 = pBR322; track 2 = pDB1; track 3 = pDB92; track 4 = pDB65; track 5 = pDB38; track 6 = pDB63; track 7 = pDB95; track 8 = pDB1Δ5, track 9 = pDB1Δ7.

The polypeptide of Mr 38000 (candidate ORF3 product) is not visible in track 2 as expected, although track 7 clearly shows the presence of a truncated version of this protein.

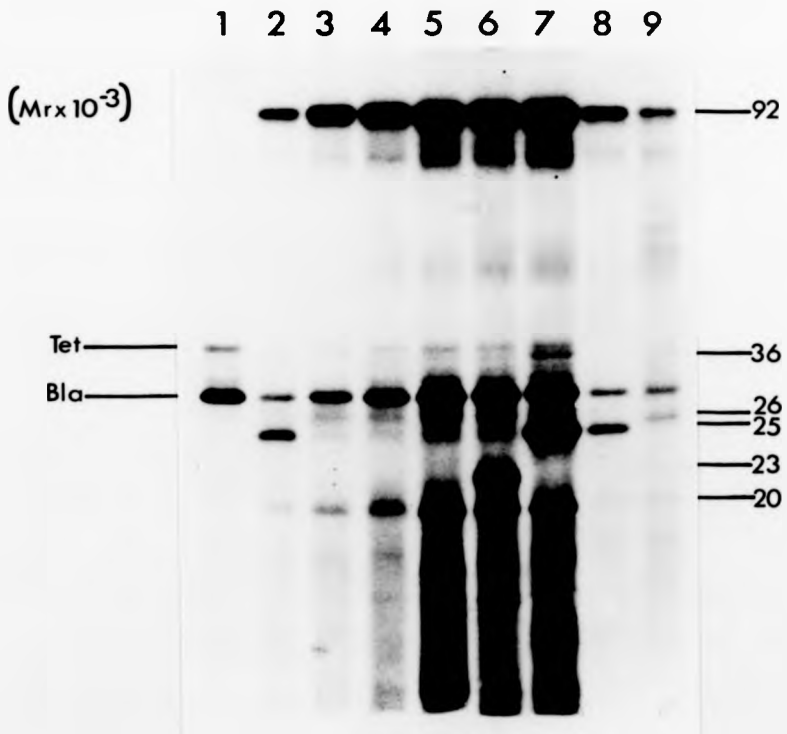
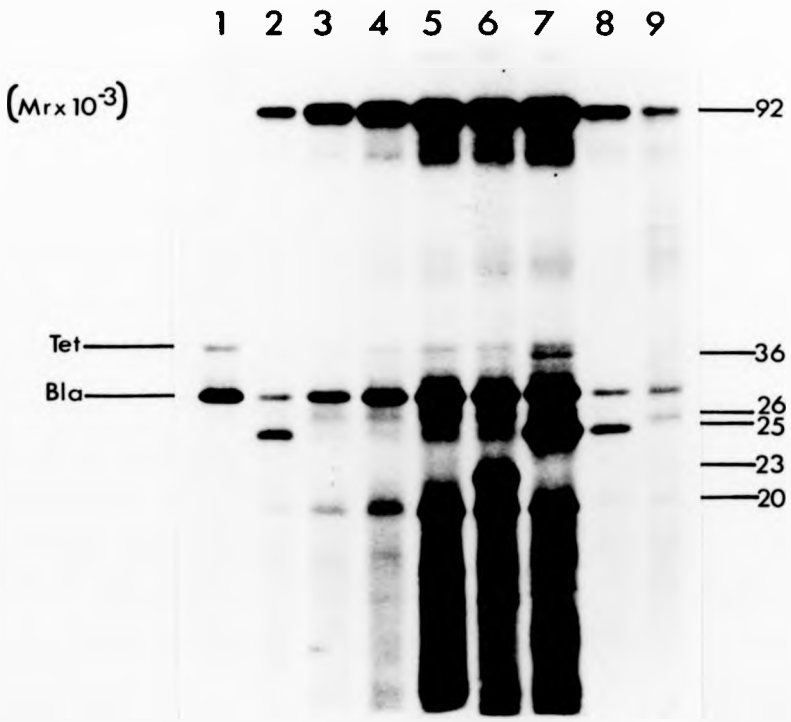


Figure 6:2:2 Autoradiogram of ^{35}S-methionine labelled maxicell samples of pBR322, pDB1, pDB92, pDB65, pDB38, pDB95, pDB1Δ5 and pDB1Δ7.

Samples were analysed by SDS-PAGE using an 11% (w/v) linear gel and subsequent fluorography. Track 1 = pBR322; track 2 = pDB1; track 3 = pDB92; track 4 = pDB65; track 5 = pDB38; track 6 = pDB63; track 7 = pDB95; track 8 = pDB1Δ5, track 9 = pDB1Δ7.

The polypeptide of Mr 38000 (candidate ORF3 product) is not visible in track 2 as expected, although track 7 clearly shows the presence of a truncated version of this protein.



of the ORF3 candidate product as expected. However, a polypeptide of Mr 36000 is observed and it is possible that this is a truncated version of the ORF3 product. Since no ORF1 candidate protein is visible in the autoradiograms shown in figures 6:2:1 and 6:2:2, it is impossible to interpret the effects of Tn*1000* insertion on expression of ORF1. It was noted however that the polypeptide of Mr 92000, like the ORF4 candidate product, remained unaffected by the transposon insertions.

Figure 6:2:2 also shows the results of maxicell experiments on plasmids pDB1Δ5 and pDB1Δ7. The former plasmid, in which the 3' end of ORF3 has been deleted, still produces the ORF2 candidate product, but fails to make both the ORF3 candidate product and the polypeptide corresponding to the *tet* gene product. Similarly, in plasmid pDB1Δ7, deletion of ORF3 and the 3' end of ORF2 leads to loss of both ORF2 and ORF3 candidate products but causes the synthesis of a new polypeptide of Mr 26000. It is presumed that this polypeptide is the result of an in-frame fusion between ORF2 and the *tet* gene as described in section 6:3:1.

6:2:3 The search for the ORF1 product

In an attempt to identify the ORF1 product, several measures were taken to alter the electrophoresis system, and to change the *in vivo* labelling process using methods successfully used to identify other proteins.

Firstly, autoradiograms were overexposed and maxicell samples electrophoresed through polyacrylamide gels of varying concentrations (10-15% (w/v)) but neither procedure revealed an ORF1 candidate protein. It is known that some hydrophobic proteins may aggregate under the electrophoresis conditions used, so that they fail to enter the separating gel (Stoker *et al.*, 1984). Although the protein predicted by

the ORF1 DNA sequence is not hydrophobic (G. Salmond, pers. comm. & Appendix III), several steps were taken to alter the conditions of SDS-solubilisation in the maxicell procedure. The SDS-sample buffer used to lyse the cells was varied in terms of the concentration of SDS (up to 5% w/v) in the presence or absence of β-mercaptoethanol. Also the temperature and duration of the solubilisation step was varied. Maxicells were solubilised at temperatures of 25°C, 50°C, 75°C, and 100°C for various time intervals (2 min to 30 min). The temperature of 37°C was also used for solubilisation, since there are examples of proteins visible after solubilisation at this temperature, which are not visible after boiling. Such proteins include the lactose permease protein (Teather *et al.*, 1978), the glycerol phosphate permease (Larson *et al.*, 1982), and the *rodA* gene product (Stoker *et al.*, 1983). These proteins are all located in the cytoplasmic membrane and the failure to detect these proteins is due to protein aggregation upon boiling in SDS solution (Teather *et al.*, 1978; Stoker *et al.*, 1983). None of these steps however, revealed a possible ORF1 product.

Apart from changing conditions which may affect the electrophoresis of the samples, alterations were also made to the conditions under which the plasmid-coded proteins were labelled, since these may also affect the stability of the ORF1 product. For example, the protein may be very unstable, being degraded before it can be detected by SDS-PAGE (Stoker *et al.*, 1984). To reduce this possibility, protease inhibitors such as tosyl-L-lysine chloromethylketone (TLCK) and phenylmethylsulphonyl-fluoride (PMSF) were added to the maxicell samples at various stages after labelling, but prior to cell lysis. The protease inhibitors were used at a final concentration of 3 mM and 1 mM respectively. However samples treated in this way, again failed to reveal a candidate ORF1

product (data not shown). Although protease inhibitors such as these are very specific, such measures were included in the successful identification of the *secY* (*prlA*) gene product (Ito, 1984). Unfortunately, a combination of several procedures, some of which are discussed in this section, were used by Ito, and it is not known exactly which of the procedures was responsible for the appearance of the SecY protein.

Maxicells were also labelled with ^{35}S-methionine for short periods of time only (5 mins or less), so that detection of the ORF1 product was not obscured by the continued synthesis of more stable proteins, but this appeared to have no effect (data not shown).

Finally, the Zubay *in vitro*, transcription-translation system was used (section 2:29) to programme the synthesis of polypeptides from pDB1, because often, peptides which are unstable *in vivo* appear to be more stable in this system (Pratt, 1984). No ORF1 candidate protein was observed (figure 6:2:3), but again the presence of the Mr 92000 protein was noted.

6:3 Discussion

6:3:1 Identification of the *ftsE* gene product

Evidence presented in chapters 4 and 5 showed that ORF2 was *ftsE*; and the DNA sequence predicted that the deduced ORF2 product was a polypeptide of Mr 24439. A polypeptide of this relative mobility was indeed observed when maxicells of pDB1/pDG1 were investigated (section 6:2:1). The results obtained using the Tn*1000* insertion plasmids also

Figure 6:2:3 Autoradiogram of ^{35}S-methionine labelled proteins synthesised from plasmid pDG1 using the Zubay *in vitro* transcription-translation system.

Zubay samples were analysed by SDS-PAGE using an 11% (w/v) linear gel. Track 1 = standards; track 2 = no added DNA; track 3 = plasmid pDG1 DNA. The polypeptide of Mr 72000 is often observed in Zubay samples (C. Jones, pers. comm.).

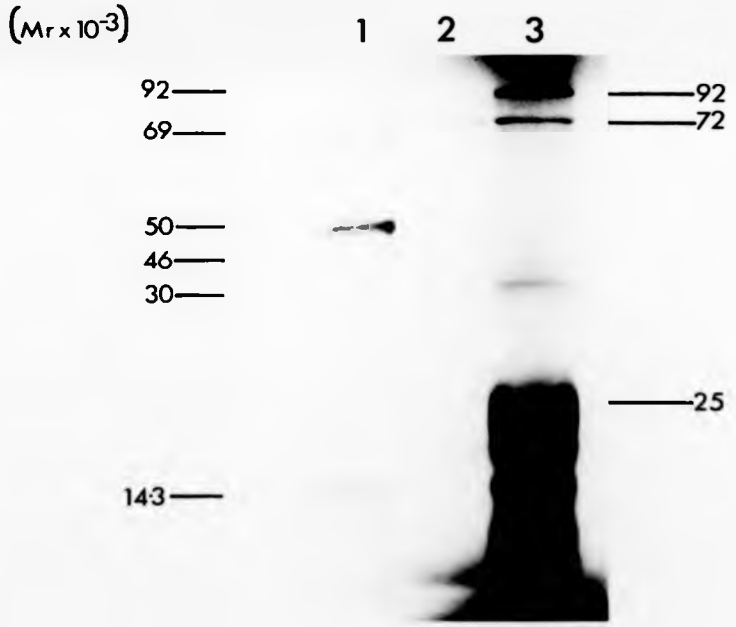


Figure 6:2:3 Autoradiogram of ^{35}S-methionine labelled proteins synthesised from plasmid pDG1 using the Zubay *in vitro* transcription-translation system.

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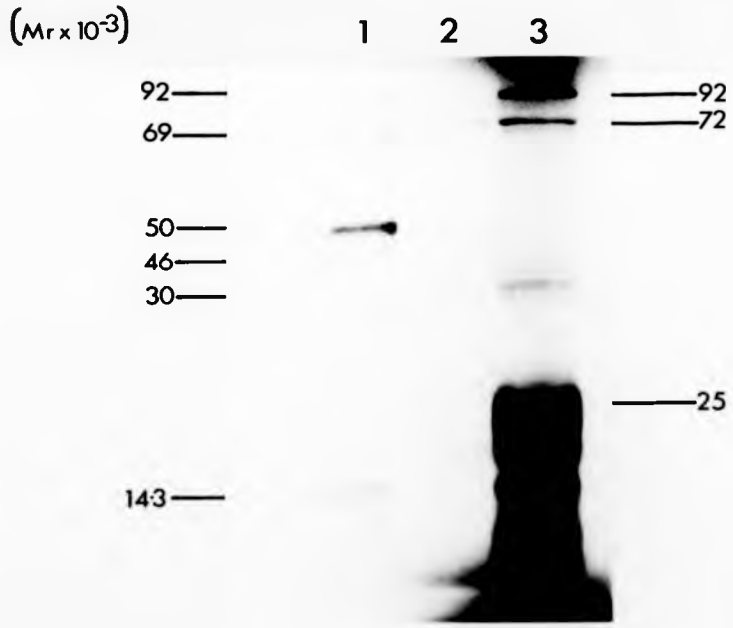


suggest that this polypeptide is the ORF2 product. The insertions 92, 65 and 38 were shown to inactivate the *ftsE* gene and thus it would be expected that the *ftsE* gene product would not be expressed from plasmids carrying these insertions. The results described in section 6:2:2 confirm this prediction. Perhaps the most convincing evidence that the Mr 25000 polypeptide observed is in fact FtsE, is that insertion 63 (which maps at the 3'-end of ORF2), produces a truncated version of this protein, (Mr 23000).

Finally, the deletion plasmid pDB1Δ7 was shown to produce a protein of Mr 26000, slightly increased in molecular weight over that of the ORF2 product. This was surprising because during the construction of pDB1Δ7 (section 5:2:1), the 3' end of ORF2 was removed using the *Sph*I restriction site at position 3260, and thus the resultant protein, if stable, should have shown a decrease in relative mobility. However, close inspection of the sequences involved in this construction, revealed that a fusion protein of Mr 26000 could be produced from this plasmid, as a result of an in-frame fusion between the 3' end of ORF2 and the *tet* gene. The complete nucleotide sequence of the *tet* gene was recently revised (Peden, 1983) and this was used to show how the fusion protein is generated (Figure 6:3:1). Therefore, in view of the evidence presented in this chapter, the polypeptide of Mr 25000 is identified as the *ftsE* gene product.

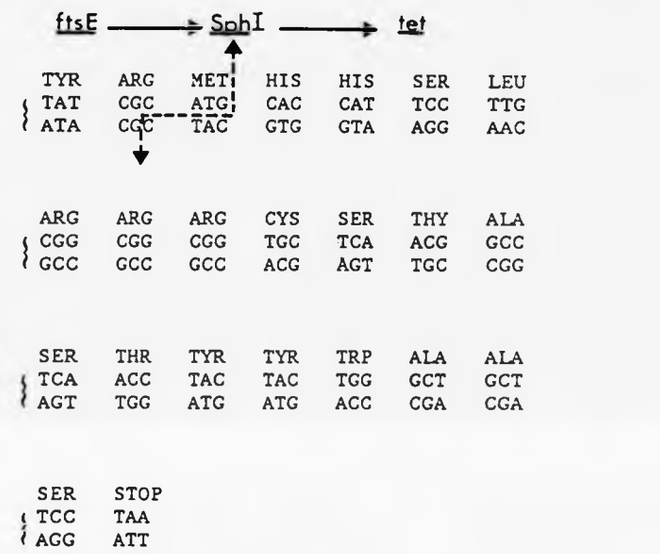
6:3:2 Identification of the *ftsX* gene product

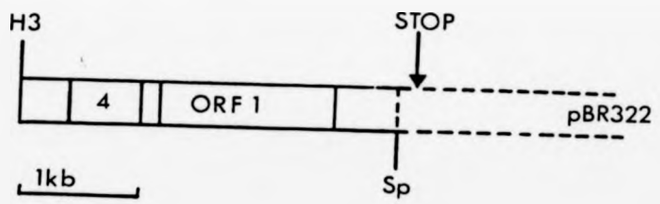
Data discussed in chapter 5 showed that ORF3 was a novel cell division gene, *ftsX*. Maxicell experiments revealed two proteins of Mr 38000 and Mr 36000, either of which could be the ORF3 product of predicted molecular weight 38543. The Mr 36000 protein does not always appear in

Figure 6:3:1 Diagram to indicate the way in which the putative ORF2 fusion protein could be generated.

In plasmid pDB1Δ7, ORF2 and the *tet* gene of pBR322 are joined at the *Sph*I site (see section 5:2:3). The reading frame continues into the *tet* gene but terminates after 20 codons, as indicated.

The predicted fusion protein has an Mr of 25365 which is greater than that of the ORF2 product (Mr 24439). When analysed using SDS-PAGE the polypeptides are observed to be of Mr 26000 and Mr 25000 respectively (figure 6:2:2).





the maxicell experiments. One possibility is that the two polypeptides represent precursor and processed forms of ORF3 protein, but this is unlikely because inspection of the protein sequence (Appendix I) shows no signal sequence (Michaelis & Beckwith, 1982). It is possible however that the Mr 36000 polypeptide is a preferred breakdown product of the Mr 38000 protein. Thus the Mr 38000 protein is believed to be the ORF3 product. The results obtained from maxicells of the Tn*1000* insertion plasmids support this hypothesis since all the insertions which inactivate *ftsX* gene expression also cause the disappearance of this protein. Insertion 95 which is located at the 3′ end of ORF3 causes the production of a protein of Mr 36000, which could represent a truncated version of the Mr 38000 protein.

Plasmid pDB1Δ5 also fails to produce the ORF3 presumptive protein, and the *tet* gene product. This was anticipated because the plasmid no longer complemented *ftsX* mutations (section 5:2:2), and during its construction, the 3′ end of ORF3 and the 5′ end of the *tet* gene, were removed. No truncated ORF3 product was observed and it is therefore presumed that such a fusion protein is unstable or comigrates with a protein of a lower relative mobility. Thus from the evidence presented here, the polypeptide of MR 38000 is identified as FtsX.

6:3:3 Coordinate expression of *ftsE* and *ftsX*

Tn*1000* insertions into ORF1 or ORF2 showed polar effects on the expression of ORF3 as measured by complementation of the *ftsX* mutation carried by strain JS10 (section 4:2:3). These findings are supported by the results of maxicell experiments presented in this chapter, which showed that the *ftsX* gene product was not observed unless the *ftsE* gene product was also produced. These data suggest that *ftsE* and *ftsX*

constitute a single transcriptional unit.

6:3:4 The possible identity of the Mr 92000 polypeptide

In spite of the measures taken to modify the maxicell procedure and to alter the conditions of gel electrophoresis, a candidate ORF1 product was not found. Only two other polypeptides besides the identified FtsE and FtsX were observed. These included the Mr 20000 polypeptide presumed to be the ORF4 product and the unidentified polypeptide of relative mobility 92000-98000. The DNA sequence of the 4.5 kb fragment (Appendix I) did not predict a protein of this molecular weight and it is difficult to see where, on the plasmid, such a large protein could be encoded.

One possibility was that the protein was the result of a fusion between one of the ends of the 4.5 kb fragment and the *tet* gene. However this was precluded by the construction of a plasmid pDB2, in which the 4.5 kb fragment was inserted in the opposite orientation to pDB1. Maxicells containing pDB2 also revealed the Mr 92000 protein.

Variations in the labelling and PAGE analysis of maxicell-coded proteins (described in section 6:2:3) did not affect the relative mobility of this large protein. Consequently, this protein could not be the result of an ionic association between two or more polypeptide molecules; nor could it be the result of interactions involving disulphide bridges. Interactions of an ionic nature, or consisting of cysteine-cysteine bonding, are destroyed by boiling in SDS and β-mercaptoethanol respectively (Hames & Rickwood, 1981). Such an interaction could however be covalent in nature. The identification of the ORF1 product and the origin of the Mr 92000 protein are described in chapter 7.

CHAPTER 7

Identification of the ORF1 gene product

7:1 Introduction

The results of maxicell labelling experiments presented in chapter 6, identified the gene products encoded by the 4.5 kb *Hin*dIII fragment. The observed gene products were correlated with the predicted proteins deduced from the sequence data (chapter 6). The sequence indicated the position of an ORF running from 1147 to 2460 which could encode a polypeptide of Mr 48117, and although the other proteins predicted by the sequence were observed no candidate ORF1 product was identified.

Moreover, a polypeptide of Mr 92000 (approximately) was consistently produced from the 4.5 kb fragment, but its source remains unknown. One possibility which would explain both observations, would be that the Mr 92000 protein was somehow encoded by ORF1. Although Tn*1000* insertions in ORF1 have already been isolated as described in chapter 4, both insertions carried by plasmids pDB92 and pDB65, were located at the 3' end of ORF1. It was therefore considered important to obtain an insertion which was more promoter-proximal within ORF1, in an attempt to identify the origin of the Mr 92000 protein. In order to achieve this, two approaches were taken.

Firstly, attempts were made to obtain a Tn*1000* insertion which caused the disappearance of the Mr 92000 protein. Such an insertion mutant would then be characterised and the location of the Tn*1000* element within pDB1 ascertained. This would identify the origin of the unidentified protein. This experiment involved the isolation of large numbers of Tn*1000* insertions in pDB1 and the subsequent screening of these isolates for loss of the Mr 92000 protein in maxicells.

The second approach involved the construction of an ORF1 insertion mutation *in vitro*. A suitable restriction site within ORF1 was selected and a DNA fragment inserted at that site in an attempt to stop transcription and translation of ORF1. The advantage of this approach is that the exact point of insertion within ORF1 is known. The resultant plasmid would then be analysed in maxicells to investigate the effect of the insertion on the gene product profile.

The DNA fragment inserted into ORF1 was the so-called "interposon" or "omega (Ω) fragment" as described by Prentki and Krisch (1984). This fragment was used because it contains well-defined transcription/translation stop signals, it has several defined restriction sites and it has the added advantage of a selectable phenotype. The interposon carries genes for spectinomycin-resistance (Sp) and streptomycin-resistance (Sm) and thus the insertion event can be positively selected.

7.2 Results

7:2:1 Use of maxicells in the identification of the Mr 92000 protein

Strain DW214, an F^+ strain carrying pDB1, was mated with the maxicell strain CSH26ΔF6 (Sm^r) as outlined in section 2:9. Transconjugants which were Sm^rAp^r were presumed to contain pDB1 carrying a Tn*1000* insertion based on the rationale discussed in chapter 3. About 100 such transconjugants were purified, and 60 of these selected for analysis in maxicells. Due to the large numbers of samples involved in this experiment, the routine maxicell-labelling procedure (section 2:22) was modified. Instead of labelling the usual 10 ml cultures, only 1.5 ml cultures were used and all manipulations were performed in 1.5 ml

Eppendorf tubes. Samples were then treated as usual for SDS-PAGE. Figures 7:2:1(a) shows an autoradiogram of 14 such samples. Due to the modifications to the routine method, the background contamination of host cell proteins was generally greater than was usually obtained. However, certain differences between the Tn*1000* samples and the pDB1 control sample could be observed. Several samples, including sample 184 shown in figure 7:2:1(a), were selected for further analysis.

When maxicells of isolate 184 were repeated, the Mr 92000 protein was clearly absent from the protein profile (shown later in figure 7:2:2(c)). Plasmid DNA was prepared from this isolate and restriction mapped as described in detail in section 4:2:2. This plasmid, designated pDB184, was found to contain a single Tn*1000* insertion at position 1400 (1.4 kb) within ORF1. Figure 7:2:1(b) indicates the relative position of this insertion within the operon and shows the DNA restriction fragments produced when pDB184 was digested with the restriction enzyme *Sal*I.

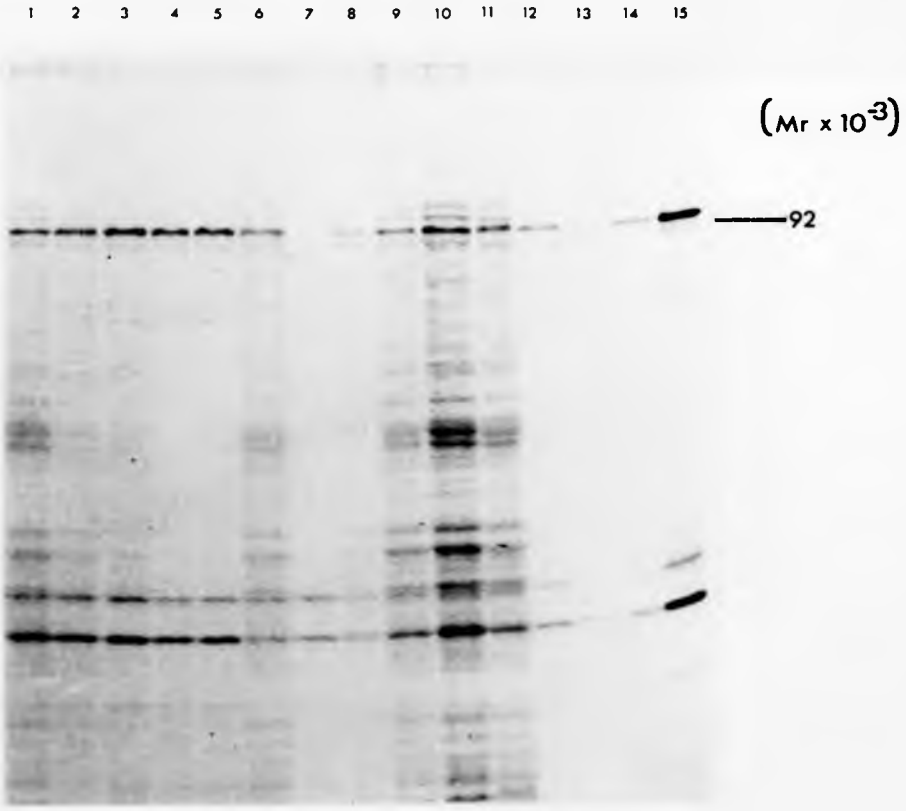
7:2:2 Construction of plasmid pDG1Ω4 and its analysis in maxicells

The major steps in the construction of pDG1Ω4 are presented in figure 7:2:2(a). In order to insert the interposon into ORF1, a suitable restriction site was chosen and this was the *Stu*I site at position 1300 (1.3 kb) (section 3:2:3). The *Stu*I restriction enzyme cuts the DNA to give blunt ends, which was convenient for this construction and this *Stu*I site was located towards the 5' end of ORF1. Thus, plasmid pDG1 was linearised with the restriction enzyme *Stu*I, ready for insertion of the interposon.

The interposon was on a plasmid pHP45Ω (Prentki & Krisch, 1984), which

Figure 7:2:1(a) Autoradiogram of ^{35}S-methionine labelled maxicell samples of random Tn*1000* insertions in pDB1.

Samples were analysed by SDS-PAGE using an 11% (w/v) gel. Tracks 1-14 = isolates 178 to 191 respectively; – track 15 = pDB1. Isolate 184 (track 7) clearly shows the absence of the Mr 92000 polypeptide.



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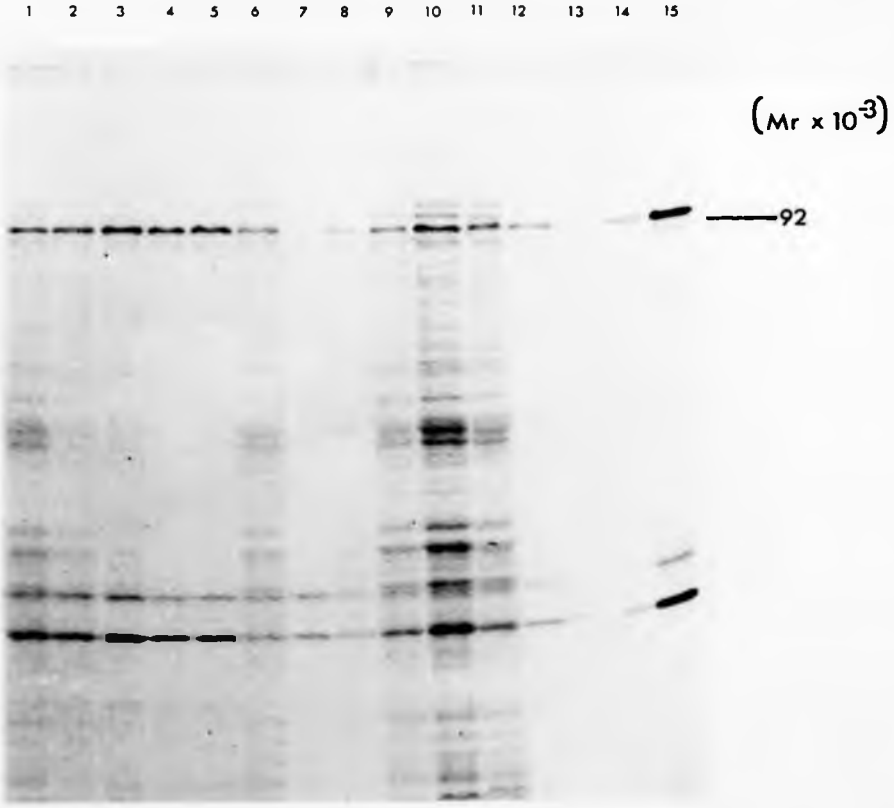
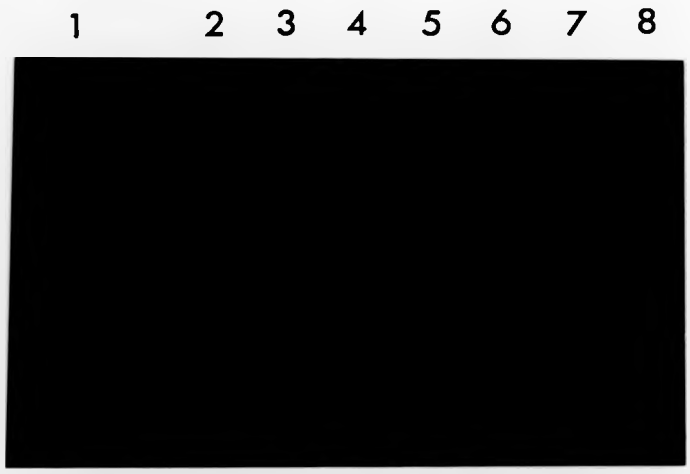


Figure 7:2:1(b) Agarose gel electrophoresis of plasmid pDB184 digested with *Sal*I and a diagram to show the relative position of Tn*1000* insertion 184 within the 4.5 kb *Hin*dIII fragment.

Track 1 = λ digested with *Hin*dIII; tracks 2-8 = plasmids pDB1, pDB184, pDB92, pDB65, pDB38, pDB63 and pDB95 respectively, digested with *Sal*I.

Insertion a = 92; b = 65; c = 38; d = 63; e = 95 and f = 184. Insertion f is oriented γ to δ relative to the 4.5 kb fragment (0 to 4.5 kb).



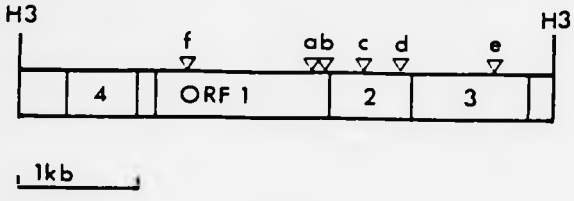
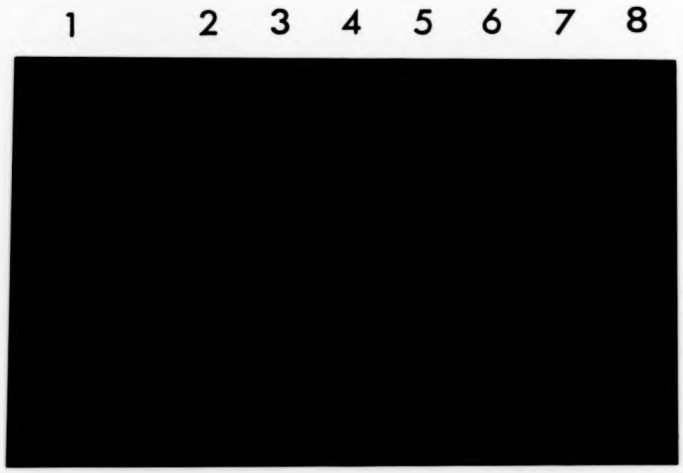


Figure 7:2:1(b) Agarose gel electrophoresis of plasmid pDB184 digested with *Sal*I and a diagram to show the relative position of Tn*1000* insertion 184 within the 4.5 kb *Hin*dIII fragment.

Track 1 = λ digested with *Hin*dIII; tracks 2-8 = plasmids pDB1, pDB184, pDB92, pDB65, pDB38, pDB63 and pDB95 respectively, digested with *Sal*I.

Insertion a = 92; b = 65; c = 38; d = 63; e = 95 and f = 184. Insertion f is oriented γ to δ relative to the 4.5 kb fragment (0 to 4.5 kb).



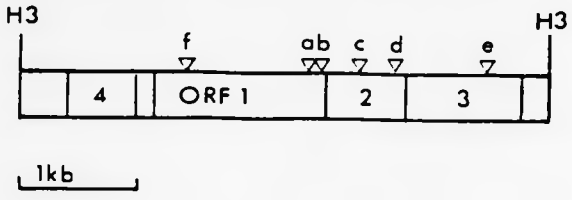
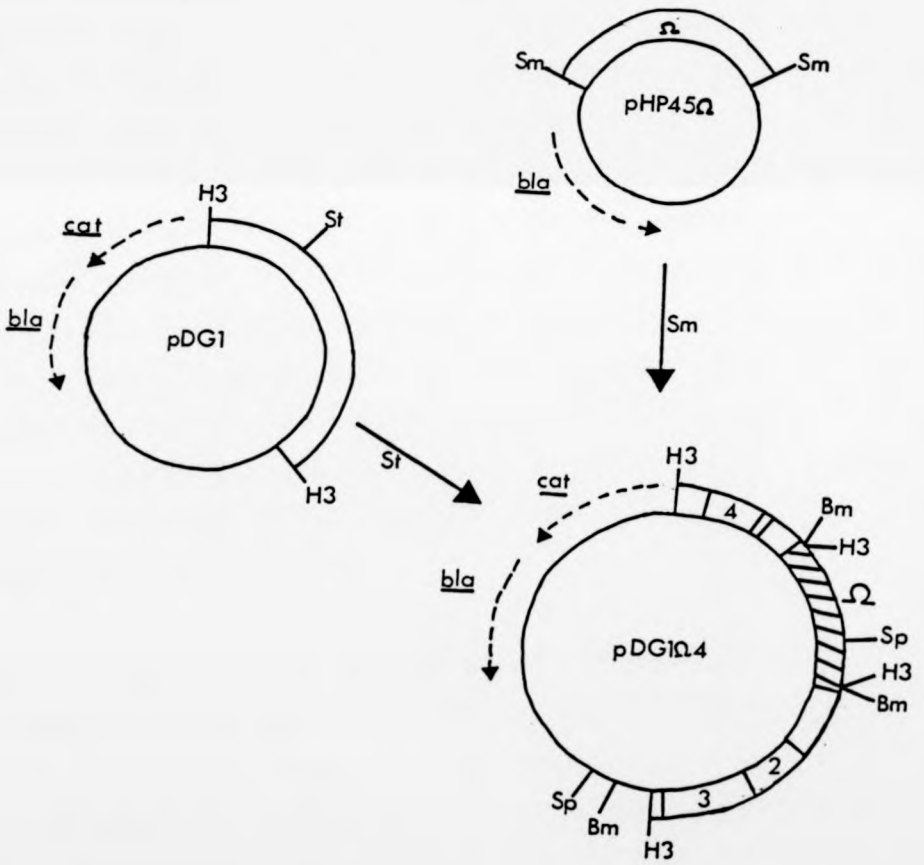


Figure 7:2:2(a) Diagram to indicate the main steps in the construction of plasmid pDG1Ω4.

See text for details.



also carries an antibiotic-resistance gene, for Ap. A restriction map of this plasmid and a diagrammatic representation of the interposon are shown in figure 7:2:2(b). The interposon can be removed from the plasmid by digestion with several restriction enzymes, as indicated. For the purpose of this construction the *Sma*I sites were used because this enzyme also cuts to produce blunt ended fragments, and thus the interposon could be ligated into the *Stu*I site in pDG1, without recourse to further manipulation of the DNA fragments. Therefore, 0.5 μg of *Stu*I digested pDG1, and 2 μg of *Sma*I digested pHP45Ω were ligated together as outlined in section 2:20, and the ligation mix was used to transform the *recA* strain DH1, selecting for Sp^rCm^r colonies.

Five such transformant colonies were obtained and growth on the following antibiotics was tested: Ap (50 μg ml^{-1}), Cm (50 μg ml^{-1}), Sm (20 μg ml^{-1}), Sp (20 μg ml^{-1}), and Tc (10 μg ml^{-1}). Two colonies were found to have the required $Ap^rCm^rSm^rSp^rTc^s$ phenotype and the plasmid from one of these was selected for further study. The plasmid designated pDG1Ω4, was restriction mapped using the restriction enzymes *Hin*dIII, *Eco*RI, *Sph*I and *Bam*HI (section 2:17) and was found to be as predicted (figure 7:2:2(a)).

The plasmid was also used to transform the strain CSH26ΔF6 to Ap-resistance and transformants were used to programme protein synthesis in maxicell experiments. Figure 7:2:2(c) shows an autoradiogram of such a maxicell sample and it can be seen that the Mr 92000 protein is missing when compared with the pDB1 products. As in the case of pDB184 maxicells, the Mr 92000 protein was the only protein which was missing.

Figure 7:2:2(b) Restriction map of plasmid pHP45Ω showing important features of the Ω fragment.

The position of the Ω fragment on plasmid pHP45Ω is indicated. This fragment carries genes for resistance to Sm and Sp, and is flanked by short inverted repeats carrying T4 transcription-termination signals (single line ——), translational stop signals (▨▨▨▨▨▨), and a polylinker (■■■■■) with sites for E1, Sm and Bm.

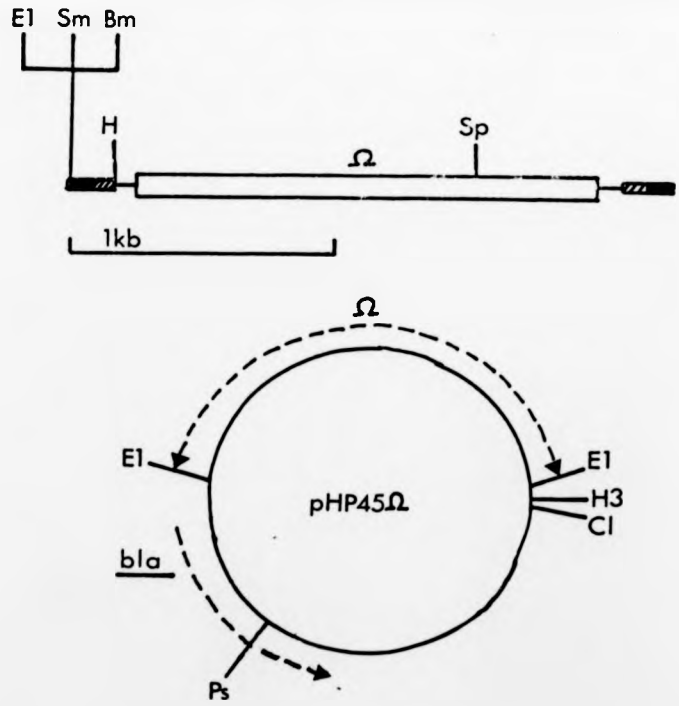


Figure 7:2:2(c) Autoradiogram of ^{35}S-methionine labelled maxicell samples of pBR322, pDB1, pDG1Ω4 and pDB184.

Samples were analysed by SDS-PAGE using an 11% (w/v) linear gel. Track 1 = pBR322; track 2 = pDB1, track 3 = pDG1Ω4; track 4 = pDB184. The position of the Mr 92000 polypeptide is indicated and this protein is absent from tracks 3 and 4. A longer exposure of this autoradiogram is also shown and the ORF3 and *tet* gene products are just visible in track 4.

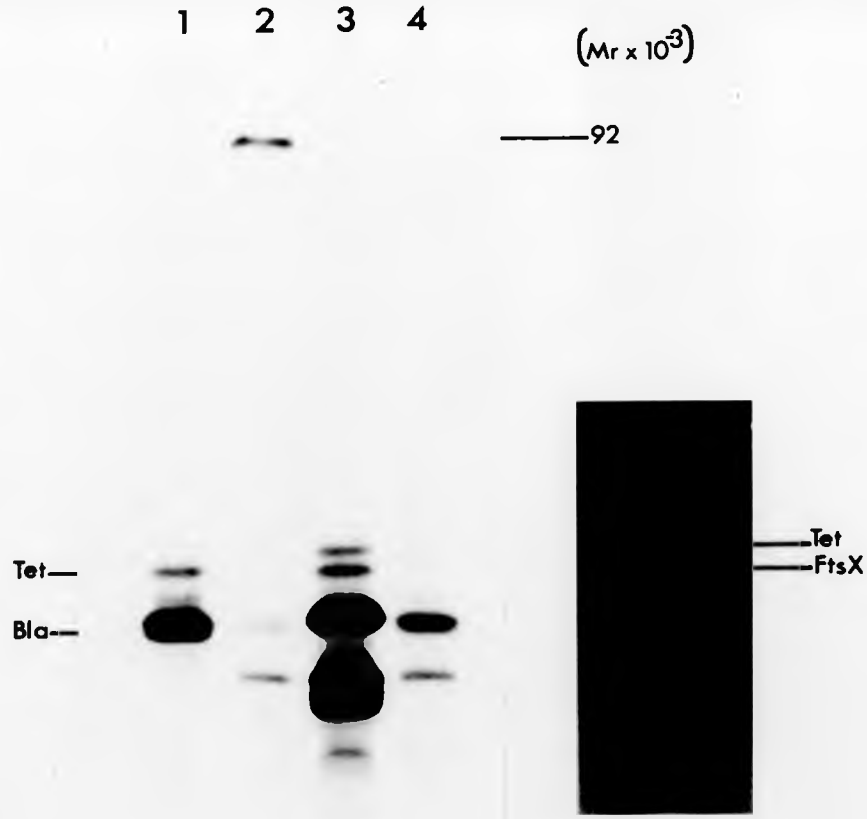
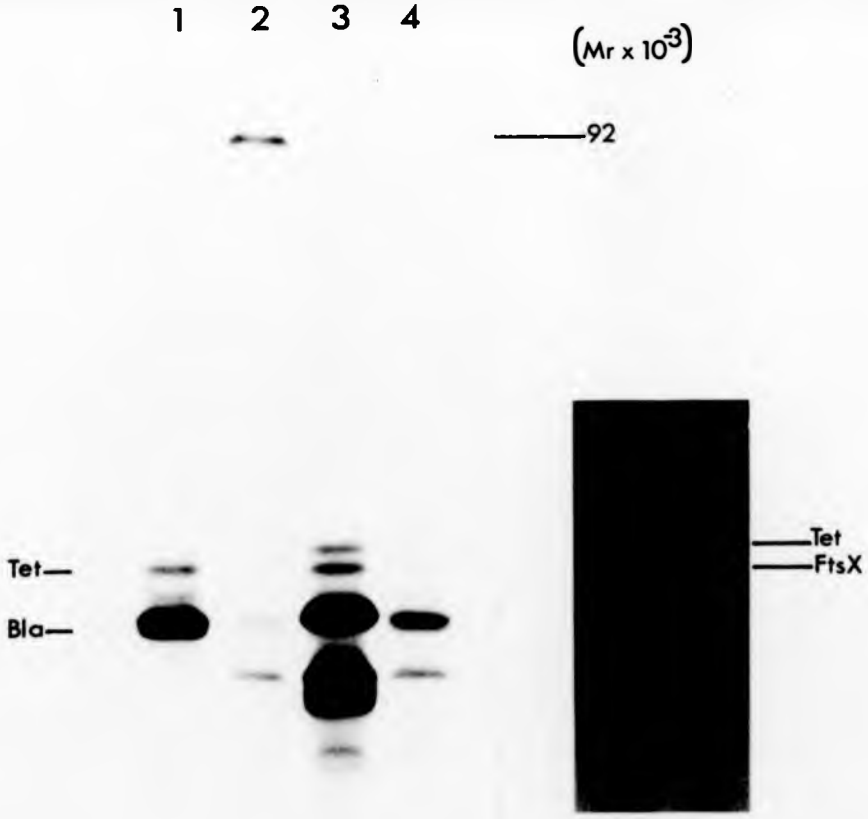


Figure 7:2:2(c) Autoradiogram of ^{35}S-methionine labelled maxicell samples of pBR322, pDB1, pDG1Ω4 and pDB184.

Samples were analysed by SDS-PAGE using an 11% (w/v) linear gel. Track 1 = pBR322; track 2 = pDB1, track 3 = pDG1Ω4; track 4 = pDB184. The position of the Mr 92000 polypeptide is indicated and this protein is absent from tracks 3 and 4. A longer exposure of this autoradiogram is also shown and the ORF3 and *tet* gene products are just visible in track 4.



7:2:3 Complementation data for pDB184 and pDG1Ω4

Plasmids pDB184 and pDG1Ω4 were each used in the transformation of the *recA*-derivatives of the five cell division mutants MFT1181, TOE22, TC35, JS10 and OV32. The resultant Ap^r transformants were tested for temperature-sensitivity and a summary of the results is presented in table 7:2:3(a).

The data indicate that both plasmids, like pDB1, were able to complement all five strains at the restrictive temperature. In order to be certain that the degree of complementation observed for plasmids pDB184 and pDG1Ω4 was equal to that of pDG1, viable count data was obtained at the permissive and restrictive temperatures, and is presented in table 7:2:3(b).

7:2:4 Further restriction analysis of the Tn*1000*-containing plasmids

Tn*1000*-containing plasmids, pDB92 and pDB65, were shown to carry insertions in the 3' end of ORF1 (chapter 4) but did not appear to affect expression of the Mr 92000 polypeptide (chapter 6). In view of these facts, attempts were made to locate the insertions more precisely and to determine whether the insertions were actually in the 3' end of ORF1 or the 5' end of ORF2.

To do this, the two *Pst*I restriction sites of the 4.5 kb fragment were used (positions 2300 and 2700). Figure 7:2:4(a) shows the position of the *Pst*I sites relative to the junction between ORF1 and ORF2. When plasmid pDB1 was digested with *Pst*I, the DNA fragments shown in figure 7:2:4(b) were observed. A DNA fragment corresponding to the 0.4 kb internal *Pst*I fragment was clearly seen. When plasmid pDB92 was similarly digested, this 0.4 kb fragment was destroyed due to the

Table 7:2:3(a) Complementation data for pDB184 and pDG1Ω4

	pBR325	pDG1	pDB184	pDG1Ω4
MFT1181*recA*	-	+	+	+
TOE22*recA*	-	+	+	+
TC35*recA*	-	+	+	+
JS10*recA*	-	+	+	+
OV32*recA*	-	+	+	+

\+ indicates complementation in a *recA* background

\- indicates no complementation

Table 7:2:3(b) Viable count data for pDB184 and pDG1Ω4

Strain	Plasmid	Percentage colony formation at restrictive temperature
MFT1181*recA*	-	<0.01
	pBR322	<0.01
	pDB1	100
	pDB184	77
	pDG1Ω4	33
OV32*recA*	-	<0.01
	pBR322	<0.02
	pDB1	82
	pDB184	85
	pDG1Ω4	64
JS10*recA*	-	<0.01
	pBR322	<0.01
	pDB1	50
	pDB184	45
	pDG1Ω4	63

Figure 7:2:4(a) Diagram to show the positions of Tn*1000* insertions 92, 65, and 38 in detail.

Insertion a = 92; insertion b = 65; insertion c = 38. The positions of the A1 and Ps restriction sites are indicated relative to the start of ORF1.

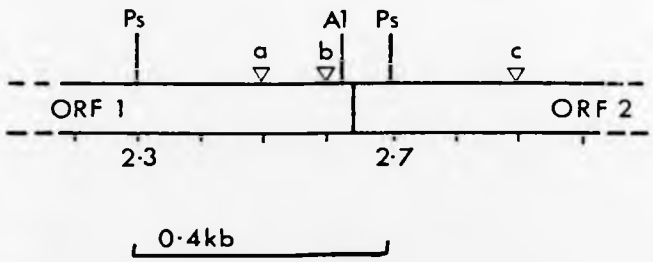


Figure 7:2:4(b) Agarose gel electrophoresis of plasmids pDB1, pDB92 and pDB38 digested with *Pst*I.

Samples were analysed on a 0.6% (w/v) agarose gel. Track 1 and 5 = λ DNA digested with *Hin*dIII; tracks 2-4 = pDB1, pDB92 and pDB38 respectively, digested with *Pst*I. The 0.4 kb *Pst*I fragment is missing from track 3.

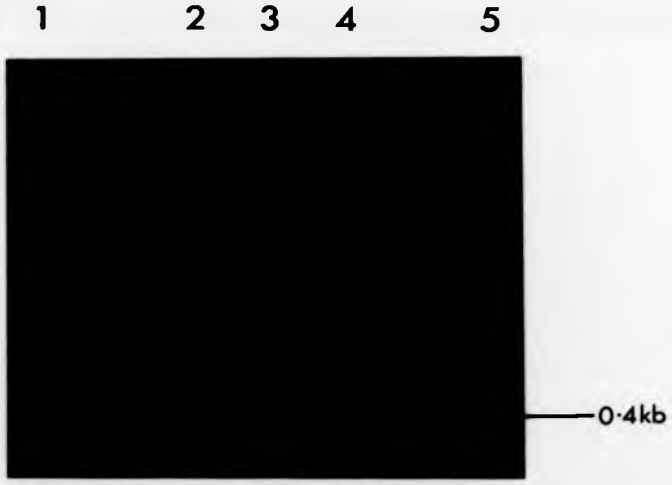
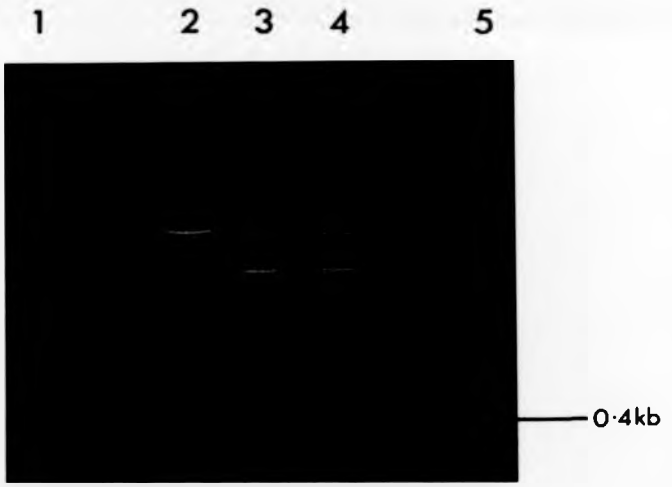


Figure 7:2:4(b) Agarose gel electrophoresis of plasmids pDB1, pDB92 and pDB38 digested with *Pst*I.

Samples were analysed on a 0.6% (w/v) agarose gel. Track 1 and 5 = λ DNA digested with *Hin*dIII; tracks 2-4 = pDB1, pDB92 and pDB38 respectively, digested with *Pst*I. The 0.4 kb *Pst*I fragment is missing from track 3.



position of the Tn*1000* fragment. The digestion of pDB65 also showed this (data not shown), but digestion of plasmid pDB38 showed that the *Pst*I fragment was intact, suggesting that insertion 38 was outside this fragment as was indicated by previous restriction mapping (section 4:2:2). Thus the digests showed that insertions 92 and 65 must be located between positions 2300 and 2700, 0.3 kb of which is ORF1.

A second attempt was made to locate the point of insertions more accurately using the unique *Ava*I restriction site at position 2630. The position of this site relative to the ORF1/ORF2 junction is also shown in figure 7:2:4(a). Unfortunately due to the presence of several (unmapped) *Ava*I sites within the Tn*1000* element, it was impossible to conclude whether the insertion lay 5′ or 3′ to this restriction site.

7:3 Discussion

7:3:1 The location of insertions 92 and 65 within ORF1

Data presented in this chapter showed that although an ORF1 candidate protein of the predicted Mr was not observed, ORF1 appears to encode part, or all, of the Mr 92000 polypeptide.

Previous experiments described in chapter 4 suggested that it was unlikely that ORF1 encoded this polypeptide because Tn*1000* insertions in the 3′ end of this gene did not affect the size of this protein. Careful restriction mapping using several restriction endonucleases, located insertions 92 and 65 to positions 2.5 kb and 2.6 kb respectively, and in this chapter it was shown that the insertions must lie within the 0.4 kb *Pst*I fragment 0.3 kb of which is ORF1. Attempts

to further locate the insertions using the *Ava*I restriction site at position 2630 failed due to the presence of several unmapped *Ava*I sites within Tn*1000*. Since *Ava*I sites were found very close to both ends of the transposon, it was impossible to determine on which side of the *Ava*I site on the 4.5 kb fragment, the insertions were located.

Irrespective of the inaccuracies inherent in restriction mapping, the precise location of the insertions was impossible due to the unavailability of a detailed restriction map of the Tn*1000* element. A fairly preliminary restriction map was described by Guyer (1978) and is generally adequate for mapping Tn*1000* insertions (Buxton & Drury, 1983; DeLencastre *et al.*, 1983; Jones & Holland, 1984). However the available restriction map is not detailed enough for fine-structure mapping and thus for the *accurate* positioning of insertions, the DNA sequence is required. Several regions of the 5.7 kb transposon have been sequenced (Reed *et al.*, 1979; Reed, 1981; Reed *et al.*, 1982), but the complete DNA sequence has not been published. If this information became available it should be possible to clone a DNA fragment containing the end of the Tn*1000* element and adjacent DNA, and to sequence through the insertion site to locate its position precisely. For example, Guyer (1983) has reported the existence of an *Sst*I site 0.078 kb from the δ end of the Tn*1000*, and it might be possible to use this restriction site as described.

Such an experiment would be important in the investigation of the ORF1 protein because if insertions 92 and 65 are found to be located in ORF1, as predicted, then interesting questions are raised as to how the ORF1 protein is assembled.

7:3:2 Co-transcription of genes in the cell division operon

Experiments described in this chapter showed that insertional inactivation of ORF1 did not appear to affect expression of downstream genes, *ftsE* and *ftsX*. It was shown in chapters 4 and 6 that *ftsE* and *ftsX* were co-transcribed, and the question was raised as to whether or not ORF1 was also part of the transcriptional unit.

Maxicells which show the gene products of both pDB184 and pDG1Ω4 (figure 7:2:2(c)) show quite clearly that the synthesis of proteins FtsE and FtsX is not affected by the transposon and interposon insertions in ORF1. It must be noted however that only the presence or absence of such proteins on polyacrylamide gels was recorded and the data were not quantitative.

Data presented in table 7:2:3(a) showed that both pDB184 and pDG1Ω4 complemented the five cell division mutants and there are several interesting conclusions to be drawn from these data. For example, the fact that both plasmids complemented OV32 means that the possibility that this strain carries a nonsense mutation in ORF1 (discussed in section 5:3:1) is no longer tenable. The possibility that OV32 is defective in ORF2 still remains, however, if it is postulated that this nonsense mutation shows polarity on expression of ORF3. This is discussed in chapter 8. Also, viable count data presented in table 7:2:3(b) gives no indication that expression of the ORF1 gene product is required for the complementation of mutations in *ftsE* and *ftsX*.

However, further experiments are required which avoid the use of multicopy plasmid vectors, in order to obtain quantitative data. These experiments could include the isolation of chromosomal gene fusions (for

review see: Silhavy & Beckwith, 1985) and the use of promoter probe vectors (Rosenberg *et al.*, 1982).

Although no evidence is presented here which indicates the involvement of ORF1 in the transcription of *ftsE* and *ftsX*, it is difficult to ignore the putative regulatory sequences found 5' to the start of ORF1. These sequences are shown in Appendix II. There is a region of tandem, overlapping, dyad symmetry 5' to the potential Shine-Dalgarno sequence (Shine & Dalgarno, 1975), of ORF1, and such dyad symmetry is commonly found in operators (Ohlendorf & Matthews, 1983; Pabo & Sauer, 1984). In Appendix II, the region is compared with the regulatory region of the *trp* operon (Yanofsky *et al.*, 1981) and the similarity is quite strong.

Moreover, in view of the fact that RNA processing is an important aspect of the regulation of gene expression (Higgins & Smith, 1986), it is possible that several messenger RNA transcripts are produced from the cell division genes, and may be involved in the fine tuning of the expression of these genes. This represents a potentially exciting possibility, especially as the RNA transcripts may be found to extend beyond the operon to include other genes within the cluster, such as the adjacent *rpoH* gene, located immediately downstream of *ftsX* (section 3:2:4). This has also been discussed by Crickmore and Salmond (1986), who show that there are a number of putative RNA stem-loop/hairpin structures in the intercistronic region between *ftsX* and *rpoH*. It is already known that several *rpoH* transcripts which extend some distance upstream of *rpoH*, can be seen *in vitro* (C. Gross, pers. comm.).

It is difficult to speculate further without accurate mapping of the RNA transcripts in this region, and this is currently being investigated in this laboratory. It is not known whether or not ORF1 is an essential

gene involved in cell division and experiments are in progress to test this. However, the position of ORF1 within the *ftsE*, *ftsX* operon suggests that this is likely, and thus ORF1 is tentatively designated *ftsY* in keeping with current nomenclature.

7:3:3 How could a polypeptide of Mr 92000 be encoded by ORF1?

It is difficult to see how a gene of known sequence, with a protein-coding capacity of Mr 48000, could encode a polypeptide of Mr 92000. The actual size of this protein is in fact variable when using different gel electrophoresis systems but is usually in the region of Mr 90000 to Mr 100000, and is routinely estimated as being of Mr 92000.

One explanation for this observation is that this large protein is a dimer of the ORF1 product. Thus the monomeric form of ORF1 (Mr 48000) is produced, but only the dimeric form is observed on polyacrylamide gels. Of course, this protein need not be a dimer (although the relative sizes of the polypeptides may predispose one to expect this), but a multimer of the ORF1 product and some other polypeptide species.

Many other, more elaborate possibilities can also be put forward to explain the existence of this large protein, involving the deletion and addition of various protein fragments. For example, it may be that the 3' end of ORF1 is not required for the formation of the large protein, and therefore Tn*1000* insertions which appear to map in this region (such as 92 and 65) would have no effect on the appearance of the protein. It is also a possibility that two or more messenger RNA transcripts are ligated together. Obviously it is futile to imagine more complex explanations while some quite basic questions remain unanswered.

Firstly, it is not known whether or not insertions 92 and 65 are actually found in ORF1, and the answer to this will probably require DNA sequence analysis. Also, it is important to determine the actual size of the ORF1 monomer before it is assembled into the Mr 92000 protein. No such monomeric species was observed (chapter 6) and maxicell analysis of both pDB184 and pDG1Ω4 (figure 7:2:2(c) did not reveal a truncated version of ORF1 or any other novel protein species. Perhaps this is not surprising since, in the case of pDB184, the Tn*1000* insertion is located very promoter-proximal within ORF1, and close inspection of pDG1Ω4 shows that transcription would be terminated to produce a similarly short reading frame. Therefore, in both cases, the small peptide fragment produced, even if stable, would be very difficult to detect.

Thus, very little is known about the processes involved in the assembly of the Mr 92000 protein, but it is known that such processes must also occur in the *in vitro* transcription-translation system (section 6:2:3) since this procedure also reveals the Mr 92000 protein. Also it has been shown that covalent bonds must be employed in the formation event(s), since procedures which disrupt ionic and disulphide interactions do not affect the appearance of this protein in maxicells (chapter 6).

Two other reported examples of such protein-to-protein ligation events exist. The first was reported by Hendrix and Casjens (1974), who showed that two λ bacteriophage coded head proteins, pE and pC, became fused during bacteriophage λ head assembly. The authors used tryptic digests of various radioactively-labelled λ head proteins to show that pE and pC sequences were covalently joined together in equimolar amounts in the resultant fusion protein. Since 1974, it appears that no further data

has been published (Georgopoulos *et al.*, 1983), although this "novel reaction" was potentially very exciting (Hendrix & Casjens, 1974).

The second, better-documented example, is that of the lectin, concanavalin A. Extensive cDNA cloning, sequencing and peptide mapping studies, have led to the proposal that mature concanavalin A is the result of post-translational transposition and ligation within the precursor polypeptide, (Carrington *et al.*, 1985). These authors outlined a remarkable sequence of discrete proteolytic cleavage and subsequent ligation events, to account for the formation of the protein. Such proposals have met with some consternation and alternative explanations have also been put forward (Gatehouse & Boulter, 1985) but no evidence for these alternatives is presented.

In order to ascertain whether or not the Mr 92000 polypeptide is a dimer or multimer as a result of such a protein-to-protein ligation event, several simple experiments could be tried. Tryptic digests or treatment with other proteolytic reagents (Lischwe & Ochs, 1982) could be used to identify the protein species involved in the fusion protein and also to yield preliminary data on the arrangement of these species within the fusion. It would also be interesting to investigate the possibility that this fusion protein further interacts with other polypeptides (perhaps other operon gene products) in an ionic association. It would be possible to do this by running maxicell samples on non-denaturing gels, cutting out any high-molecular weight bands and eluting them, and electrophoresing these complexes on denaturing gels to identify the gene products involved.

In conclusion, evidence has been presented here which shows that ORF1

somehow encodes the Mr 92000 protein. This may be the result of some modification of the ORF1 product and experiments suggest that this modification involves covalent bonding. Perhaps the most plausible explanation is that the Mr 92000 protein is a dimeric or multimeric protein. Other examples of protein-to-protein ligation events may have been observed or partially investigated, but not reported due to the somewhat remarkable nature of the phenomenon.

CHAPTER 8

Cloning of the mutations from MFT1181 and OV32

8.1 Introduction

Once the various cell division mutations had been assigned to different genes, it was decided to try and clone several of these mutations. The clones could then be used to locate the position of the mutation within each gene more accurately using simple subcloning techniques, and then precisely by DNA sequencing.

Recently, several other putative missense and nonsense *ftsE* mutants have been isolated (C. Bowler, pers. comm.) and it would be interesting to clone and sequence these mutations to determine where they lie within the *ftsE* gene.

Firstly, a simple, reliable method for cloning the conditionally lethal mutations was required. The *ftsE* mutations carried by strains TOE22 and TC35 had already been isolated on λ transducing phages (Salmond & Plakidou, 1984) by lysogenising *att*λΔ strains with λpGS22 and subsequent UV-induction of the phages. In order to study these phages, the cloned fragments would need to be subcloned onto plasmid vectors for routine manipulations. Other genetic techniques were also available for cloning the chromosomal mutations and these included the *in vivo* transfer of such mutations onto multicopy plasmids utilising *polA* strains (Saarilahti & Palva, 1985).

However, these genetic techniques although simple in principle, often require several successive stages of manipulation and incubation. Thus it was decided to use a more directed approach, taking advantage of the detailed restriction map and versatile molecular cloning techniques already available.

Briefly, chromosomal DNA from each mutant was digested with the restriction enzyme *Hin*dIII, and in each case DNA fragments of approximately 4.5 kb were prepared and ligated to pBR322. Selection for the 4.5 kb fragment carrying a mutation in, for example, *ftsE* was based on the biological complementation of the chromosomally-located mutation in *ftsX*, at the non-permissive temperature. This technique eliminated the requirement for a representative gene library and avoided the use of laborious screening procedures. It would also directly confirm the assignation of different cell division mutations to different genes. This method was primarily used to clone the *ftsE* mutation from MFT1181 and the nonsense mutation from OV32 which was subsequently found to be in *ftsX*.

8:2 Results

8:2:1 Cloning the MFT1181 *ftsE* mutation

In order to clone the *ftsE* mutation from MFT1181, chromosomal DNA was prepared from this strain and completely digested with the restriction enzyme *Hin*dIII. The digest was electrophoresed overnight through an agarose gel, to achieve good separation of the DNA fragments, and the digest compared with the position of the 4.5 kb *Hin*dIII fragment purified from pDG1. Figure 8:2:1 shows such a gel and indicates the region of the gel removed for elution of DNA fragments in this region.

The fragment was purified using the freeze-squeeze method and was then ligated to the *Hin*dIII restriction site of pBR322. The ligation mix was used to transform the *ftsX* mutant JS10*recA* and Ap^r colonies selected at 25^oC (the non-permissive temperature). After three days incubation at

Figure 8:2:1 Agarose gel electrophoresis of chromosomal DNA from strain MFT1181 digested with *Hin*dIII

Samples were analysed on a 0.6% (w/v) agarose gel. Track 1 = λ DNA digested with *Hin*dIII; track 2 = MFT1181 chromosomal DNA digested with *Hin*dIII. The region of the gel removed for elution of DNA fragments is indicated.

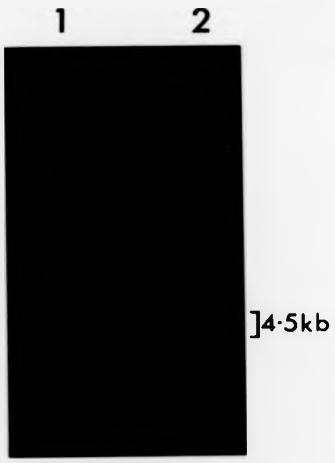
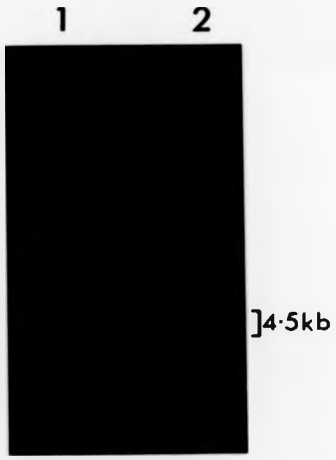


Figure 8:2:1 Agarose gel electrophoresis of chromosomal DNA from strain MFT1181 digested with *Hin*dIII

Samples were analysed on a 0.6% (w/v) agarose gel. Track 1 = λ DNA digested with *Hin*dIII; track 2 = MFT1181 chromosomal DNA digested with *Hin*dIII. The region of the gel removed for elution of DNA fragments is indicated.



this temperature, a single colony appeared and this was purified. It was presumed that this clone carried a plasmid with a 4.5 kb insert which allowed complementation of JS10, because it carried a wild-type *ftsX* gene, but also carried a mutant *ftsE* gene derived from MFT1181. The plasmid DNA was prepared from this clone and used to re-transform JS10*recA*, to confirm that the plasmid alone was responsible for growth at the restrictive temperature. This plasmid was designated pDB1181.

8:2:2 Cloning the OV32 *ftsX* mutation

A similar strategy was used to clone the nonsense mutation from OV32 into pBR322. Although it remained a formal possibility that OV32 carried a nonsense allele of *ftsE*, this was unlikely, and therefore, the ligation mix was used to transform the *ftsE* mutant MFT1181*recA* (DW52) and Ap^r colonies were selected at 42°C. After two days incubation, one colony appeared and this was purified. Plasmid isolated from this strain was used to re-transform MFT1181*recA* and was designated pDB232. This plasmid was presumed to contain the 4.5 kb *Hin*dIII fragment from OV32 carrying the OV32 *ftsX* mutation.

8:2:3 Restriction analysis of pDB1181 and pDB232

The first step in the analysis of these plasmids was to determine the restriction map in each case and compare them to that of pDB1. It was found that the 4.5 kb fragment of pDB1181 had been ligated to vector DNA in the same orientation as pDB1, whereas the 4.5 kb fragment in pDB232 had been ligated to vector DNA in the same orientation as pDB2.

Figure 8:2:3 shows several restriction digests of each of these plasmids. When digested with *Hin*dIII, both plasmids appeared to produce a single fragment of approximately 4.5 kb, as observed for pDB1. The

Figure 8:2:3 Agarose gel electrophoresis of plasmids pDB1, pDB2, pDB1181 and pDB232 digested with *Hin*dIII and *Ava*I.

Samples were analysed on a 0.6% (w/v) agarose gel. Track 1 = λ DNA digested with *Hin*dIII; track 2-4 = plasmids pDB1, pDB1181 and pDB232 respectively, digested with *Hin*dIII; tracks 5-8 = plasmids pDB1, pDB1181, pDB2 and pDB232 respectively, digested with *Ava*I.

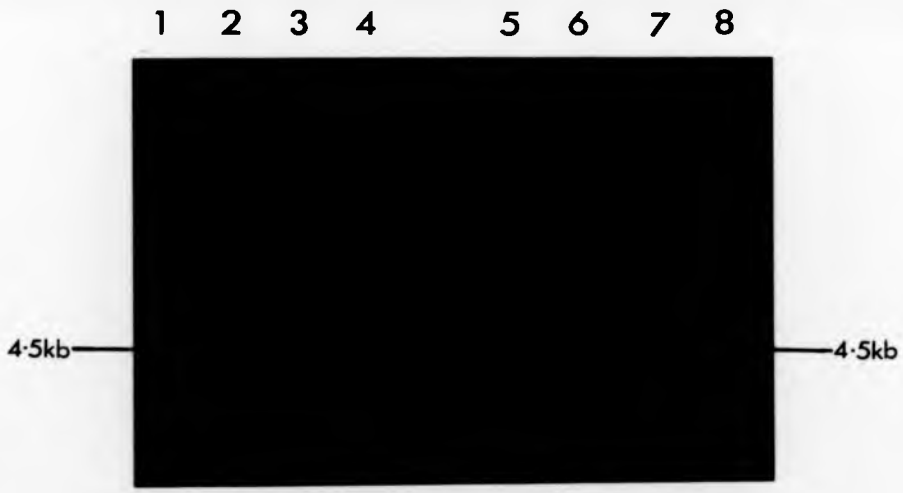
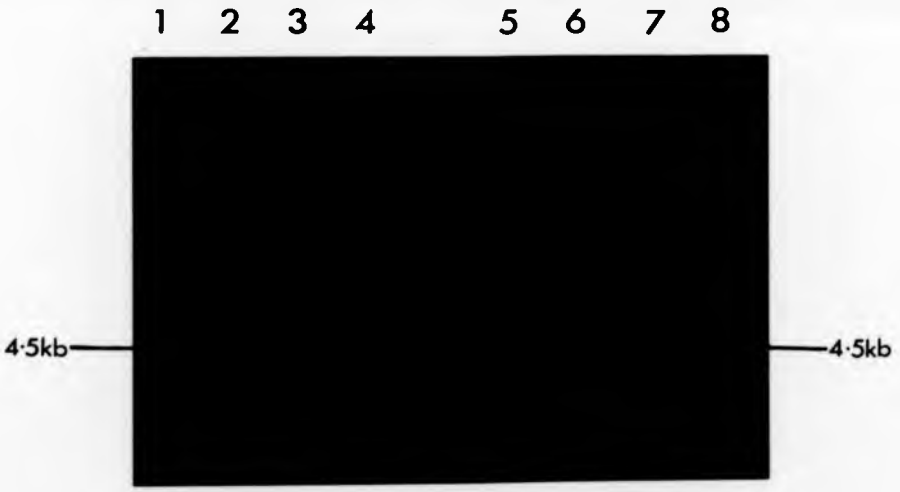


Figure 8:2:3 Agarose gel electrophoresis of plasmids pDB1, pDB2, pDB1181 and pDB232 digested with *Hin*dIII and *Ava*I.

Samples were analysed on a 0.6% (w/v) agarose gel. Track 1 = λ DNA digested with *Hin*dIII; track 2-4 = plasmids pDB1, pDB1181 and pDB232 respectively, digested with *Hin*dIII; tracks 5-8 = plasmids pDB1, pDB1181, pDB2 and pDB232 respectively, digested with *Ava*I.



4.36 kb linearised pBR322 and the 4.48 kb *Hin*dIII fragment from pDB1, appeared as a single band, and usually remain unresolved after routine electrophoresis. However, when the plasmids were digested with the restriction enzyme *Ava*I, three fragments appeared instead of the expected two. The extra fragment observed in both cases was of approximately 4.5 kb in size. Several other restriction digests indicated that this extra fragment was linearised pBR322 and that the 4.5 kb fragment from MFT1181 and from OV32 had each been cloned into a dimeric form of pBR322.

Exactly how this had occurred was unclear, because although dimeric pBR322 DNA had been used as the cloning vector, digestion with *Hin*dIII should have resolved the plasmid to its momomeric form. All restriction digests showed that the 4.5 kb fragment from MFT1181 and OV32 had in fact been cloned and to confirm this, each plasmid was probed with ^{32}P-labelled 4.5 kb fragment from pDB1, in a Southern blot experiment (data not shown).

8:2:4 Analysis of pDB1181 and pDB232 in maxicells

The plasmids were used to transform CSH26ΔF6 for maxicell analysis. Figure 8:2:4(a) shows an autoradiogram of maxicells carrying pDB1181 and it can be seen that the plasmid shows an identical gene product profile to that observed for maxicells carrying pDB1. This also confirmed that the 4.5 kb *Hin*dIII fragment from MFT1181 had been cloned.

Figure 8:2:4(a) also shows an autoradiogram of maxicells carrying pDB232 and all gene products except for the Mr 38000 FtsX protein are present. It must be noted that this protein is often difficult to see, even if the autoradiogram has been overexposed, but in this case the *ftsX*

Figure 8:2:4(a) Autoradiogram of ^{35}S-methionine labelled maxicells of pDB1181 and pDB232.

Samples were analysed by SDS-PAGE using an 11% (w/v) linear gel and subsequent fluorography. Track 1 = pBR322; track 2 = pDB1; track 3 = pDB232; track 4 = pDB1181. The presence of the Mr 38000 FtsX polypeptide can be seen in longer exposures of the autoradiograph.

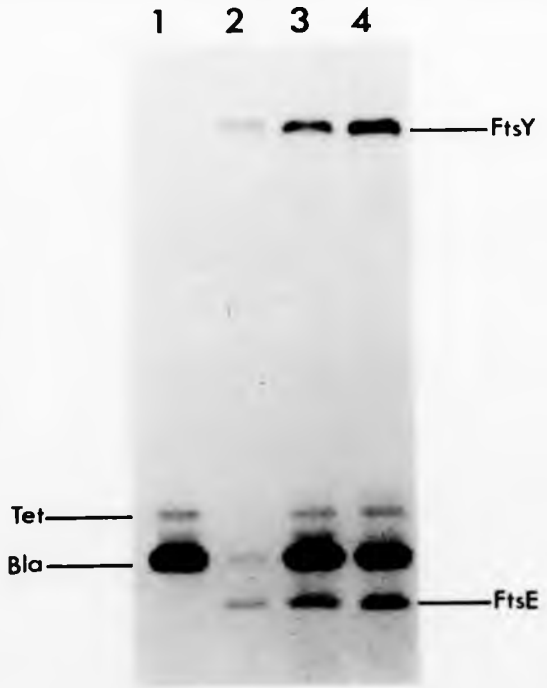
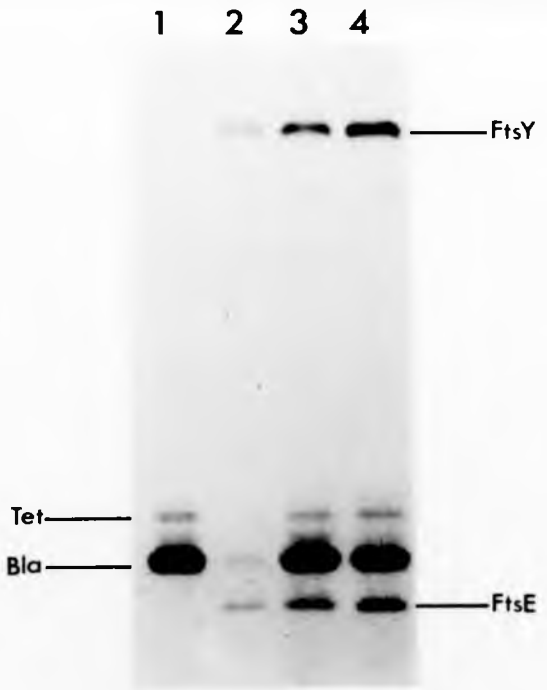


Figure 8:2:4(a) Autoradiogram of ^{35}S-methionine labelled maxicells of pDB1181 and pDB232.

Samples were analysed by SDS-PAGE using an 11% (w/v) linear gel and subsequent fluorography. Track 1 = pBR322; track 2 = pDB1; track 3 = pDB232; track 4 = pDB1181. The presence of the Mr 38000 FtsX polypeptide can be seen in longer exposures of the autoradiograph.



product appears to be missing (see also figures 8:2:4(b) & (c)). In view of the fact that the OV32 mutation is an amber mutation it is not surprising that the mutant *ftsX* product is absent, especially, as the maxicells were labelled in CSH26ΔF6 which is a *sup*^{o} strain (J. Hinton, pers. comm.).

Based on this premise, maxicells of pDB232 were also made in the strain QD*supFrecA*. This strain is a proline auxotroph and carries the *supF* gene. It was anticipated that the presence of a suppressor mutation in the maxicell host strain, would allow suppression of the OV32 amber mutation and subsequent formation of a full length FtsX. Figures 8:2:4(b) and 8:2:4(c) show the results from these experiments. It can be seen that in a *sup*^{o} strain the FtsX protein encoded by pDB232 is missing, whereas in a *supF* strain the FtsX protein reappears. Moreover, careful inspection of the pDB232 product profile in the *supF* strain, reveals the presence of an additional protein of Mr 27000 (approximately). The origin of this protein is unknown, but is discussed in section 8:3:1.

8:2:5 Complementation data for pDB1181 and pDB232

The plasmids pDB1181 and pDB232 were used to transform *recA*-derivatives of the five cell division mutants and the transformants tested for temperature-sensitivity. Tables 8:2:5(a) and (b) show the results of these experiments.

The data show that pDB1181, which was selected by complementation of JS10, also complemented OV32 as expected, but failed to complement all three *ftsE* mutants. In the case of pDB232, the data are more complicated. The plasmid was selected by complementation of the *ftsE*

Figure 8:2:4(b) Autoradiogram of ^{35}S-methionine labelled maxicells of pDB1 and pDB232 in *sup*^{o} and *supF* maxicell strains.

Samples were analysed by SDS-PAGE using an 11% (w/v) linear gel and subsequent fluorography. Tracks 1 and 2 = pDB1 maxicells (*supF*); tracks 3 and 4 = pDB232 maxicells (*sup*^{o}); tracks 5 and 6 = pDB232 maxicells (*supF*). Tracks 1, 3 and 5 = samples solubilised at 37^{o}C for 1 h; tracks 2, 4 and 6 = samples boiled for 1 min. The position of an unidentified polypeptide of Mr 27000 is indicated. Figure 8:2:4(c) is a longer exposure of the same autoradiograph.

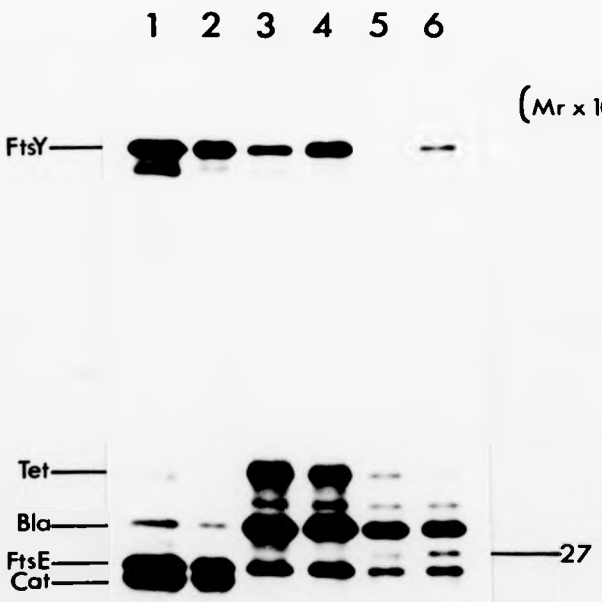


Figure 8:2:4(b) Autoradiogram of ^{35}S-methionine labelled maxicells of pDB1 and pDB232 in *sup*^{o} and *supF* maxicell strains.

Samples were analysed by SDS-PAGE using an 11% (w/v) linear gel and subsequent fluorography. Tracks 1 and 2 = pDB1 maxicells (*supF*); tracks 3 and 4 = pDB232 maxicells (*sup*^{o}); tracks 5 and 6 = pDB232 maxicells (*supF*). Tracks 1, 3 and 5 = samples solubilised at 37^{o}C for 1 h; tracks 2, 4 and 6 = samples boiled for 1 min. The position of an unidentified polypeptide of Mr 27000 is indicated. Figure 8:2:4(c) is a longer exposure of the same autoradiograph.

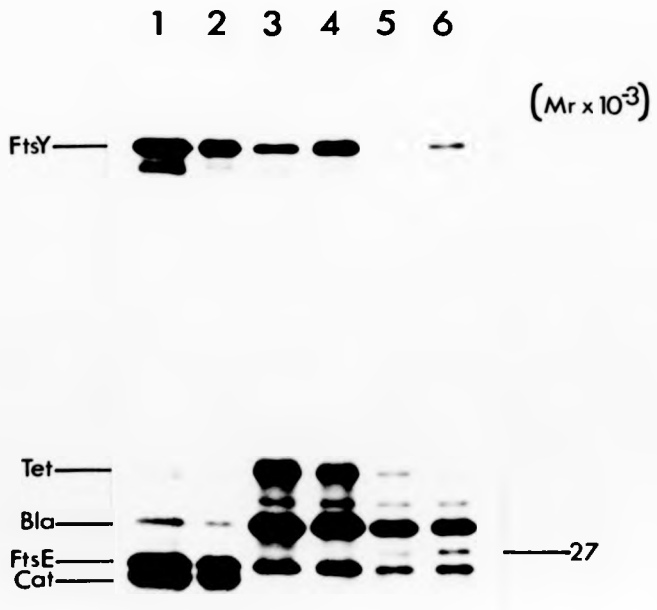


Figure 8:2:4(c) A longer exposure of autoradiogram described in figure 8:2:4(b).

Tracks 1-6 as figure 8:2:4(b). Tracks 7 and 8 = longer exposure of tracks 5 and 6. This reveals the presence of the Mr 38000 FtsX protein in the *supF* maxicell strain (tracks 7 and 8) but not in the sup^{o} strain (tracks 3 and 4). The longer exposure (approximately 3 weeks) also revealed the presence of the unidentified Mr 27000 polypeptide in pDB1 maxicells (tracks 1 and 2).

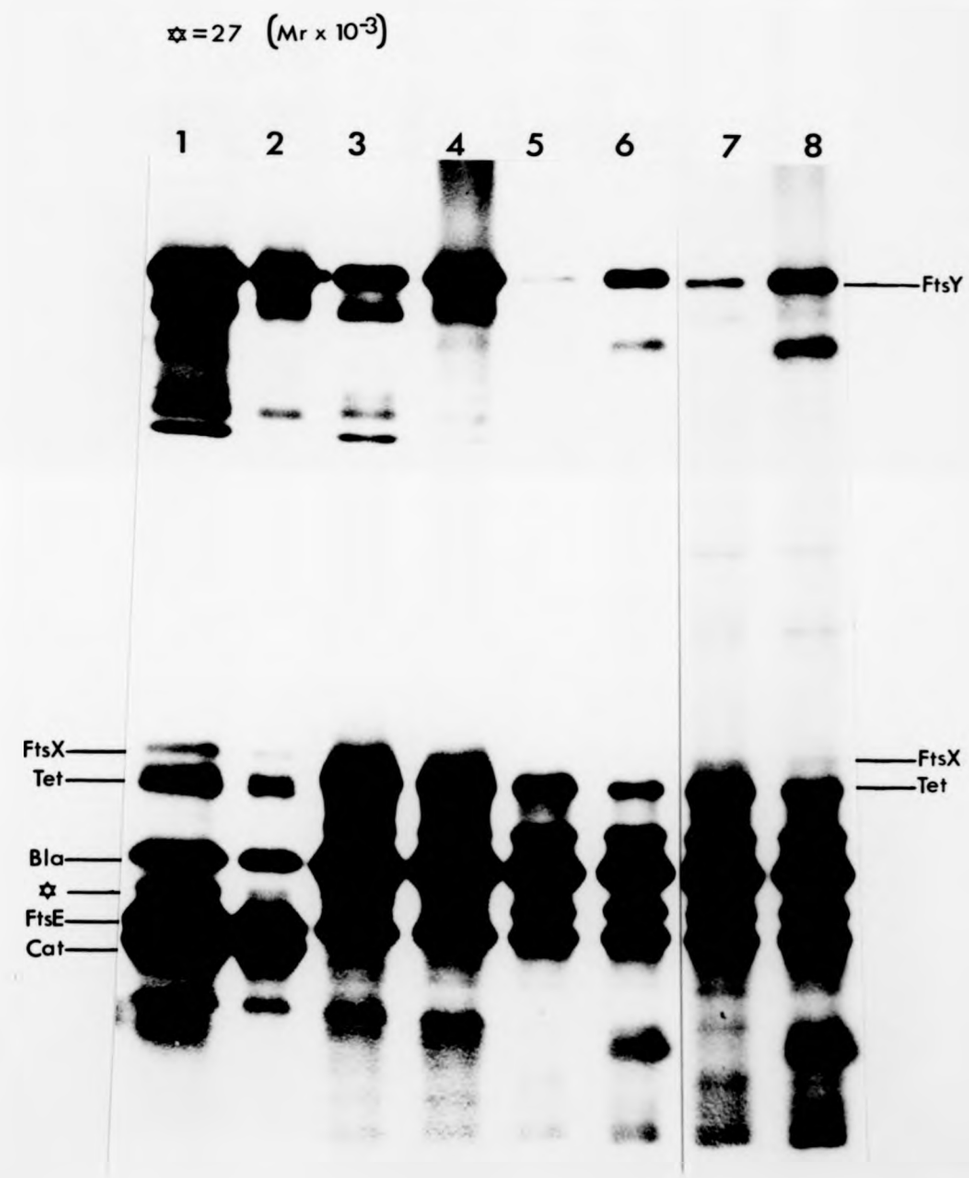


Figure 8:2:4(c) A longer exposure of autoradiogram described in figure 8:2:4(b).

Tracks 1-6 as figure 8:2:4(b). Tracks 7 and 8 = longer exposure of tracks 5 and 6. This reveals the presence of the Mr 38000 FtsX protein in the *supF* maxicell strain (tracks 7 and 8) but not in the sup^{o} strain (tracks 3 and 4). The longer exposure (approximately 3 weeks) also revealed the presence of the unidentified Mr 27000 polypeptide in pDB1 maxicells (tracks 1 and 2).

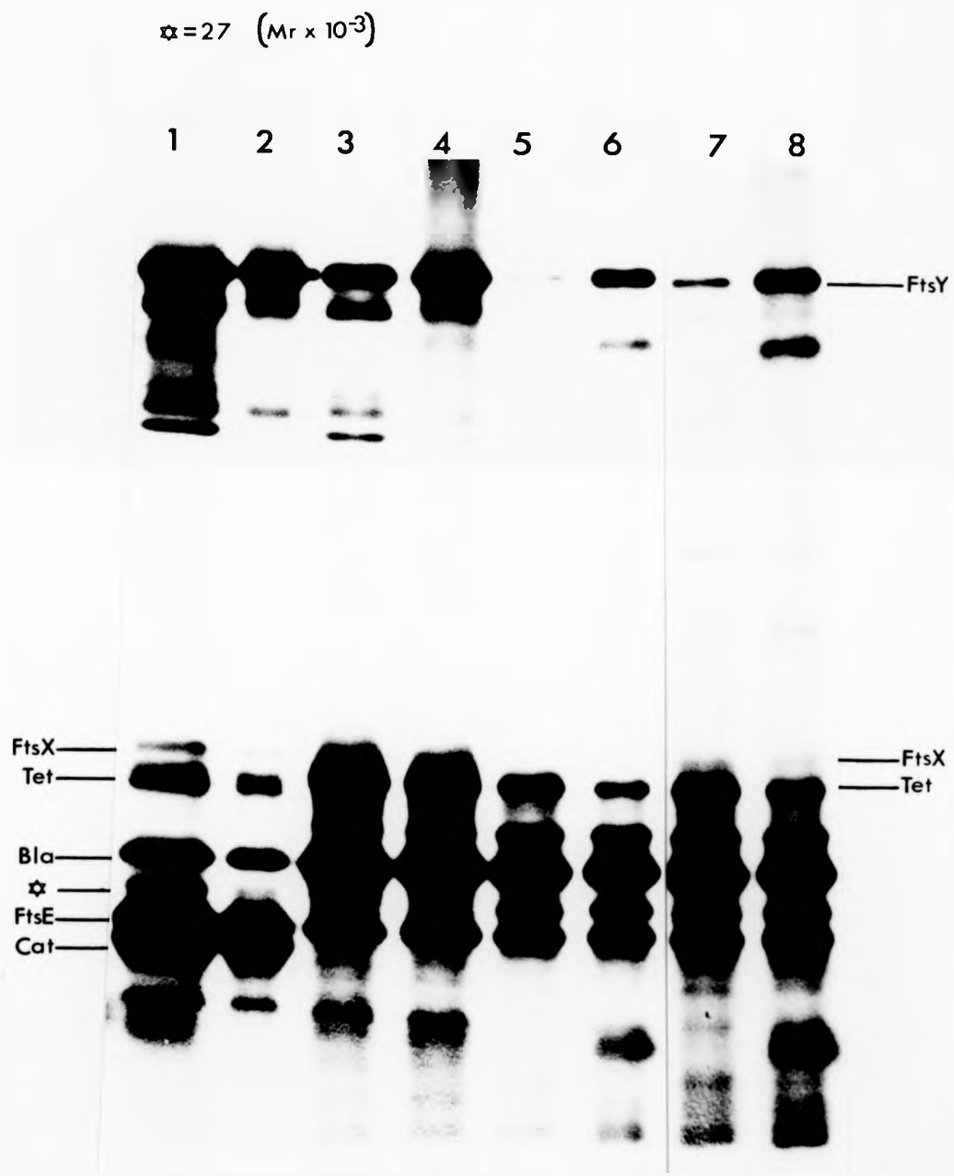


Table 8:2:5(a) Complementation data for pDB1181 and pDB232

	pDB1181	pDB232
MFT1181	-	+
TOE22	-	+
TC35	-	+
JS10	+	?
OV32	+	-

\+ indicates complementation in a *recA* background

\- indicates no complementation

? indicates some complementation observed

Table 8:2:5(b) Viable count data for pDB1181 and pDB232

Strain	Plasmid	Percentage colony formation at restrictive temperature
JS10recA	-	< 0.01
JS10recA	pDB1	74
JS10recA	pDB1181	60
JS10recA	pDB232	0.3

mutant MFT1181 and also complemented the other two *ftsE* mutants. The plasmid did not complement OV32 as expected, but showed some degree of complementation of JS10.

8:2:6 Localisation of the mutations within each gene

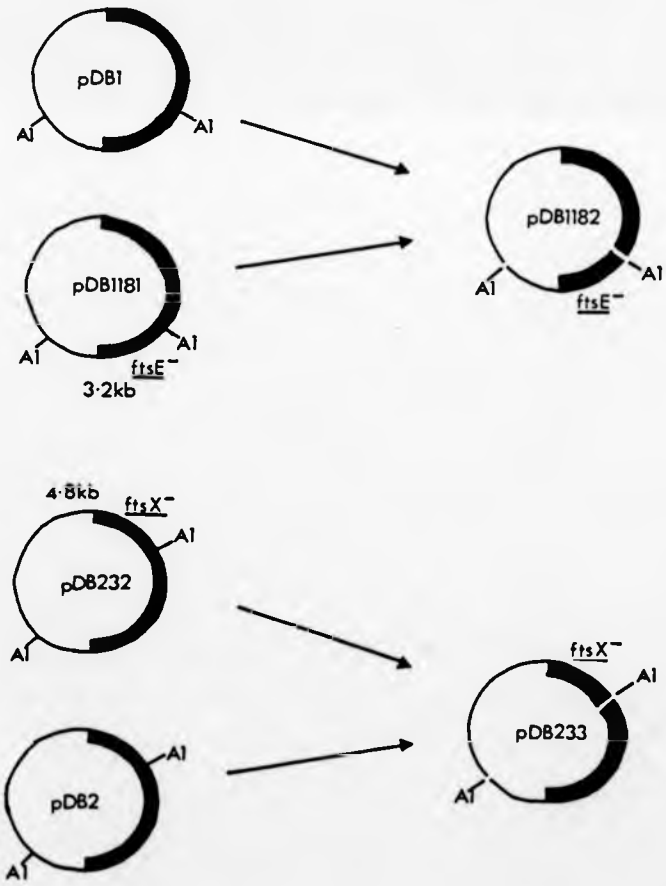
In order to further locate the position of the two mutations within each gene, several subclonings were attempted.

Firstly, the unique *Ava*I site at position 2630 was used. Figure 8:2:6 shows how this site divides the 4.5 kb fragment into two smaller fragments of 2.6 kb, carrying ORF4 and ORF1, and 1.9 kb, carrying *ftsE* and *ftsX*. Figure 8:2:3 showed the results obtained when pDB1181 and pDB232 were digested with *Ava*I. In the case of pDB1181, the 3.2 kb fragment was purified and ligated to the complementary fragment from pDB1, and in the case of pDB232, the 4.8 kb fragment was purified and ligated to the complementary fragment from pDB2. Use was made of the fact that *Ava*I recognises degenerate sequences and therefore will produce DNA fragments with different termini. Only some of the possible combinations of termini produced by this enzyme will be compatible for ligation. It was noted that the two *Ava*I restriction sites in pDB1 (or pDB2) were composed of different recognition sequences and therefore digestion with *Ava*I generated two sets of compatible termini. Thus, when the fragments were ligated together, it was fortuitous that (theoretically) the fragments could be ligated to each other in only one orientation, as indicated in figure 8:2:6. Consequently the two halves of the operon, one half from the parent plasmid (pDB1 or pDB2) and the other from the plasmid carrying the mutation, were ligated together.

The ligation mixes were used to transform CSH26ΔF6, and in each case a

Figure 8:2:6 Diagram to show the construction of plasmids pDB1182 and pDB233.

The *Ava*I site in the 4.5 kb fragment which cuts the operon at the start of ORF2 was used to construct plasmids pDB1182 and pDB233 as shown.



single colony was purified. Plasmid DNA was prepared and digested with *Hin*dIII and *Ava*I to confirm the identity of the products of the subcloning. The plasmids were designated pDB1182 and pDB233 as appropriate. Both plasmids were used to transform the cell division mutants and the complementation data obtained was shown to be identical to that of the parent plasmids pDB1181 and pDB232, (see tables 8:2:5(a) and (b)).

This subcloning avoided the problem of having a dimeric plasmid vector, and should allow further subcloning of small DNA fragments carrying the mutations, for ease of sequencing.

8:3 Discussion

8:3:1 Methods used to analyse the cloned mutations

Experiments outlined in this chapter described a simple but reliable method for cloning mutations from the cell division mutants. Using this method, the *ftsE* and *ftsX* mutations from MFT1181 and OV32 respectively, were cloned; and recently the *ftsX* mutation from JS10 was also cloned in this way but has not been characterised.

Experiments also described in this chapter showed how the maxicell technique could be used to examine the polypeptides produced from genes which carry nonsense mutations. In the *sup*^{o} strain CSH26ΔF6, maxicells of pDB232 carrying an amber mutation in *ftsX* showed no FtsX protein, whilst in the *supF* strain, the FtsX protein could be observed (figures 8:2:4(b) & (c)). This suggests that strain OV32 carries a nonsense mutation in the *ftsX* gene, and that in the presence of a suitable

suppressor mutation the wild type FtsX protein is produced. Figures 8:2:4(b) and (c) also showed that the presence of the *supF* mutation in the maxicell strain caused the synthesis of a novel polypeptide of Mr 27000. This protein was also present in the product profile of *supF* maxicells carrying pDB1. It is possible that the suppressor mutation has allowed expression of an ORF which is usually interrupted by a nonsense mutation.

This method could be used in the identification of gene products for which amber mutants are available. The λ UV-infection labelling technique as reviewed by Stoker *et al*. (1984), has often been used to identify gene products in this way, but has the disadvantage of having relatively high background labelling of host and/or phage λ proteins, which may obscure smaller or poorly-labelled proteins encoded by cloned DNA fragments. The maxicell system has a comparatively low background contamination of host proteins and therefore this technique would be more suitable in most cases.

It appears that maxicells can be made in virtually any *recA* strain with careful handling and comparisons made between maxicells made in different strains show few differences. Theoretically, such experiments should be carried out in isogenic strains with only single genetic differences between them. The results observed, could therefore be attributed to single genetic changes. For the purposes of this study, however, the use of a *supF* strain as opposed to a *sup*^{o} strain was sufficient to demonstrate the re-appearance of the FtsX protein.

The complementation data presented in tables 8:2:5(a) and (b) correspond well to that expected for plasmids pDB1181 and pDB232, except for one

surprising result. It appeared that pDB232 showed some complementation of the *ftsX* mutant JS10*recA*. Such an observation could be explained in several ways.

The most likely explanation was that strain JS10 had some suppressor mutation in its genetic background, and this was found to be the case (table 2:1). JS10 carries the suppressor mutation *supE* which might allow the inefficient suppression of the *ftsX* nonsense mutation on pDB232, sufficient at least to produce some wild-type functional FtsX protein. Due to the differences in type and efficiency of suppressor mutations (Smith, 1979), the *supE* mutation might only allow a low level of suppression and therefore only partial complementation of the JS10 mutation would be observed.

A simple experiment to test this hypothesis, would be to transduce the *ftsX* mutation from JS10 into a *sup*^{o} strain, via the closely-linked Tn*10* insertion zhf-1::Tn*10* (Salmond & Plakidou, 1984), and to use this strain to test for complementation by pDB232. This should determine whether or not the presence of the *supE* mutation is responsible for the partial complementation observed.

A similar situation appears to have occurred when cloning a putative *ftsE* nonsense mutation which as yet remains uncharacterised. This *ftsE* nonsense mutation was cloned, surprisingly, by complementation of the *ftsE* mutation and maxicell analysis of this clone showed that in a *sup*^{o} genetic background FtsE was missing but in a *supF* background, FtsE appeared (data not shown).

It therefore seems probable that the partial complementation of JS10 by

pDB232 was due to the presence of *supE*. Perhaps these observations suggest a modification to the cloning method described here, for use in cloning nonsense mutations. Selection for complementation at the restrictive temperature should be carried out in the presence of a suitable suppressor. This would be important for example in the cloning of *ftsE* nonsense mutations which might also affect expression of the downstream *ftsX*.

Other hypotheses can also be made to explain the complementation of JS10 by pDB232. One could invoke the existence of some interaction between an OV32 FtsX truncate and a thermally unstable form of FtsX from JS10, to produce a functional protein complex. No such FtsX truncate has been observed in maxicells (figures 8:2:4(b) & (c)) and it is not known whether FtsX functions as a monomer or multimer; therefore such suggestions are highly speculative.

One other important point to note at this stage is that once again the complementation tests were performed using multicopy plasmids and thus the results may not reflect the situation in the 'normal' bacterial cell.

8:3:2 Use of the cloned mutations for future work

Restriction analysis, complementation tests, maxicell experiments and Southern blotting, all confirmed that the MFT1181 and OV32 mutations had been successfully cloned. Fragments carrying these mutations were subcloned with a view to obtaining a defined DNA fragment small enough to sequence quickly. This would allow the precise location of the mutation within the gene.

Now that several *ftsE* mutants have been isolated, one could determine whether the mutations are scattered throughout the gene or are clustered within specific regions. If the protein has more than one function, perhaps two or more classes of mutation could be identified, as have been found for the RecA protein (Kawashima *et al.*, 1984).

Close inspection of the FtsE protein sequence has already revealed at least two regions or domains, both of which show spectacular homology with sequences from other better-characterised proteins (Higgins *et al.*, 1986). A description of these findings and a discussion of their significance is presented in chapter 9.

CHAPTER 9

The subcellular location of the proteins of the *ftsE* operon

9:1 Introduction

The FtsE and FtsX proteins were identified as polypeptides of Mr 25000 and Mr 38000 respectively (chapter 6) and experiments presented in this chapter, describe how these proteins were localised within the cell.

Analysis of the FtsE protein sequence revealed some interesting homologies with several other proteins. Protein sequences were compared using the DIAGON programme (Staden, 1982) and protein comparisons in the Doolittle (1981) database were done using the Wilbur and Lipman (1983) method. There is strong homology between FtsE and certain proteins known to be components of periplasmic binding protein-dependent transport systems. These include the MalK and PstB proteins of *E. coli* and the HisP and OppD proteins of *Salmonella typhimurium* which have been extensively characterised (Hobson *et al.*, 1984; Amemura *et al.*, 1985; Higgins *et al.*, 1985; Surin *et al.*, 1985; Ames, 1986).

These transport systems are multi-component and consist of a substrate-binding protein located in the periplasm as well as several membrane-associated components. The question then arose as to whether or not FtsE could be involved in transport processes also? Recently however, two other proteins, not involved in such processes, were found to share this striking homology. These were the HlyB protein, a component of the Haemolysin secretory apparatus in *E. coli* (Mackman *et al.*, 1985; Holland *et al.*, 1986), and the NodI protein, involved in nodulation in *Rhizobium leguminosarum* (Evans & Downie, 1986).

All these proteins and several others not mentioned here, represent a family of closely related proteins from various bacterial species which

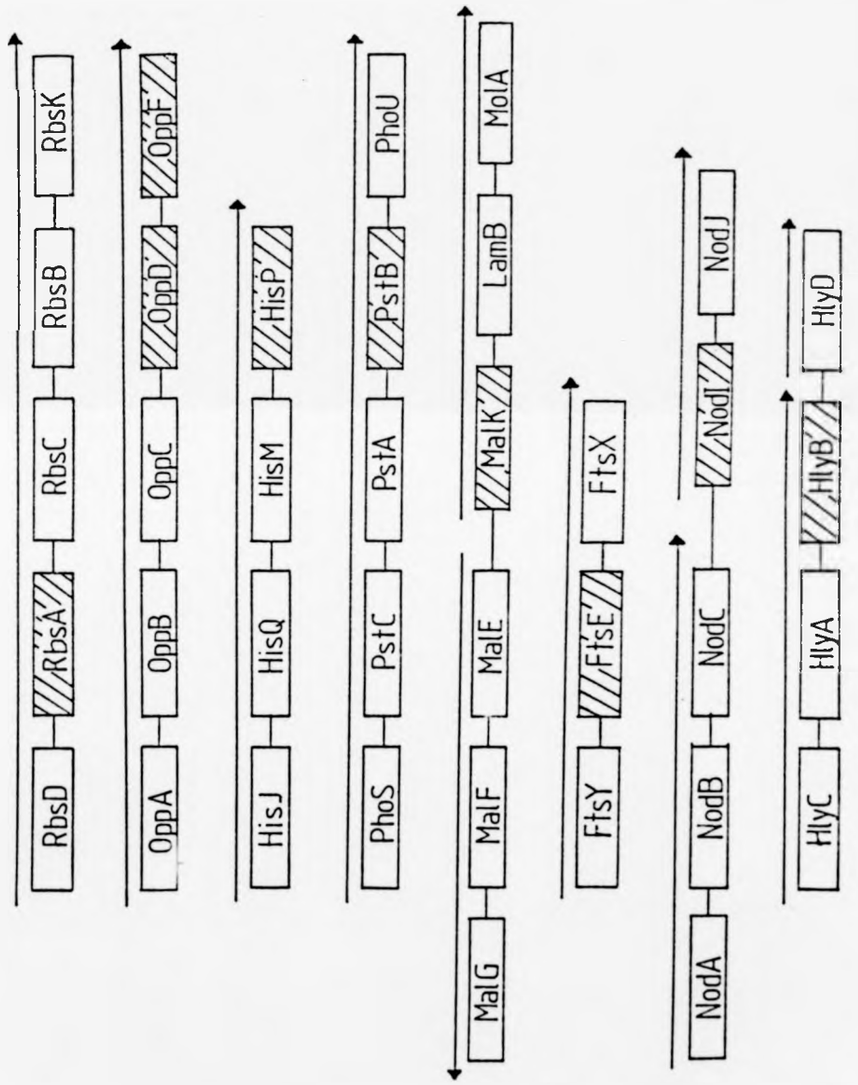
probably share a common evolutionary origin (Higgins *et al.*, 1986). There is no evidence to suggest that the FtsE, HlyB or NodI proteins are part of periplasmic transport processes especially as the other proteins encoded within the same operons (figure 9:1(a)), show little or no resemblance to the other components of the binding protein-dependent systems (Dassa & Hofnung, 1985; Higgins *et al.*, 1986). Inspection of the protein sequence of the Fts proteins reveals that none of these appears to have a signal sequence (Michaelis & Beckwith, 1982) which is required for translocation of all periplasmic proteins and outer-membrane proteins in Gram-negative bacteria (Michaelis & Beckwith, 1982; Pugsley & Schwartz, 1985). Thus it is unlikely that one of the Fts proteins is a periplasmic binding protein.

The striking homology between these proteins is indicated in figure 9:1(b) and consists of two strongly conserved domains. The first domain is amino-terminal and has the consensus sequence G/A X X G X G K T/S, flanked by regions of hydrophobicity. A domain of this general sequence can be found in an extremely wide range of proteins (Gill *et al.*, 1986). It has been suggested that this is the consensus sequence for a nucleotide binding site (Moller & Amons, 1985). This sequence is also found in the ORF1 (*ftsY*) gene product (figure 9:1(c)).

Several of the nine proteins shown in figure 9:1(b), have been shown to bind ATP (Higgins *et al.*, 1985) and it seems highly probable that ATP-binding, and perhaps ATP-hydrolysis plays a role in the function of all these proteins. Of the extensive list of proteins now identified as containing a nucleotide-binding consensus sequence (Gill *et al.*, 1986), only the nine proteins presented here have so far been shown to share additional conservation of amino acid sequence, which extends over

Figure 9:1(a) The location of certain ATP-binding proteins with respect to adjacent genes.

The arrows indicate the direction and extent of transcription. Genes encoding the ATP-binding proteins are hatched. Abbreviations: Rbs = ribose transport; Opp = oligopeptide permease; His = histidine transport; Pst = phosphate transport; Mal = maltose transport; Fts = cell division; Nod = nodulation in *Rhizobium*; Hly = haemolysin synthesis and secretion. The diagram is not drawn to scale. Taken from Higgins *et al.*, 1986.



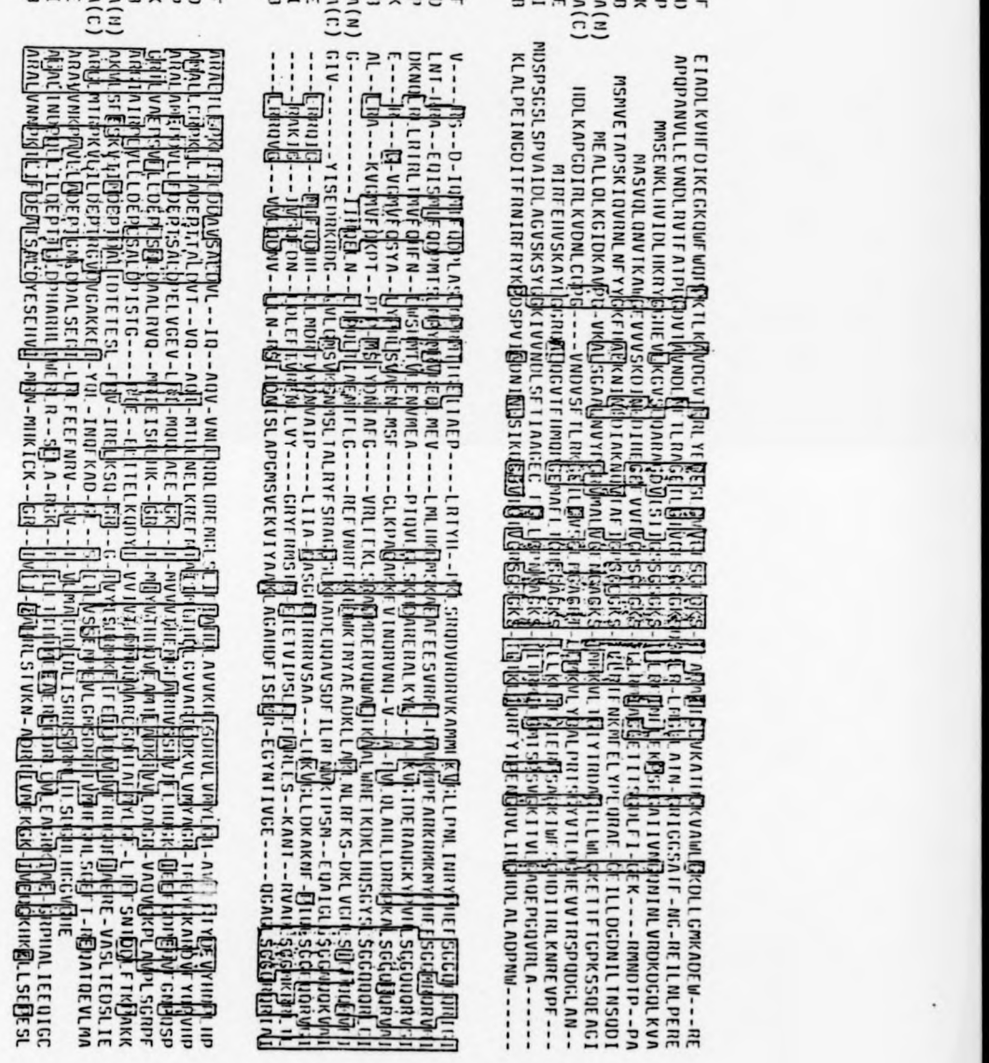


Figure 9:1(b) Sequence homology between ATP-binding proteins.

Proteins are aligned to illustrate their homology. Conserved amino acids are boxed. The sequences shown are OppF and OppD = *S. typhimurium* oligopeptide permease; HisP = *S. typhimurium* histidine transport; MalK = *E. coli* maltose transport; PstB = *E. coli* phosphate transport; RbsA = *E. coli* ribose transport; FtsE = *E. coli* cell division; NodI = *Rhizobium leguminosarum* nodulation; HlyB = *E. coli* haemolysin secretion. Taken from Higgins *et al.*, 1986.

Figure 9:1(c) Consensus sequence for a nucleotide-binding site as seen in FtsY and FtsE proteins.

The nucleotide-binding site in FtsY protein is located between residues 293 and 313 and in FtsE protein, between residues 32 and 52. Taken from Gill *et al.*, 1986.

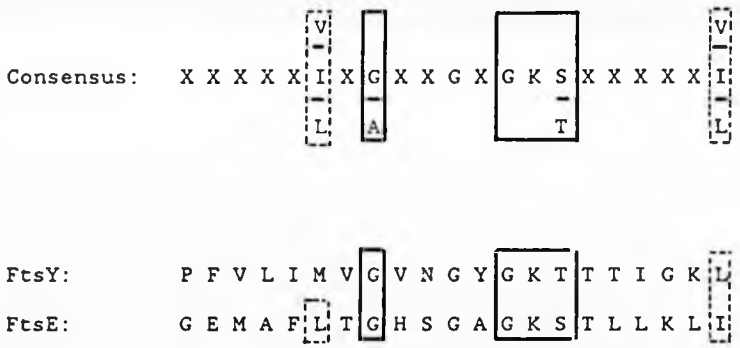
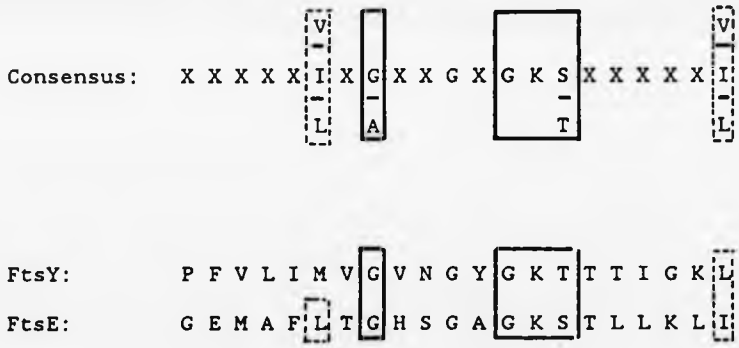


Figure 9:1(c) Consensus sequence for a nucleotide-binding site as seen in FtsY and FtsE proteins.

The nucleotide-binding site in FtsY protein is located between residues 293 and 313 and in FtsE protein, between residues 32 and 52. Taken from Gill *et al.*, 1986.



almost the entire length of each protein.

A second domain strongly conserved in all nine proteins listed in figure 9:1(b), extends over 35 amino acids. Initially this domain was ascribed a role in substrate binding (Walker *et al.*, 1982; Higgins *et al.*, 1985). Recent findings however, suggest that an alternative function for this domain is in membrane binding, because several members of this protein family have now been found to be associated with the inner membrane. These include: HisP (Ames & Nikaido, 1978), MalK (Bavoil *et al.*, 1980), OppD (Higgins *et al.*, 1985) and PstB (Surin *et al.*, 1985). Recently, HlyB has also been shown to be associated with the inner membrane (N. Mackman, pers. comm.). It was therefore important to investigate the localisation of FtsE and the other proteins in the Fts operon.

Many proteins have now been ascribed a subcellular location within the *E. coli* cell. Proteins can be localised to the cytoplasm, periplasm, the cytoplasmic (inner) membrane and the outer membrane. Centrifugation allows the division of a bacterial cell lysate into two fractions, a pellet which is composed of the bacterial cell envelope (inner and outer membranes, and cell wall components), and a supernatant containing cytoplasmic and periplasmic components. Several methods now exist which enable the separation of the inner and outer membranes and these include: isopycnic centrifugation in sucrose gradients (Osborn & Munson, 1974), electrophoresis (Joseleau Petit & Kepes, 1975) and selective solubilisation in detergent (Schnaitman, 1971; Filip *et al.*, 1973). Periplasmic proteins can be released using osmotic shock procedures (Neu & Heppel, 1965). The relative merits of these methods have been discussed (Silhavy *et al.*, 1983) and, in general, findings from such experiments involving the disruption of cells to yield subcellular

fractions should be treated with caution. Nevertheless such experiments are useful and have been used with great success.

In this study, the maxicell procedure was used to preferentially label plasmid coded proteins and the cell envelopes were prepared as described in sections 2:25 and 2:27. Further fractionation techniques were then used to locate the individual proteins within the bacterial cell envelope, largely based on the differential solubility of inner and outer membrane proteins in several detergents. This technique (Filip *et al.*, 1973) is more applicable for use with maxicell cultures. The results of each experiment are presented and discussed individually in the following sections, and the general conclusions to be made from the fractionation techniques employed, are described in section 9:6.

9:2 Preparation of cell envelopes from maxicells carrying pDG1/pDB1

9:2:1 Results

Maxicell samples of pDG1/pDB1 were made and cell envelopes were prepared as described in section 2:25. The centrifugation step in this procedure yielded a pellet consisting of cell envelope particles and a supernatant containing cytoplasmic and periplasmic proteins. The fractions were prepared in phosphate buffer and 10 mM magnesium sulphate ($MgSO_4$) (Churchward & Holland, 1976) and analysed using SDS-PAGE. Subsequent staining of the gels with Coomassie brilliant blue (section 2:24:3), revealed different protein profiles for each fraction and figure 9:2:1(a) is an autoradiogram showing the proteins coded by pDG1 distributed between the envelope and soluble fractions. Figure 9:2:1(b) also shows the effect of successive washing procedures, on the proteins

Figure 9:2:1(a) Autoradiogram of ^{35}S-methionine labelled maxicells of pDG1 separated into soluble and envelope fractions.

Samples were analysed by SDS-PAGE using an 11% (w/v) gel and subsequent fluorography. Track 1 = pDG1 maxicells; track 2 = soluble fraction; track 3 = envelope fraction. Subcellular fractions were prepared in the presence of Mg^{++}.

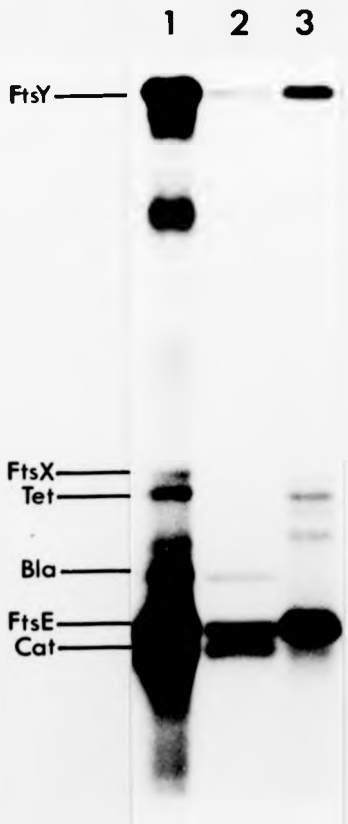


Figure 9:2:1(a) Autoradiogram of ^{35}S-methionine labelled maxicells of pDG1 separated into soluble and envelope fractions.

Samples were analysed by SDS-PAGE using an 11% (w/v) gel and subsequent fluorography. Track 1 = pDG1 maxicells; track 2 = soluble fraction; track 3 = envelope fraction. Subcellular fractions were prepared in the presence of Mg^{++}.

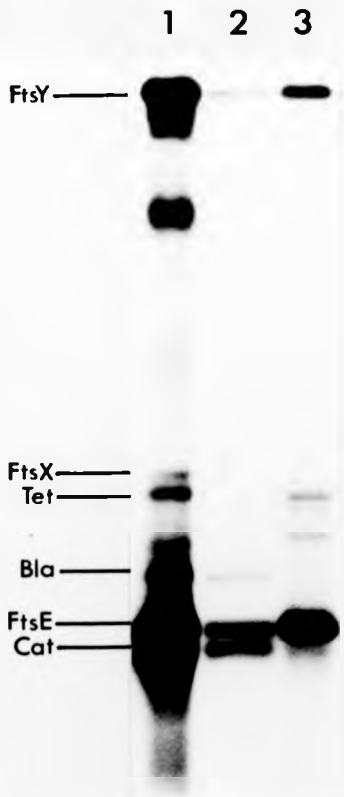


Figure 9:2:1(b) Autoradiogram of ^{35}S-methionine labelled maxicells of pBR322 and pDB1 separated into soluble and envelope fractions.

Samples were analysed by SDS-PAGE using a 10-30% (w/v) gradient gel. Track 1 - pBR322 maxicells; track 2 - soluble fraction; track 3 - envelope fraction.

Track 4 - pDB1 maxicells; tracks 5-7 - soluble fraction of pDB1 maxicells; tracks 8-10 - envelope fraction of pDB1 maxicells after three successive washes during the preparation. Subcellular fractions were prepared in the presence of Mg^{++}.

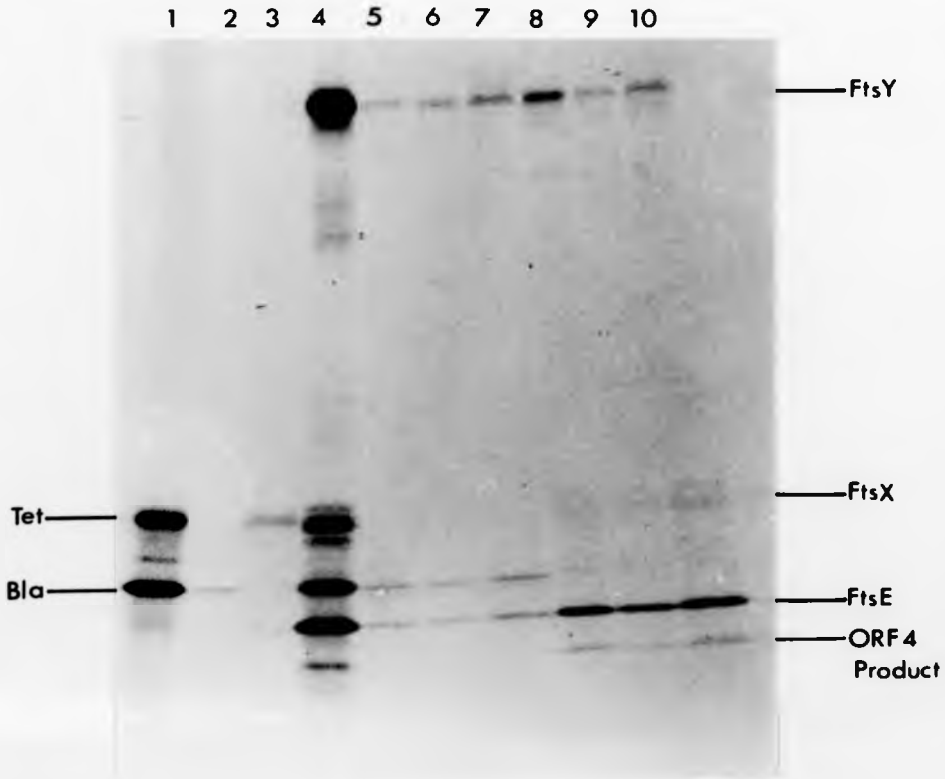
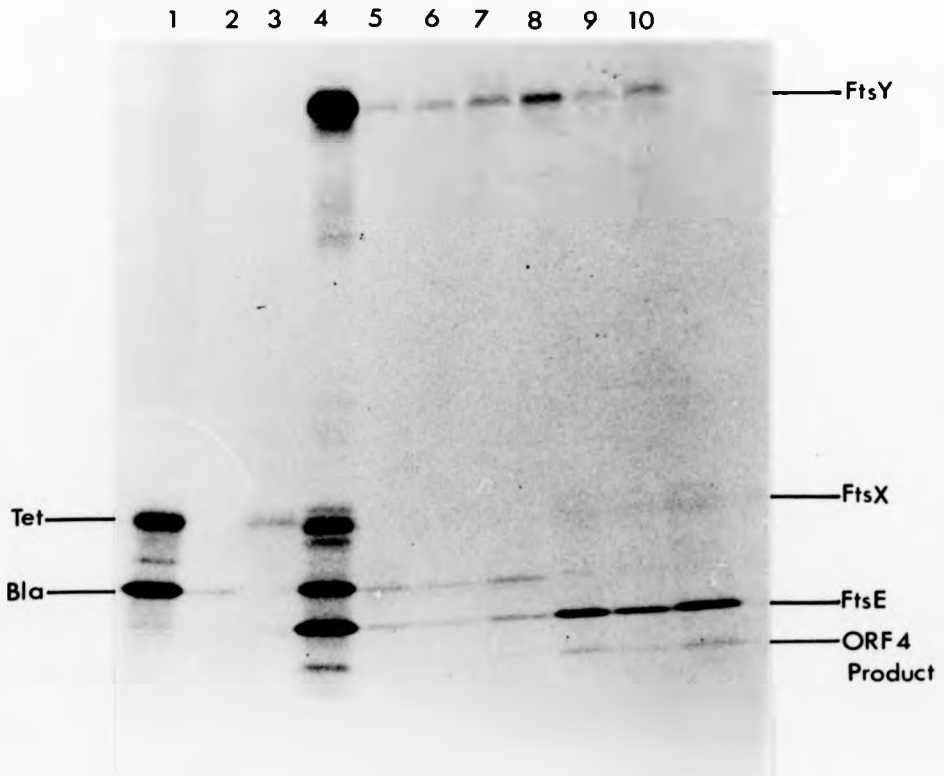


Figure 9:2:1(b) Autoradiogram of ^{35}S-methionine labelled maxicells of pBR322 and pDB1 separated into soluble and envelope fractions.

Samples were analysed by SDS-PAGE using a 10-30% (w/v) gradient gel. Track 1 = pBR322 maxicells; track 2 = soluble fraction; track 3 = envelope fraction.

Track 4 = pDB1 maxicells; tracks 5-7 = soluble fraction of pDB1 maxicells; tracks 8-10 = envelope fraction of pDB1 maxicells after three successive washes during the preparation. Subcellular fractions were prepared in the presence of Mg^{++}.



found in the envelope fraction. It was seen that the washes did not affect the distribution of the protein and there was no reduction in the affinity of any of the proteins for the envelope fraction.

The relative distribution of the labelled proteins between soluble and envelope fractions was as follows:

(a) The Mr 92000 and Mr 25000 proteins corresponding to FtsY and FtsE respectively, were located mainly in the envelope fraction, and a smaller proportion of each was found in the soluble fraction.
(b) The Mr 38000 FtsX protein was found exclusively in the envelope fraction.
(c) The Mr 20000 ORF4 product was also found exclusively in the envelope fraction.

Other proteins coded by pDG1, namely those encoded by vector DNA, were also fractionated in these experiments and provided internal controls or standards. The products of the Ap-resistance gene (Bla), the Cm-resistance gene (Cat) and the Tc-resistance gene (Tet) have been localised to specific subcellular compartments previously, as discussed by Stoker *et al.* (1983b), and Silhavy *et al.* (1983). In this study, the periplasmic Bla and the cytoplasmic Cat proteins were both located in the soluble fraction as expected (figure 9:2:1(a)), whereas the inner membrane-associated Tet protein was found exclusively in the envelope fraction. Thus, the observed fractionation of these three proteins corresponds well with that found previously.

9:2:2 Discussion

The data show that all four proteins encoded by the 4.5 kb *Hin*dIII

fragment were found associated with the bacterial cell envelope. The possibility that any of these proteins were "contaminating" the envelope fraction was eliminated by the repeated washing of the membranes, to remove loosely associated proteins. It can be seen that these measures did not reduce the association of any of the proteins with the cell envelope. However, approximately one third of the total labelled FtsY and FtsE proteins seen in such experiments was found in the soluble fraction, whereas the *ftsX* and ORF4 products were found exclusively in the envelope fraction. There are several possible explanations for these observations.

Firstly, the use of sonication to break open the cells prior to centrifugation (section 2:25) is often thought to be the cause of a 20-30% contamination of soluble fractions with membrane-associated proteins (Churchward & Holland, 1973). Perhaps the sonication procedure was too harsh for the membrane-association of FtsY and FtsE in these experiments. However, this finding is specific to these proteins because no such contamination of the soluble fraction was found in the case of FtsX and Tet, which were both found exclusively in the membrane fraction. One way of avoiding such disruptive treatment would be to use the method of spheroplasting (Osborn *et al.*, 1972) which has been found to be less harsh (J. Pratt, pers. comm.).

Secondly, the use of multicopy plasmid vectors and the consequent overproduction of plasmid-coded proteins may have affected the localisation of the proteins in some way. For example, it is possible that there are only a limited number of sites in the membrane at which FtsY and/or FtsE can be inserted. Therefore, when these sites are saturated, excess protein would accumulate in the cytoplasm. This type

of explanation emphasises the dangers in drawing conclusions from data obtained using artificial systems. One reported example of such an artifact is the accumulation of precursor Bla protein in the inner membrane of mini-cells (Silhavy *et al.*, 1983).

An experiment to investigate the possibility that overproduction of FtsY or FtsE leads to their aberrant translocation within the cell, would be to repeat the above experiments using the 4.5 kb fragment sub-cloned into a low copy number vector. Low copy number vectors such as pLG338 (Stoker *et al.*, 1982) have already been successfully used to localise certain *E. coli* membrane proteins (N. Mackmann, pers. comm.). This type of experiment could also be undertaken using a temperature-inducible vector such as pOU71 (Larson *et al.*, 1984). At a growth temperature of 30°C, the copy number of this plasmid is one. The copy number increases linearly with temperature (above 37°C) reaching up to 1000 copies per cell at 42°C. The 4.5 kb fragment could be sub-cloned into such a vector, and maxicells labelled first at 30°C and then at increasing growth temperature before sonication and fractionation. It should therefore be possible to follow the localisation of the proteins before, during, and after they are overproduced.

A third explanation for the observed protein localisations is that the presence of some FtsY and FtsE protein in the soluble fraction is not an artifact. Some of the protein may accumulate in the cytoplasm, for example prior to insertion into the membrane. One way to test this hypothesis would be to pulse chase maxicells carrying pDG1 with ^{35}S-methionine and to prepare envelopes and soluble fractions at various time intervals after labelling. It might be possible to follow proteins first seen in the cytoplasm, being "chased" into the membrane. Such an

experiment would probably require several refinements, but pulse chase and subsequent fractionation procedures are practical, as has been shown previously (Jackson *et al.*, 1986).

The various explanations presented here are currently being tested.

9:3 Separation of envelopes into inner and outer membrane fractions

9:3:1 Results

The various methods now available for the further fractionation of bacterial cell envelopes were listed in section 9:1. The separation of inner and outer membrane proteins on the basis of solubility in the detergent sodium lauryl sarkosinate (sarkosyl) (Churchward & Holland, 1973; Filip *et al.*, 1973) is convenient for use with the maxicell procedure and the small culture volumes involved. This technique has been used to successfully identify the locations of several proteins (Spratt, 1977; Jones & Holland, 1985; Jackson *et al.*, 1985, 1986; Mackmann *et al.*, 1985) and was used to separate inner and outer membrane proteins of cell envelopes prepared from pDG1 maxicell cultures (figure 9:3:1(a)). It can be seen that none of the Fts proteins were found in the outer membrane fraction, and that all the proteins were located in the inner cytoplasmic membrane. These results were highly reproducible.

Internal controls in this experiment were the Tet protein from pDG1, found in the inner membrane as expected, and the outer membrane proteins, OmpA and LamB. The OmpA protein is encoded by plasmid pTU101 (Bremer *et al.*, 1980)and the fractionation results are shown in figure 9:3:1(c). The maltose and bacteriophage λ receptor protein LamB is

Figure 9:3:1(a) Autoradiogram of ^{35}S-methionine labelled maxicells of pDG1 separated into fractions enriched for inner and outer membranes.

Samples were analysed by SDS-PAGE using an 11% (w/v) gel and subsequent fluorography. Track 1 = pDG1 maxicells; track 2 = soluble fraction; track 3 = envelope fraction; track 4 = inner membrane-enriched fraction; track 5 = outer membrane-enriched fraction. All subcellular fractions were prepared in the presence of Mg^{++}.

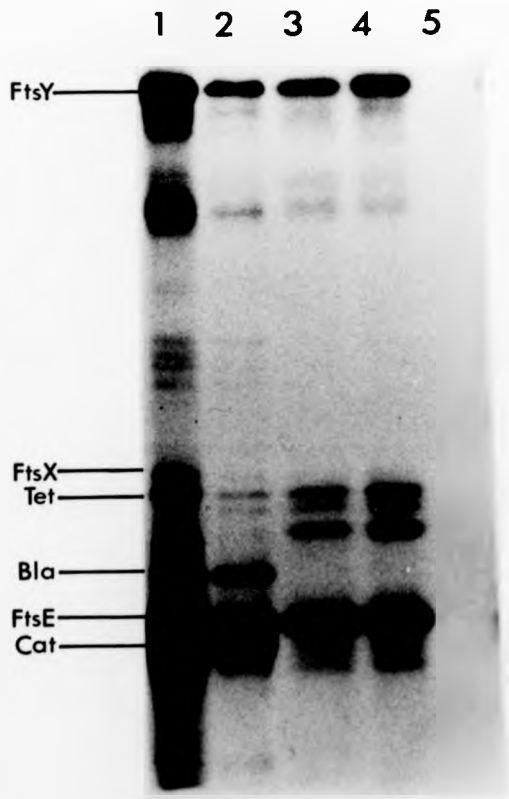


Figure 9:3:1(a) Autoradiogram of ^{35}S-methionine labelled maxicells of pDG1 separated into fractions enriched for inner and outer membranes.

Samples were analysed by SDS-PAGE using an 11% (w/v) gel and subsequent fluorography. Track 1 = pDG1 maxicells; track 2 = soluble fraction; track 3 = envelope fraction; track 4 = inner membrane-enriched fraction; track 5 = outer membrane-enriched fraction. All subcellular fractions were prepared in the presence of Mg^{++}.

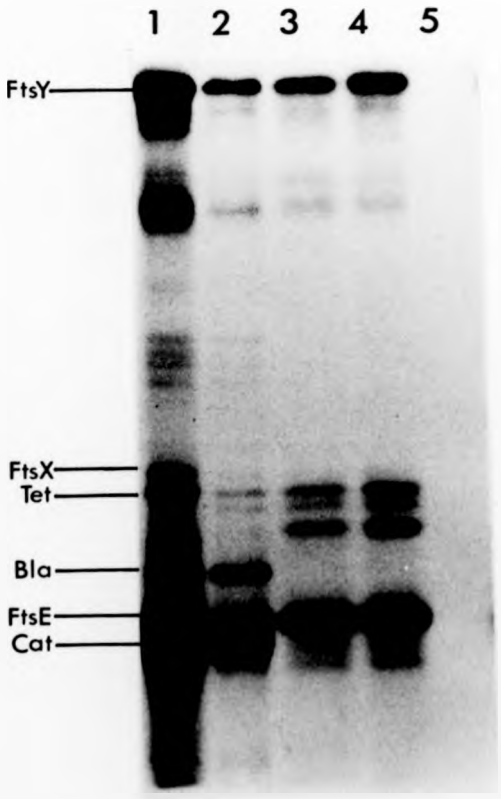


Figure 9:3:1(b) Autoradiogram of ^{35}S-methionine labelled maxicells of pHCP2 separated into fractions enriched for inner and outer membranes.

Samples were analysed by SDS-PAGE using an 11% (w/v) gel and subsequent fluorography. Track 1 = pHCP2 maxicells; track 2 = soluble fraction; track 3 = envelope fraction; track 4 = inner membrane-enriched fraction; track 5 = outer membrane-enriched fraction. Subcellular fractions were prepared in the presence of Mg^{++}.

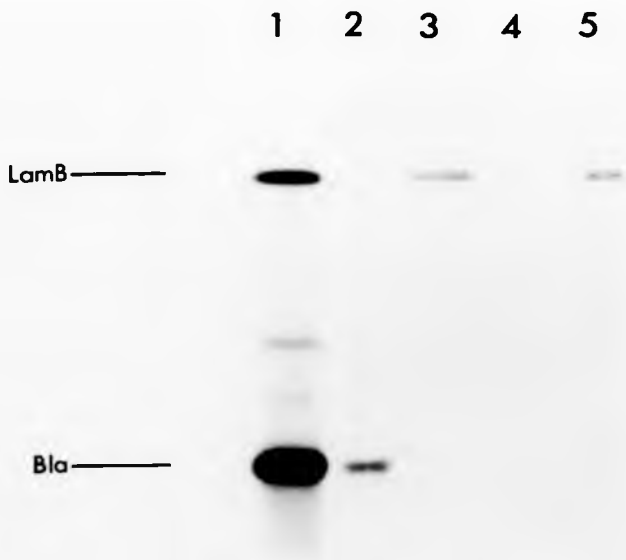
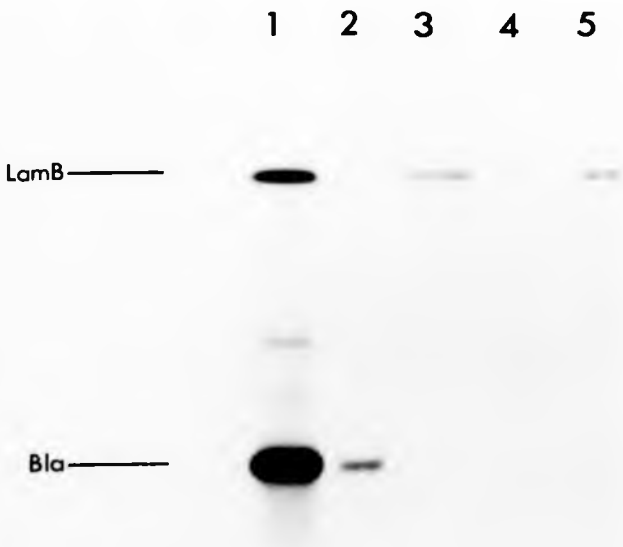


Figure 9:3:1(b) Autoradiogram of ^{35}S-methionine labelled maxicells of pHCP2 separated into fractions enriched for inner and outer membranes.

Samples were analysed by SDS-PAGE using an 11% (w/v) gel and subsequent fluorography. Track 1 = pHCP2 maxicells; track 2 = soluble fraction; track 3 = envelope fraction; track 4 = inner membrane-enriched fraction; track 5 = outer membrane-enriched fraction. Subcellular fractions were prepared in the presence of Mg^{++}.



encoded by pHCP2 (Clement *et al.*, 1982), and maxicells carrying this plasmid were fractionated as seen in figure 9:3:1(b). Both proteins are found mainly in the outer membrane fraction except for a small proportion in both cases which is found in the inner membrane fraction.

9:3:2 Discussion

These results showed that none of the proteins encoded by pDG1 were found associated with the outer membrane and this suggested that the four Fts envelope-associated proteins are located in the inner (cytoplasmic) membrane. Proteins of known location within the cell (Tet, OmpA and LamB) were all localised as expected in these experiments although a small degree of contamination of the inner membrane fractions with the outer membrane protein, OmpA was observed. Such observations have also been made previously by Halegoua and Inouye, (1979), who showed that under certain conditions, precursor forms of OmpA usually associated with the outer membrane were nevertheless soluble in sarkosyl.

The localisation of all four proteins encoded by the 4.5 kb fragment, to the inner membrane was not surprising because none of the genes for these proteins appeared to have a signal sequence (Appendix I). Therefore it was unlikely that they were translocated across the inner membrane. The possibility remained, however, that one or more of these proteins was somehow located in the periplasm. This was tested and the results are described in section 9:4.

Figure 9:3:1(c) Autoradiogram of ^{35}S-methionine labelled maxicells of pTU101 separated into fractions enriched for inner and outer membranes.

Samples were analysed by SDS-PAGE using an 11% (w/v) gel and subsequent fluorography. Track 1 = soluble fraction of pTU101 maxicells; track 2 = envelope fraction; track 3 = inner membrane-enriched fraction; track 4 = outer membrane-enriched fraction. Subcellular fractions were prepared in the presence of Mg^{++}.

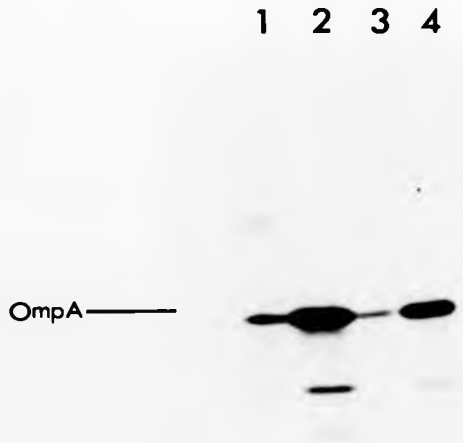
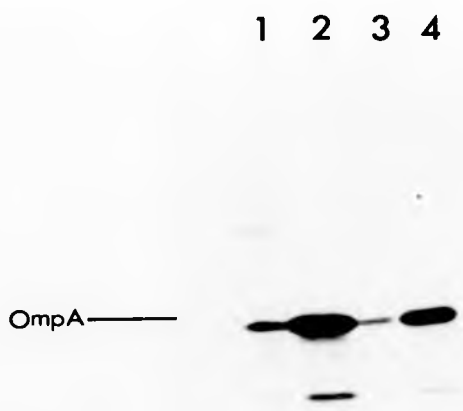


Figure 9:3:1(c) Autoradiogram of ^{35}S-methionine labelled maxicells of pTU101 separated into fractions enriched for inner and outer membranes.

Samples were analysed by SDS-PAGE using an 11% (w/v) gel and subsequent fluorography. Track 1 - soluble fraction of pTU101 maxicells; track 2 - envelope fraction; track 3 - inner membrane-enriched fraction; track 4 - outer membrane-enriched fraction. Subcellular fractions were prepared in the presence of Mg^{++}.



9:4 Isolation of periplasmic proteins

9:4:1 Results

The soluble fraction of a cell lysate contained both cytoplasmic and periplasmic proteins and therefore it was possible that the small amounts of FtsY and FtsE found in this fraction (section 9:2:1) were actually located in the periplasm. Periplasmic proteins were prepared using the osmotic shock procedure of Neu and Heppel (1965). This technique was adapted for use with labelled maxicell cultures as described in section 2:28.

Figure 9:4:1 is an autoradiogram of pDG1 maxicells and periplasmic fraction. It was observed that the periplasmic protein Bla, which is usually present in the soluble fraction, was located in the periplasmic fraction. A very small proportion of the FtsE and Cat protein was also observed.

9:4:2 Discussion

Previous studies have demonstrated that all periplasmic proteins and outer membrane proteins in Gram-negative bacteria require an amino-terminal signal sequence for translocation (Pugsley & Schwartz, 1985). None of the Fts proteins appear to have such a sequence, and therefore it is probable that the small proportion of FtsE observed in the periplasm (figure 9:4:1), is the result of cell lysis occurring during the osmotic shock procedure. The presence of a similar proportion of the cytoplasmic Cat protein, supports this suggestion. Other workers have experienced similar 'contamination' problems when attempting to isolate periplasmic fractions in this way (C. Jones, pers. comm.). Thus, results obtained using this technique are sometimes variable and

Figure 9:4:1 Autoradiogram of ^{35}S-methionine labelled periplasmic proteins of maxicells carrying pDG1.

Samples were analysed by SDS-PAGE using an 11% (w/v) gel and subsequent fluorography. Track 1 = pDG1 maxicells; track 2 = periplasmic fraction.

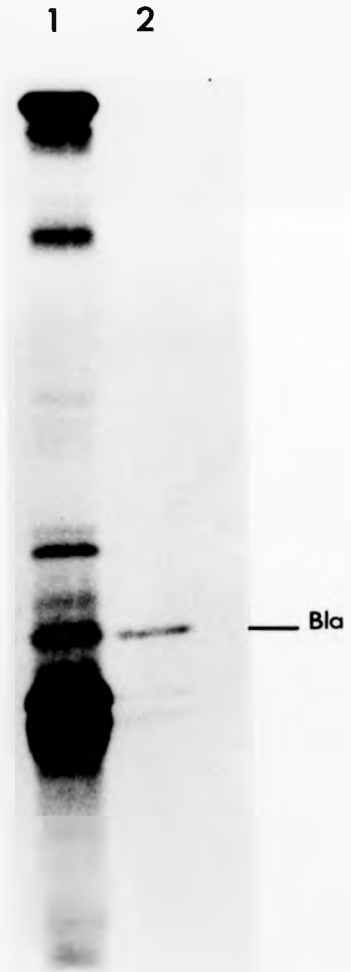
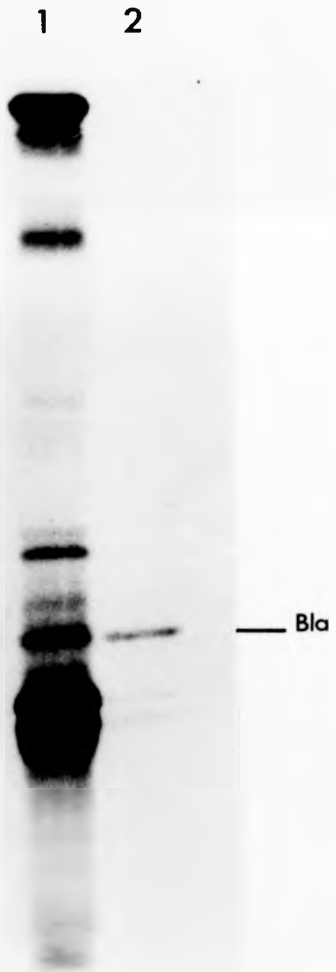


Figure 9:4:1 Autoradiogram of ^{35}S-methionine labelled periplasmic proteins of maxicells carrying pDG1.

Samples were analysed by SDS-PAGE using an 11% (w/v) gel and subsequent fluorography. Track 1 = pDG1 maxicells; track 2 = periplasmic fraction.



therefore it is unlikely that any of the proteins encoded by the 4.5 kb fragment are located in the periplasm.

9:5 Effect of divalent ions on the localisation of the Fts proteins

9:5:1 Results

The localisation of the proteins in the inner membrane was further investigated using a variety of washing procedures. Filip *et al.*, (1973) described how the outer membrane of *E. coli* was resistant to solubilisation by the detergent sarkosyl, although the basis for this resistance was not known. Under similar conditions, the ionic detergent SDS and the neutral detergent Triton X-100 were observed to solubilise all membrane proteins. Other experiments (Filip *et al.*, 1973) indicated that solubilisation of inner membrane material with sarkosyl in the presence of Mg^{++} ions, partially protected fragile membrane-associated structures and that this was probably due to the formation of Mg^{2+}-sarkosyl crystals. This property was exploited by Jones and Holland, (1985) to prepare partially protected membranes in the presence and absence of Mg^{++} ions.

Membranes of pDG1-containing maxicells were prepared in the presence and absence of Mg^{++} ions. Figure 9:5:1(a) is an autoradiogram of various subcellular fractions of pDG1 maxicells prepared in the absence of Mg^{++}. This autoradiogram should be compared with figure 9:3:1(a), where fractions were prepared in the presence of Mg^{++}. It can be seen that in the absence of Mg^{++}, FtsY, FtsE and FtsX are no longer associated with the membrane fraction to any great extent, and are found mainly in the soluble fraction. Figure 9:5:1(b) shows results of a similar experiment

Figure 9:5:1(a) Autoradiogram of ^{35}S-methionine labelled maxicells of pDG1 separated into various subcellular fractions in the absence of Mg^{++}.

Samples were analysed by SDS-PAGE using an 11% (w/v) gel and subsequent fluorography. Track 1 = pDG1 maxicells; track 2 = soluble fraction; track 3 = envelope fraction; track 4 = inner membrane-enriched fraction. This figure should be compared with figure 9:3:1(a) which shows identical samples prepared in the presence of Mg^{++}.

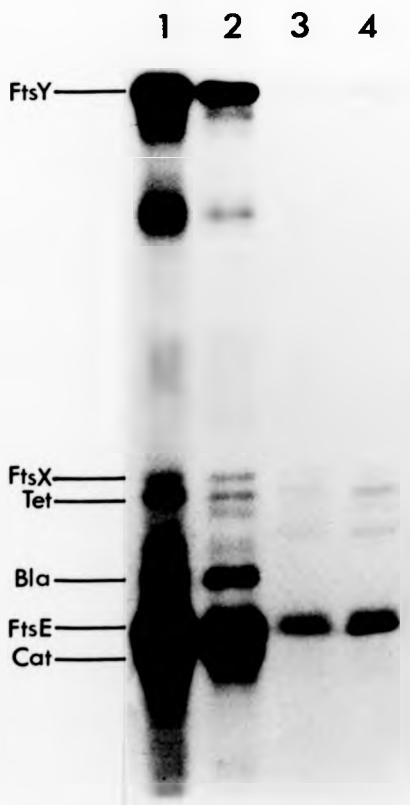


Figure 9:5:1(a) Autoradiogram of ^{35}S-methionine labelled maxicells of pDG1 separated into various subcellular fractions in the absence of Mg^{++}.

Samples were analysed by SDS-PAGE using an 11% (w/v) gel and subsequent fluorography. Track 1 = pDG1 maxicells; track 2 = soluble fraction; track 3 = envelope fraction; track 4 = inner membrane-enriched fraction. This figure should be compared with figure 9:3:1(a) which shows identical samples prepared in the presence of Mg^{++}.

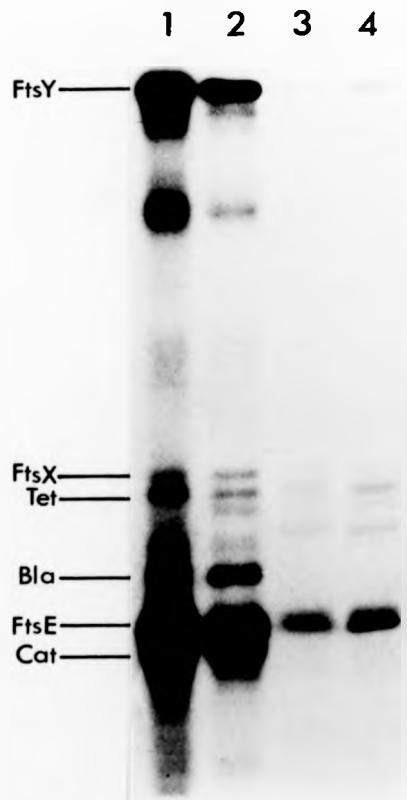


Figure 9:5:1(b) Autoradiogram of ^{35}S-methionine labelled maxicells of pHCP2 separated into various subcellular fractions in the presence and absence of Mg^{++}.

Samples were analysed by SDS-PAGE using an 11% (w/v) gel and subsequent fluorography. Tracks 1 and 6 = pHCP2 maxicells; tracks 2 and 7 = soluble fraction; tracks 3 and 8 = envelope fraction; tracks 4 and 9 = inner membrane-enriched fraction; tracks 5 and 10 = outer membrane-enriched fraction. Fractions in tracks 2-5 were prepared in the presence of Mg^{++} whereas fractions in tracks 7-10 were prepared in the absence of Mg^{++}.

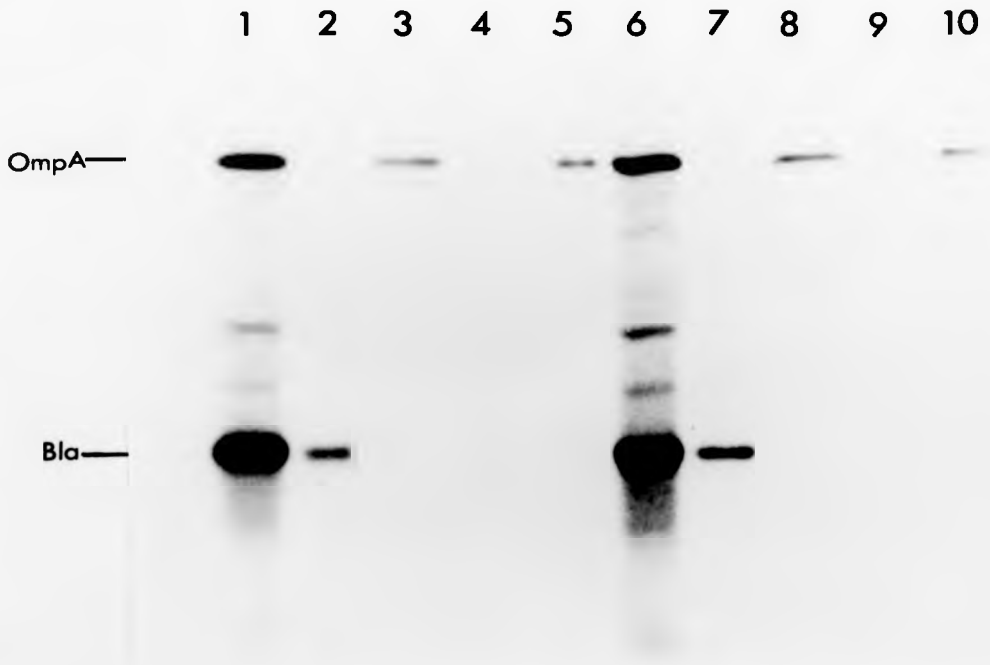
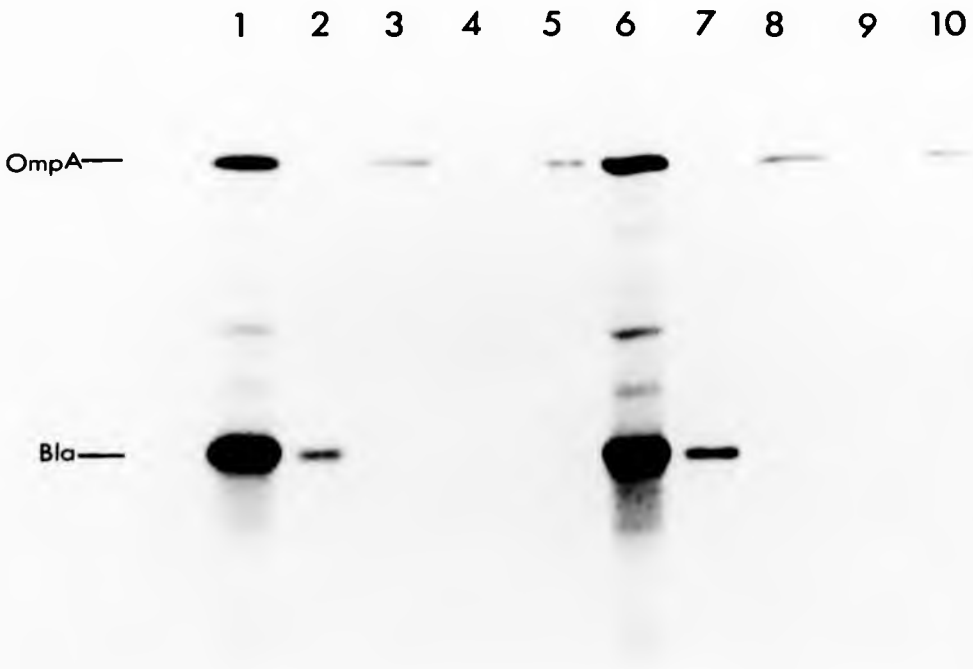


Figure 9:5:1(b) Autoradiogram of ^{35}S-methionine labelled maxicells of pHCP2 separated into various subcellular fractions in the presence and absence of Mg^{++}.

Samples were analysed by SDS-PAGE using an 11% (w/v) gel and subsequent fluorography. Tracks 1 and 6 = pHCP2 maxicells; tracks 2 and 7 = soluble fraction; tracks 3 and 8 = envelope fraction; tracks 4 and 9 = inner membrane-enriched fraction; tracks 5 and 10 = outer membrane-enriched fraction. Fractions in tracks 2-5 were prepared in the presence of Mg^{++} whereas fractions in tracks 7-10 were prepared in the absence of Mg^{++}.



involving pHCP2 encoding the LamB protein. The fractions prepared in the presence and absence of 10 mM Mg^{++} appeared identical.

Conditions of low ionic strength have been shown to be important in achieving good separation of inner and outer membranes (Osborn *et al.*, 1972; Filip *et al.*, 1973). However, the distribution of the Fts proteins between the fractions was not affected by changing the concentration of sodium chloride (NaCl) in the preparation buffer (data not shown).

9:5:2 Discussion

In section 9:2:1 it was seen that the FtsY, FtsE and FtsX proteins were mainly membrane-associated but that a small proportion of FtsY and FtsE was also found in the soluble fraction (figure 9;2:1(a)). When membranes were prepared in the absence of Mg^{++}, the affinity of these proteins for the membrane was reduced (figure 9:5:1(a)). However, when membranes of pHCP2-maxicells were prepared in a similar way, the distribution of proteins encoded by this plasmid was unaffected.

The removal of proteins from the envelope by alteration of the preparation buffer may reflect the type of membrane association involved. Relatively weak forces, such as ionic or hydrogen bonding are affected by factors such as temperature, ionic strength and pH of the environment (Salton, 1971). For example, the Mr 92000 SecA protein was located mainly in the membrane in the presence of 100 mM NaCl but mainly in the cytoplasm in buffers of lower ionic strength (Oliver & Beckwith, 1982) and these workers concluded that SecA was a peripheral membrane protein. The association of SecA with the inner membrane was also reduced in the absence of 10 mM Mg^{++} (Jones & Holland, 1985) since the

presence of Mg^{++} ions is thought to stabilise certain 'membrane-binding' proteins (Salton, 1971) as distinct from integral membrane proteins. In the absence of the stabilising effect of Mg^{++}, loosely-associated membrane proteins are probably removed during successive washing procedures. During the initial separation of soluble and envelope fractions, such membrane proteins are collected in the soluble fraction.

In this study, the presence of Mg^{++} ions appeared to preserve the affinity of the Fts proteins for the inner membrane, perhaps in a similar way to that of SecA. However, it must be noted that the concentrations of Mg^{++} and NaCl used may not have been optimal for the interaction of the proteins with the membrane and this should be taken into consideration.

9:6 Concluding remarks

All the experiments presented in this chapter were based on the assumption that plasmid-coded proteins labelled in maxicells are translocated in a similar way to that of normal cells. Several objections have been raised to the use of these standard fractionation techniques (Silhavy *et al.*, 1983; Pugsley & Schwartz, 1985) although the use of 'control' proteins (section 9:2 and 9:3) and cautious interpretation of data, has allowed the localisation of many proteins in this way. Recently, the use of more sophisticated techniques involving immunogold-labelling (Roth, 1983) have also been used to determine protein location within cells (Walker & Bleesley, 1983).

The data presented in this chapter indicated that all four proteins

encoded by the 4.5 kb fragment were located in the envelope fraction (section 9:2) and more precisely, in the inner membrane (section 9:3). Although FtsX and the ORF4 product were found exclusively associated with the inner membrane, the FtsE and FtsY proteins were also found in lesser amounts in the soluble fraction. The membrane associations are highly reproducible, but is is possible that the cytoplasmic localisation is an artifact, as discussed in section 9:2:2.

The fractionation of the Fts proteins appears to be unusual because it is affected by the presence of Mg^{++} ions, but not by the ionic strength of the preparation buffer. The findings suggest that these proteins are peripherally associated with the inner membrane, perhaps in a way similar to that of SecA (Oliver & Beckwith, 1982).

The finding that FtsE was membrane-associated was perhaps not surprising because, as discussed in section 9:1, all the proteins which share homology with FtsE (presented in figure 9:1(b)) have also been found associated with the membrane. However it is interesting that all three Fts proteins, and the ORF4 product are membrane-located. Hydropathy plots can sometimes be useful as a general indication of hydrophobicity of a given protein, or in the identification of hydrophobic domains, which may indicate a membrane location for that protein. The hydropathy plots for all four proteins are presented in Appendix III according to the method of Kyte and Doolittle (1982). The computer programme continuously determines the average hydrophobicity of the protein as it advances from the amino- to carboxy-terminus. Where the hydropathy of a given amino acid segment averages a high positive value, there is a high probability that it respresents a membrane spanning region (Kyte & Doolittle, 1982). The hydropathy plots show that FtsX and the ORF4

product are comparatively hydrophobic except for a hydrophilic amino-terminus. This may suggest that the membrane-association observed for these two proteins is integral in nature. The FtsY and FtsE proteins, however, do not appear hydrophobic overall, but have several hydrophobic domains throughout. This also correlates with the observed membrane-association of these two proteins.

In section 9:1, it was noted that FtsE was a member of a family of nine related proteins which showed strong homology (Higgins *et al.*, 1986). It was also noted that several of these proteins were located in the inner membrane and some of these were also associated with other proteins encoded within the same operon. For example, the HisP protein was found to be associated with HisJ (Ames & Spudich, 1976) and the MalK protein was found to interact with MalG (Shuman & Silhavy, 1981). See also figure 9:1(a).

The observation that FtsE was also found in the inner membrane (section 9:3:1:) raises the question as to whether FtsE might also be associated with adjacent gene products, for example, FtsY or FtsX. Preliminary evidence for such an interaction has been obtained from recent experiments in this laboratory (not presented in this thesis). The fractionation experiments described in this chapter involved the use of maxicells of pDG1. Similar experiments were carried out using maxicells containing other plasmids deleted for either *ftsY*, *ftsE* or *ftsX*. Such experiments suggested a possible interaction between the FtsE and FtsY proteins.

In order to study such an interaction genetically, it should be possible to isolate mutations in *ftsY* which suppress *ftsE* mutations. This

strategy has been used to successfully identify new components of the export machinery in *E. coli* (Brickman *et al.*, 1984; Beckwith & Ferro-Novick, 1986) and to study the interaction between components of the maltose periplasmic binding protein-dependent transport system. Treptow and Shuman (1985) isolated two classes of extragenic suppressor of mutations in the maltose-binding protein; and these mapped in the *malF* and *malG* proteins. MalF and MalG are membrane components of this transport system. MalG has also been shown to interact with MalK (Shuman & Silhavy, 1981). This approach has shown that these components function as a membrane complex in maltose transport and thus could be an important approach in the investigation of the functional interactions of the Fts proteins.

The results presented in this chapter suggested that the Fts proteins are all associated with the inner membrane. Removal of Mg^{++} from the preparation buffer causes a substantial reduction in the membrane affinity of all these proteins, but especially the FtsY protein. These data indicate a 'loose' or peripheral association of the proteins with the membrane. Such preliminary investigations provides a direction for further investigations into the roles played by FtsY, FtsE and FtsX in cell division because, although no function can be ascribed to these proteins, they may constitute a functional complex. The Fts proteins may interact in the membrane in a way similar to that observed for the His proteins of *S. typhimurium* or the Mal proteins of *E. coli* (section 9:1). Alternatively, the Fts proteins may be part of an active focus for many proteins involved in cell division (the hypothetical 'septalsome': see section 1:5) envisaged by Holland and Jones (1985).

CHAPTER 10

General Discussion

10:1 A summary of the major results

The major aim of this project was to characterise the essential genes located in the 76-minute region of the *E. coli* chromosome. Several essential genes including two cell division genes *ftsE* and *ftsS*, and the gene for the control of the heat shock regulon (*rpoH*) were located in this region (sections 1:19 and 1:20). A study of the genes in this region gave the following results:

(i) Mutations in *ftsE* were complemented by a 4.5 kb *Hin*dIII fragment which was subcloned from λpGS22 (section 3:2:2).

(ii) DNA sequence analysis of the 4.5 kb fragment showed several features suggestive of an operon (section 4:1:1). Three, closely arranged genes which had the same predicted direction of transcription, were shown to have a potential regulatory region 5' to the start of the first gene. This region showed considerable homology with the promoter/operator region of *E. coli trp* operon (Appendix II).

(iii) Tn*1000* mutagenesis and deletion studies showed that *ftsE* was the second gene in the group and that transposon insertions in ORF1 also affected expression of the *ftsE* gene downstream. Similarly, the third gene in the group was shown to be a novel cell division gene, *ftsX* and strains OV32 and JS10 were found to be defective in this gene (Chapter 5). Transposon insertions in ORF1 and *ftsE* also prevented expression of *ftsX*. This suggested that the three genes were co-transcribed and may represent an operon, and thus, the first gene was tentatively designated *ftsY*.

(iv) Comparison of the DNA sequence of the 4.5 kb fragment and the published sequence of the *rpoH* gene (Landick *et al.*, 1984), indicated an identical overlap of 66 bp. Restriction analysis of pLC31-16, a plasmid which complemented mutations in *ftsE* and *rpoH* confirmed the suggestion that the *ftsX* and *rpoH* genes are adjacent in the *E. coli* chromosome (section 3:2:4).

(v) The polypeptides encoded by *ftsE* and *ftsX* were identified by labelling with ^{35}S-methionine in maxicell experiments (Chapter 6). FtsE and FtsX were identified as proteins of Mr 25000 and Mr 38000 respectively. Both estimates compared favourably with those predicted by analysis of the sequence data.

(vi) The use of both *in vivo* and *in vitro* insertion mutagenesis demonstrated that the *ftsY* gene encoded a polypeptide of approximately twice the predicted relative molecular mass (Chapter 7). It is possible that the protein is a dimeric form of FtsY or a multimer of FtsY and some other protein species.

(vii) Subcellular fractionation studies showed that the FtsY, FtsE and FtsX proteins were mainly associated with the inner (cytoplasmic) membrane (section 9:3:1). A small proportion of FtsY and FtsE proteins was also found in the cytoplasm. The affinity of the Fts proteins for the membrane was shown to be reduced in the absence of Mg^{++}, suggesting that the membrane-association was peripheral in nature (section 9:5:1).

(viii) Detailed amino acid sequence analysis revealed that FtsE was a member of a family of nine related proteins which show strong homology (section 9:1). Several of these proteins are known to be located in the inner membrane and to bind nucleotides. The homology between these proteins consists of two strongly conserved domains. One domain is amino-terminal and contains the consensus sequence for a nucleotide binding site whereas the function of the other domain is more speculative.

10:2 Possible significance of the results

10:2:1 A new cell division operon

The most significant finding presented in this study was the identification of a novel cell division operon in the 76-minute region of the *E. coli* chromosome. In sections 1:19 and 1:20, several essential genes were described which were found to map in this region. These included the cell division genes *ftsE* and *ftsS*, the gene for control of the heat shock regulon (*rpoH*), *dnaM* and *fam*. Experiments described in chapter 3 confirmed that the position of the Fts operon was immmediately upstream of *rpoH*, and recently it has been shown that *fam* is an allele of the *rpoH* gene (Crickmore & Salmond, 1986). The exact positions of *ftsS* and *dnaM* are currently being investigated. It is possible that other previously unidentified genes also map in this region upstream of the Fts operon.

Localised mutagenesis experiments have been successful in the isolation of mutations in this 76-minute region, and several of these map in the *ftsE* gene (C. Bowler, pers. comm.). Further experiments are required to

obtain mutations in new genes including ORF4 and FtsY. It is imperative to establish whether or not *ftsY* is an essential gene and studies are in progress to test this.

In section 1:7, a gene cluster was described as a group of two or more genes of related function which constitute more than one independent operon. Several observations in this study suggest that the genes in the 76-minute region may represent a new morphogene cluster. These observations include the close association of five or six genes, involved in essential cellular processes and the existence of individual promoters for these genes. The *rpoH* gene possesses its own promoter as was shown by Neidhardt *et al.*, (1983a). The Fts operon must contain at least two promoters, one of which is located 5′ to the start of the *ftsY* gene (Appendix II) and one which allows co-transcription of *ftsE* and *ftsY* (Chapter 7). Close inspection of the DNA sequence of the operon (Appendix I) reveals that a gap of only two base pairs exists between the end of *ftsY* and the start of *ftsE*. Therefore, the *ftsE* promoter must be located in the coding region of the *ftsY* gene. This is analagous to the situation in the 2′-minute morphogene cluster, where one of the *ftsZ* gene promoters was located in the coding sequence of the upstream *ftsA* gene (see section 1:11).

A detailed analysis of the activity of the promoters in the Fts operon and the determination of the RNA transcripts produced in this region are now required. Such studies would be invaluable in order to begin to understand the regulation of the operon, and they might also reveal the significance (if any) of the close proximity between the *rpoH* gene and the Fts operon. Similar questions have been posed many times previously for genes of related function found in other clusters. Is there some

higher order of gene regulation which has not yet become apparent? Or is this clustering the result of unknown evolutionary forces once important in the development of the organism, but which are now obsolete? Many groups of investigators would be interested in the answer to these questions.

10:2:2 The nature of the FtsY protein

Identification of the Fts gene products showed that FtsE was a polypeptide of Mr 25000 and FtsX was a polypeptide of Mr 38000. These estimates correspond well with those predicted by analysis of the sequence data. However, in Chapter 7 it was conclusively shown that the *ftsY* gene encoded a polypeptide of Mr 92000, an estimate which was at least twice that predicted by the sequence data. As discussed in section 7:3:3, it is difficult to imagine how a gene of known sequence with a protein-coding capacity of Mr 48000 could encode a polypeptide of Mr 92000. It was therefore proposed that the large protein was either a dimer of FtsY or a multimer of FtsY and some other polypeptide species. Thus, very little is known about the processes involved in the assembly of the Mr 92000 protein but investigation of these processes should prove very interesting and will hopefully reveal more information about the functional significance of this phenomenon.

Several experiments were described in Chapter 7, which should ascertain whether or not the FtsY protein is dimeric. Other important experiments would include the possible identification of the FtsY monomer (Mr 48000). In theory it should be possible to identify the FtsY monomer before it is assembled into its larger form. This would probably require the radiolabelling of the protein under certain conditions such as a specific pH or in the presence of a particular chromosomal

mutation. It would also be interesting to discover why it is necessary for the FtsY monomer to be assembled into a larger protein. There are many possible reasons why the FtsY protein is required in a dimeric or multimeric form and one of these is discussed later.

10:2:3 The Fts proteins and the septalsome

Of the results described in this study the finding which has led to most speculation, is the localisation of the FtsY, FtsE and FtsX proteins to the inner membrane. Other essential division proteins have also been found associated with the inner membrane, such as FtsZ and FtsI (see also section 1:15), but some proteins such as FtsA are cytoplasmically located. Thus it is perhaps surprising that all three Fts proteins encoded by the operon were found to be membrane proteins. A clue to the reason for this may be provided by the FtsE protein. As discussed in section 9:1, analysis of the FtsE protein sequence revealed some interesting homologies with several other proteins. Strong homology is observed between FtsE and certain proteins known to be components of periplasmic binding protein-dependent transport systems (figure 9:1(b)). Two proteins which were not involved in such processes were also found to share this striking homology. These were the *HlyB* protein, a component of the haemolysin secretory apparatus in *E. coli* (Holland *et al.*, 1986) and the NodI protein involved in nodulation in *R. leguminosarum* (Evans & Downie, 1986). Each of the nine proteins indicated in figure 9:1(b) possess a sequence for a nucleotide-binding site (figure 9:1(c)), and are located in the inner membrane. Moreover, the gene for each of these proteins is located in an operon of several genes of related function (figure 9:1(a)). Some of these proteins, such as HisP and MalK have been well characterised and it has been shown that these proteins interact with other proteins encoded by their respective

operons to form a functional unit in the membrane. It seems probable therefore, that the Fts proteins also interact in the membrane to play an essential role in division.

Thus, FtsY, FtsE and FtsX proteins could be part of the putative septalsome. In section 1:15 the possible role of the FtsZ protein in the organisation and activation of the septum components into a septalsome was described, although the regulation of the septalsome is unknown. The scene is now set for the identification of the septalsome components and the discovery of their interaction during division. It is presumed that such components would include the essential division proteins, FtsA, FtsI and EnvA and perhaps FtsY, FtsE and FtsX also.

When Jones and Holland (1985) showed that both SfiA and FtsZ proteins were membrane located, these workers also showed that a small proportion of each was also found in the cytoplasm, and that preparation of the cell envelopes in the absence of Mg^{++} reduced the affinity of these proteins for the membrane. In section 9:1 similar findings were described for FtsY, FtsE and FtsX and several possible experiments for this were discussed. One possibility was that prior to insertion into the membrane, these proteins are present in the cytoplasm perhaps in some latent or inactive form. These proteins would then be inserted into the membrane when conditions in the cell were favourable for division and septation could take place.

If the FtsY, FtsE and FtsX proteins are indeed involved in the septalsome, what could be their function? Obviously, there is very little information known at present, but once again the FtsE protein and its striking homology with other membrane proteins could provide a clue.

The FtsE protein could utilise its nuclotide-binding site for the binding and hydrolysis of ATP to provide energy. Presumably, much energy will be required for septum formation and FtsE could be a key protein in the provision of this. No other division protein for which the DNA sequence is known has revealed a nucleotide-binding site (G. Salmond, pers. comm.). Interestingly, the FtsY protein also contains a nucleotide-binding site (figure 9:1(c)), and if as discussed earlier, the FtsY protein functions as dimer, the nucleotide-binding capacity of this protein is effectively doubled. A similar proposal has been made for the HlyB protein involved in the secretion of haemolysin in *E. coli* K-12 (Mackman *et al.*, 1985). The *hlyB* gene was shown to encode a polypeptide of Mr 66000 and also, under certain conditions a polypeptide of Mr 46000. This latter protein appears to be the result of an in-frame internal translational start rather than a degradation product of the larger protein. Thus two HlyB proteins are produced each containing a nucleotide-binding site and therefore it has been proposed that this protein helps provide the energy required for secretion of haemolysin (N. Mackman, pers. comm.).

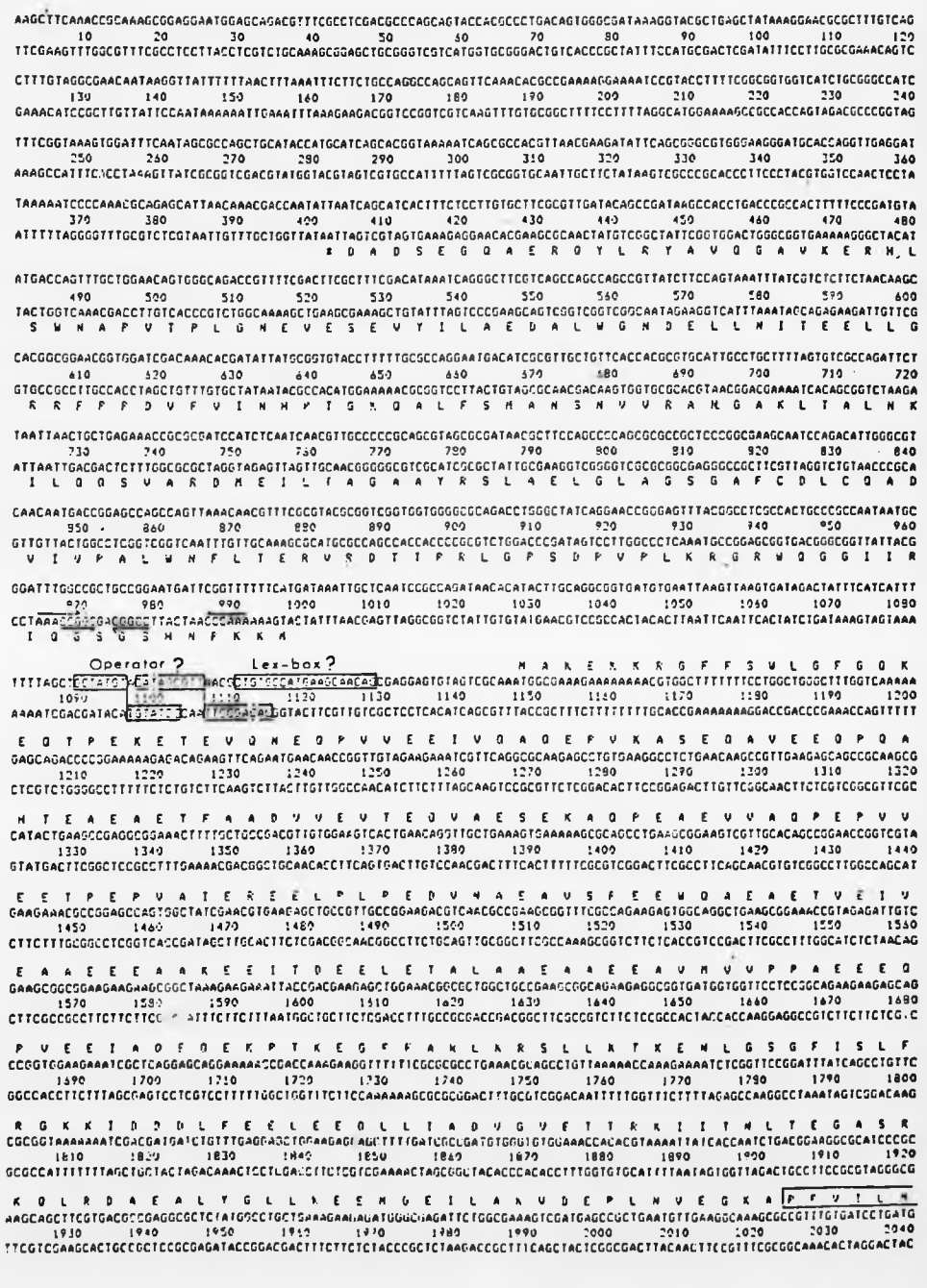
The possible location of the FtsY, FtsE and FtsX proteins in the septalsome could be tested in several ways. It should be possible to isolate extragenic suppressors of mutations in *ftsY*, *ftsE* or *ftsX*. These suppressor mutations would provide the first direct evidence for an association between these recently-identified Fts proteins and other cell division proteins. The use of immunogold-labelling to confirm the subcellular location of specific proteins was discussed in section 9:6. The use of these techniques should allow the more precise positioning of the Fts proteins within the envelope and might even provide evidence for the existence of the septalsome.

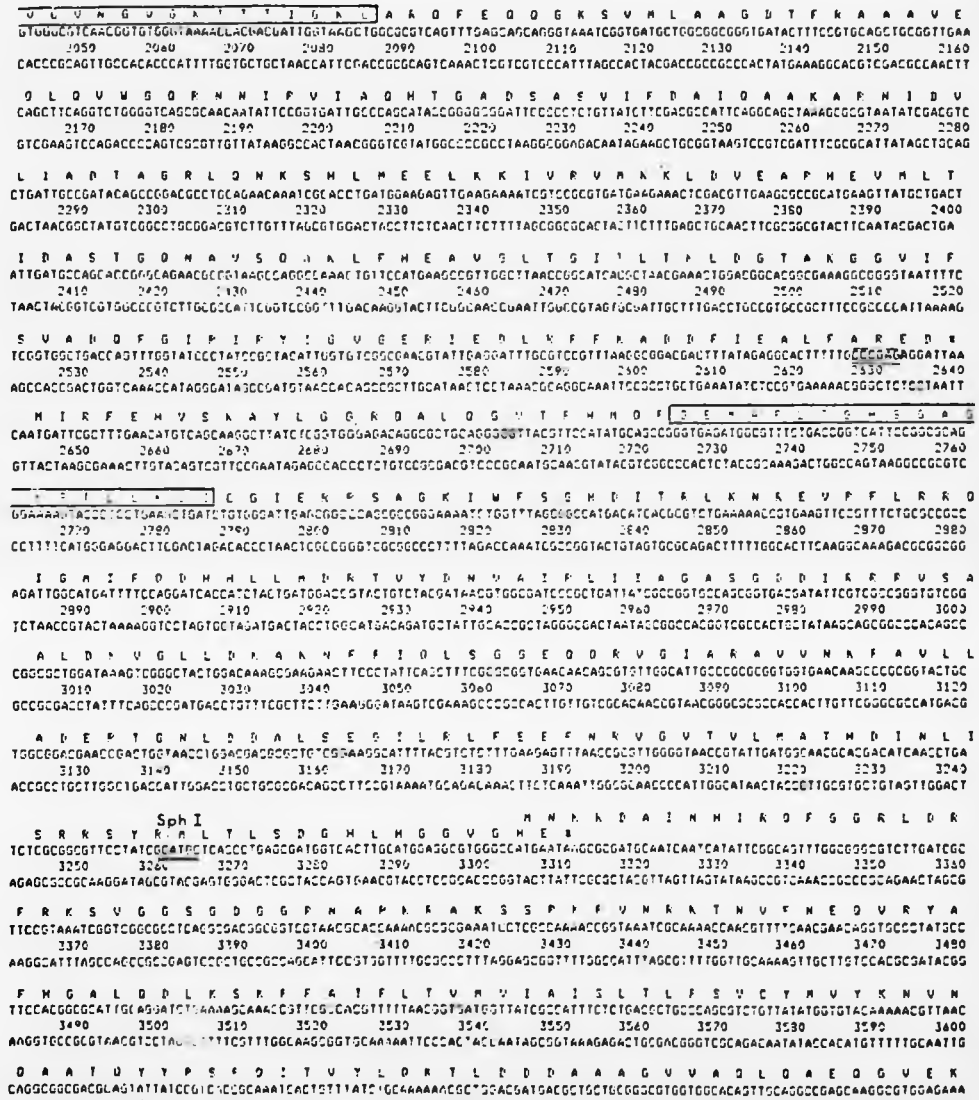
In conclusion, the results presented in this study have stimulated interest in a group of essential genes in the 76-minute region which appear to represent a new morphogene cluster. This chapter has summarised the findings of this work and has discussed their general significance with regard to future investigation. Unfortunately, such discussion does not provide evidence for the function of the Fts proteins in division, but without such speculation, we would not know which questions to ask nor which to answer.

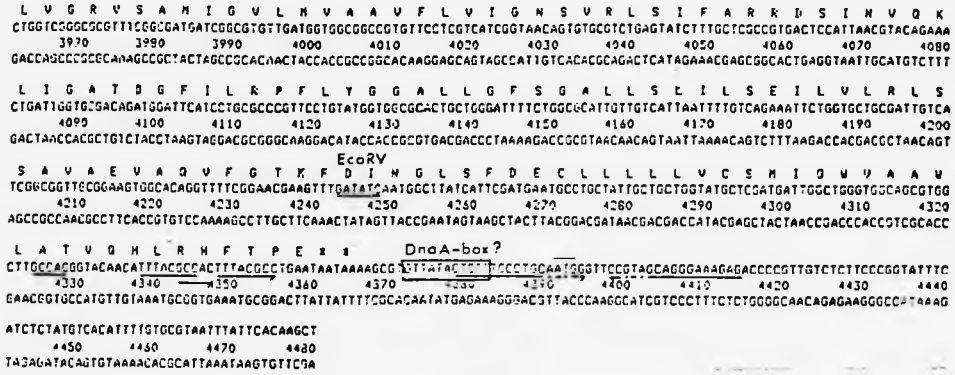
Appendix I Complete nucleotide sequence of the 4.5 kb *Hin*dIII fragment.

DNA sequencing and analysis were performed as described in section 3:2:3. ORF1 runs from base 1147 to 2640. ORF2 runs from base 2643 to 3311. ORF3 runs from base 3304 to 4365. ORF4 runs in the opposite direction from base 999 to 401.

Boxed DNA sequences are either regions of dyad symmetry, a potential LexA-binding site or a potential DnaA-box, as indicated. The significance (if any) of these regions is unknown. Important restriction sites used in the construction of plasmids pDB1Δ5 and pDB1Δ7 are also shown.

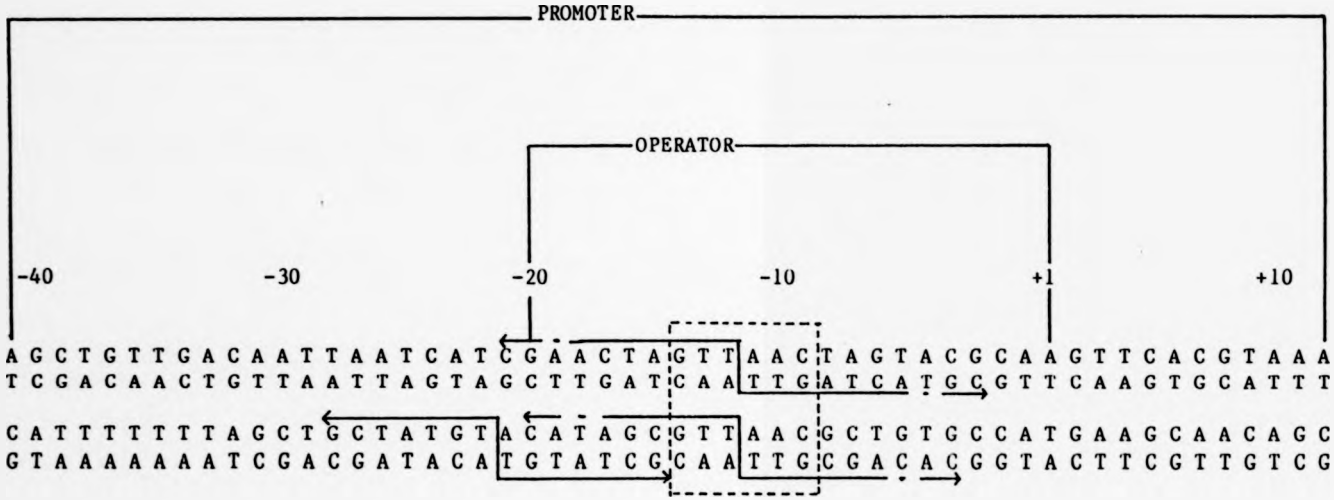

Operator ?
Lex-box?


Sph I


EcoRV
DnaA-box?

Appendix II Comparison of the tryptophan (*trp*) promoter/operator with the Fts putative operator.

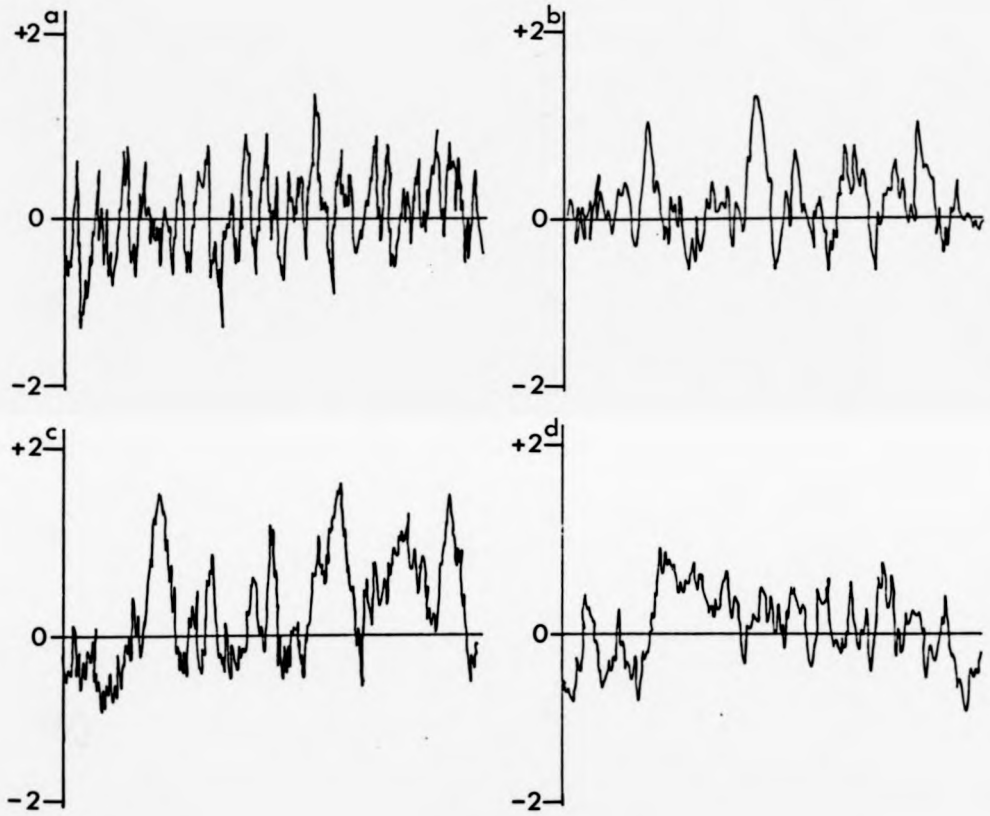
The upper sequence is of the *E. coli trp* regulatory region (Yanofsky *et al.*, 1981) and the lower sequence is the region 5' to the translational start of ORF1. The operator and promoter regions of the *trp* operon are indicated. The boxed sequence shows the hexanucleotide homologies in the regions of dyad symmetry.



COMPARISON OF TRP PROMOTER/OPERATOR WITH PUTATIVE "CELL DIVISION OPERATOR".

Appendix III Hydropathy plots for the proteins encoded by the 4.5 kb *Hin*dIII fragments.

The hydropathy plots were obtained as described in section 9:6, according to the method of Kyte and Doolittle (1982). a = ORF1 product (FtsY); b = ORF2 product (FtsE); c = ORF3 product (FtsX); d = ORF4 product. Each plot proceeds from left to right (amino- to carboxy-terminus). Peaks above the central line represent regions of hydrophobicity.


+2
a
0
-2
+2
b
0
-2
+2
c
0
-2
+2
d
0
-2

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