An Investigation into FtsE and the 76 Minute Morphogene Cluster in *Escherichia coli*

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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 were obtained by myself, under the supervision of Dr. G.P.C. Salmond, with the exception of those instances where the contribution of others has been acknowledged. All sources of information have been specifically acknowledged by means of reference.

Abbreviations

AMPS	Ammonium persulphate
ATP	Adenosine 5'-triphosphate
bp	Base pairs
cfu	colony forming units
CIP	Calf intestinal phosphatase
dATP	Deoxyadenosine 5'-triphosphate
Dap	Diamino pimelic acid
dCTP	Deoxycytosine 5'-triphosphate
dGTP	Deoxyguanosine 5'-triphosphate
DMF	Dimethyl formamide
dTTP	Deoxythymidine 5'-triphosphate
EDTA	Ethylene diamine tetra-acetic acid
GTP	Guanosine 5'-triphosphate
kb	Kilobase
kD	Kilodaltons
mins	Minutes
ml	Millilitres
moi	Multiplicity of infection
MOPS	3-(N-morpholino)propanesulphonic acid
NADH	Nicotine adenine dinucleotide, reduced form
PAGE	Polyacrylamide gel electrophoresis
PBP	Penicillin-binding protein
pfu	Plaque forming units
PPO	2-5 diphenyl oxazole
secs	Seconds
SDS	Sodium deodecyl sulphate

Temed	N, N, N', N', tetramethyl ethylenediamine
ul	Microlitre
um	Micrometer
UV	Ultra-violet

Amino Acid Abbreviations

A	ALA	Alanine
B	ASX	Asparagine or aspartic acid
C	CYS	Cysteine
D	ASP	Aspartic acid
E	GLU	Glutamic acid
F	PHE	Phenylalanine
G	GLY	Glycine
H	HIS	Histidine
I	ILE	Isoleucine
K	LYS	Lysine
L	LEU	Leucine
M	MET	Methionine
N	ASN	Asparagine
P	PRO	Proline
Q	GLN	Glutamine
R	ARG	Arginine
S	SER	Serine
T	THR	Threonine
V	VAL	Valine
W	TRP	Tryptophan
Y	TYR	Tyrosine
Z	GLX	Glutamine or glutamic acid

Summary

It has been thought for some time that the 76 minute region of the Escherichia coli chromosome may contain a large cluster of essential genes. The finding that the ftsYEX operon and rpoH are contiguous has given encouragement to this speculation. The extended homology of the FtsE protein with the superfamily of ATP binding cassette (ABC) proteins has created extra interest in this gene product in particular and the possibility of ascribing a function to it. This investigation into ftsE and the 76 minute region has given the results described below.

A localised mutagenesis procedure was performed on the 76 minute region and a screen for temperature-sensitive growth yielded 28 temperature-sensitive mutants. There were three classes of mutants based on their microscopic appearance at the restrictive temperature, that is filamentous, rpoH-like (having short filaments with inclusions), and pleomorphic. The mutations carried by these mutants were mapped by complementation with a range of plasmids carrying the known essential genes from the 76 minute region. Of the mutations giving rise to a filamentous phenotype, nine mutations were found to map in ftsE, five in ftsX, two appeared to carry mutations in both ftsE and ftsX, and two appeared to be complemented but were not fully mapped. All nine of the mutations giving rise to an rpoH-like phenotype were mapped to rpoH. The remaining two mutations that gave a pleomorphic phenotype were not complemented by any of the available plasmids. No temperature-sensitive lethal mutations were isolated in ftsY or ORF4, although this does not constitute proof that these genes are non-essential.

The ftsE missense mutants were analysed phenotypically and were not easily classified into groups on the basis of temperature-sensitivity, microscopic appearance, salt reversibility of phenotype, or the requirement for de novo protein synthesis for recovery of septation following a period of incubation at the restrictive temperature. The ftsE mutant alleles were cloned and the DNA sequenced. This showed the mutations to be clustered in a region of extensive homology with the ABC superfamily of proteins. Nucleotide binding of maxicell radiolabelled FtsE and FtsY proteins was investigated using dye-ligand chromatography columns. The results were very ambiguous due to the low amounts of the labelled proteins in the maxicell lysates but they are suggestive of a possible interaction between ATP and these proteins. These findings are consistent with the premise that FtsE will, like other ABC proteins, hydrolyse ATP and couple this to an essential biological process.

One of the mutations (Ts33) that was not complemented by any of the available constructs carrying DNA from the 76 minute region was mapped by transduction to the 'silent' region clockwise of 76 minutes, and appeared to lie very close to pit. It is therefore thought to define a new essential gene. This confirms the existence of more essential genes near 76 minutes. A cosmid library was constructed and ten cosmids were isolated that contained Ts33 complementary DNA.

Chapter 1: INTRODUCTION

Preface

Cell division and control of cellular morphology are essential processes in all living organisms, and as such they are of fundamental biological interest. These processes have been studied in a variety of species, both eukaryotic and prokaryotic, but there remains a large gap in our knowledge of how the coordination and integration of biochemical and genetic factors results in accurate fission and consistent morphology. One of the most widely studied organisms in this respect is Escherichia coli. This is for three main reasons: firstly that E. coli appears to have a relatively simple cell cycle and morphology; secondly that more is known about the biochemistry, physiology and genetics of this Gram-negative bacterium than any other organism; and thirdly that many techniques are already available for its manipulation and analysis. It is hoped that study of these processes in E. coli, although not necessarily directly applicable to other organisms, will provide models for, and aid our understanding of, the more complex systems of higher eukaryotes.

In this chapter various aspects of the research into E. coli cell division and morphogenesis are discussed. The first section deals with the measurement of physical parameters, observation of the cell cycle, and the incorporation of this data into models of growth. Subsequent sections deal with the involvement of cell envelope and peptidoglycan in morphogenesis, the genetic analysis of cell division, and work already

completed on the 76 minute morphogene cluster. Naturally research in all these areas has been largely concurrent and, to a greater or lesser extent, complementary, but they are being presented separately for the sake of clarity.

1.1 Physical Parameters and the Cell Cycle

Measurement of physical parameters

Much early research was devoted to analysing physical parameters such as cell length, cell volume and buoyant density, and their relation to cell cycle events and growth rates in *E. coli*. This work has served to illustrate that even in this apparently simple organism the task of measuring physical parameters and constructing accurate models is still not an easy one. The difficulties in measuring physical parameters are mainly due to the small size of *E. coli*, which is close to the resolving power of the light microscope. Most treatments of cells for viewing under the electron microscope result in shrinkage and thus measurements of lengths and diameters made from electron micrographs must be treated with caution.

The *E. coli* cell cycle is a very simple one of growth and transverse fission. *E. coli* cells are essentially cylindrical with hemispherical ends. From birth a cell elongates until it has approximately doubled in length, mass, and volume (Vincente, 1984) during which time the DNA is replicated and the two chromosomes decatenated and segregated. After doubling of length invagination of the cell envelope occurs, a septum forms, and separation ensues, giving rise to two identical daughter cells.

The width (or diameter) of cells was originally thought not to vary at all throughout the cell cycle (Marr *et al.*, 1966), but further careful microscopic studies by Trueba and Woldringh (1980) have suggested that, at least at slow growth rates, cell diameter decreases slightly as cells

elongate, until the start of invagination when the diameter increases again. They suggested that this has the effect of keeping the surface area to volume ratio constant as the cell is still accumulating mass whilst septation takes place. Cell diameter is also known to increase with increasing growth rate (Pierucci, 1978, Koppes et al., 1978).

Cell mass is thought to increase in either an exponential or a linear manner through the cell cycle. Cooper (1988a, 1988b, 1990) suggests that mass increases in an exponential or near exponential manner due to exponential increases in the major cellular components. However Kubitschek (1987) has suggested that the increase is linear due to the cyclic doubling of growth-limiting transport proteins at cell division, with fluctuation in metabolite pools exactly compensating for exponential increases in protein and other macromolecular synthesis (Kubitschek and Pai, 1988). Cell volume, by implication, also increases in an exponential or linear manner with mass as the buoyant density has been shown not to vary significantly through the cell cycle or indeed with growth rate (Martinez-Salas et al., 1981; Woldringh et al., 1981; Kubitschek, 1988).

Mean cell length is found to increase with increasing growth rate (Pritchard, 1974; Grover et al. 1977). Cell length probably increases in a bilinear fashion during the cell-cycle. In order to investigate this, Donachie et al. (1976) performed experiments with synchronised cultures, selecting small cells from slow growing cultures on sucrose gradients and transferring them into rich media in order to magnify the increases in growth rate through the cell cycle. They found that although the cell mass increased immediately on nutritional shift-up, the increase in elongation rate occurred only after the cells had reached a length that

was equivalent to double the minimum possible size of a cell, which is the predicted length of a newborn cell at zero growth rate (dubbed the "unit cell length"). Furthermore although chromosome replication terminates at about this time (Cooper and Helmstetter, 1968) termination of replication was found to be unnecessary for the doubling in elongation rate. Division did not occur until 20 minutes after the increase in elongation rate. This was found to be the case at all growth rates. Length has also been shown to increase in a bilinear fashion in the absence of a nutrient shift-up by the analysis of population length and birth length distributions in an exponentially growing population of E. coli B/r (Cullum and Vincente, 1978).

The E. coli cell-cycle

The cell-cycle can be temporally resolved into two periods: 'C' the time taken to replicate the chromosome, and 'D' the time between the termination of replication and division (Cooper and Helmstetter, 1968). Helmstetter and Pierucci (1976) measured these periods in a range of E. coli B/r sub-strains at various growth rates and found that for doubling times between 25 and 60 minutes C and D were fairly constant for each substrain, at around 42 minutes for C in all cases and between 14 and 22 minutes for D, depending on the substrain. Churchward and Bremer, (1977) found that the C period gradually lengthens with increasing doubling time from 28 minutes at a doubling time of 25 minutes, to 60 minutes at a doubling time of 120 minutes. Skarstad et al. (1985) used flow cytometry to measure the distribution of DNA in cells and compared these to the values predicted by the Cooper and Helmstetter model to determine the durations of the C and D periods. They found the C period to have a

constant duration of 43 minutes in cultures growing with a doubling time of less than one hour but that this increased in slower growing cultures. The D period in fast growing cultures was found to be 22 to 23 minutes long.

Obviously where the generation time 'T' is less than C+D minutes the C+D periods overlap. This is effected by the C periods overlapping in the sense that new replication forks are initiated before the previous rounds of replication are completed. This is known as dichotomous replication (Bird and Lark, 1968). In cultures with a generation time 'T' greater than C+D an additional period 'B' is also apparent after division but prior to the initiation of replication, leading to the suggestion of Cooper (1979) that these correspond to G1, S, and G2 phases in eukaryotic cell cycles. The presence of this G1-like period has been supported by the flow-cytometry studies of Skarstad et al. (1985)

There appear to be three major discontinuous events in a normal E. coli cell-cycle. These are: cell-division/birth; initiation of chromosome replication; and termination of chromosome replication. Many of the models that have been proposed to describe the cell-cycle are primarily concerned with linking these three events. The virtual absence of DNA-less cells in cultures of most E. coli strains implies that there must be tight control such that cells do not divide unless the chromosome has been fully replicated and decatenated.

Control of DNA replication

The replicon model for the control of replication was proposed by Jacob et al. (1963). This stated that the minimally required features for the control of replication of a stable DNA molecule (replicon) were an origin

of replication and a structural gene for an initiator of replication, coded for by the replicon. Replication could then be controlled via inhibition of initiation. They also suggested that the chromosome might be attached to the cell envelope at the origin of replication, subsequent envelope growth at this point segregating the newly replicated chromosomes. The chromosomal origin has subsequently been shown to bind to the cell membrane (Wolf-Watz and Masters, 1979), and more specifically the outer membrane both in vitro (Hendrickson et al., 1982) and in vivo (Nagai et al., 1980; Wolf-Watz, 1984), although this is only found if the DNA is hemimethylated (Ogden et al., 1988). Hendrickson et al. suggested that the outer membrane would provide a more stable attachment site than the inner membrane because of its covalent attachment to the peptidoglycan sacculus.

Considering data relating DNA concentration to growth rate in the light of the relatively fixed length of the C+D periods Donachie (1968) suggested that chromosome replication was initiated at a fixed ratio of origins of replication to cell mass. This he called the 'initiation mass' which corresponds to the mass of a new born cell when T (the doubling time) equals 60 minutes. Donachie and Begg (1970) proposed the 'unit cell' model of growth which stated that at doubling times greater than 60 minutes the cell grows in one direction from a single membrane growth site located near the pole of a new-born cell. When the cell reaches 2 unit cell lengths a second growth site is activated adjacent to the first which is now in the middle of the cell. The septation process is initiated between the growth sites at this time and takes approximately 20 minutes to complete (Donachie et al., 1976). It was proposed that these membrane

growth sites might be the same as the chromosome attachment sites proposed by Jacob et al. (1963), and that once chromosome replication was complete the ensuing division between the growth sites would ensure segregation of the sister chromosomes. Zaritsky and Pritchard (1973) suggested that the termination of chromosome replication triggered the doubling in envelope growth rate which could automatically lead to septation due to a fall in internal hydrostatic pressure as the rate of envelope growth exceeded the rate of increase in mass.

There are two models that attempt to describe control of the initiation process. The first of these is the inhibitor dilution model (Pritchard et al., 1969; Pritchard, 1984). This suggests that there is an inhibitor of initiation that is coded for by a gene near the origin of replication. This gene is transcribed only after a replication fork has passed through it, giving rise to increased levels of the gene product. During subsequent cellular growth the inhibitor is diluted out by the cell's mass and volume increase until it is at such a low level that initiation can occur again and the process is repeated. Later versions of this model invoke a continuously expressed gene whose product is fairly unstable, the cyclical variation in concentration of the inhibitor being achieved purely as a result of an increased gene dosage during the early stages of replication.

An alternative proposal, the initiator accumulation or autorepressor model, was presented by Sompayrac and Maaloe (1973). In this model the timing of initiation would be controlled by the accumulation of the initiator protein of Jacob et al. (ibid) as the cell increased in mass. The concentration of this initiator would be kept constant by the negative feedback of a repressor molecule on the operator of an operon consisting

of structural genes for the repressor and the initiator. When a sufficient number of molecules of the initiator had been accumulated they would cause initiation, and be consumed in the process. This cycle would then repeat.

Conditional mutants have been isolated that demonstrate a slow arrest of DNA synthesis. These are dnaA mutants and are known to be defective in initiation of DNA replication, which results in their ability to complete rounds of replication already in progress, but not initiate any new rounds under restrictive conditions. DnaA protein has been found to be autoregulated and rate limiting for initiation (Atlung et al., 1985a, 1985b; von Meyenburg and Hansen, 1987) but fluctuations in concentration over the cell cycle have not been found and it does not appear to be unstable (Sakakibara and Yuasa, 1982). It would seem therefore to be an ideal candidate for the initiator in an autorepressor model. For the degree of precision found in initiation, however, it is likely that many additional factors contribute to the control mechanism. For a full discussion see von Meyenburg and Hansen (1987).

Links between DNA replication and septation

Termination of DNA replication has been widely suggested as the signal temporally linking DNA replication and division. The initial evidence for this came from the experiments of Jones and Donachie (1973) who studied cells in which DNA synthesis was arrested by thymine starvation. When DNA synthesis was allowed to resume septation took place following a short period of RNA and protein synthesis that occurred around the time that the first new round of DNA synthesis terminated. This led Jones and Donachie to propose that septation initiation required the

synthesis of one or more proteins (called the 'termination protein/s') triggered by DNA replication termination.

This explanation does not satisfactorily account for the observation that some temperature-sensitive DNA synthesis mutants continue to divide, and therefore produce DNA-less cells, at the restrictive temperature (Hirota et al. 1968). These strains were found to carry dnaA^{ts} mutations which, as mentioned above, affect initiation of DNA synthesis. This led Donachie and co-workers to suggest that initiation of chromosome replication created a requirement for termination before cell division could take place (Donachie et al., 1973).

The original work of Jones and Donachie (1973) was compromised by the fact that the strains used would have been recovering from the SOS response which results in transient division inhibition (see Section 1.3), and this could have explained the apparent delay in resumption of division (Maguin et al., 1986). Jaffe et al. (1986) also demonstrated that termination was not a pre-requisite for cell division by blocking DNA elongation in the absence of the SOS response and getting the production of anucleate cells. Grossman et al. (1989) have more recently tried to distinguish between the triggering of septation by the initiation and the termination of DNA synthesis. They have shown that if newly divided cells are starved for an amino acid, cell division is still triggered during the starvation period provided that DNA replication is initiated before the start of starvation. They have found that DNA replication can be slowed by the presence of methionine in the medium during amino acid starvation and have used this to vary the time between initiation and termination of DNA replication. Cell division was found to vary with the termination

time, supporting the idea that termination of DNA replication is in some way functionally correlated with initiation of cell division.

Links between DNA segregation and septation

Another link between DNA metabolism and division has been suggested by the absence of cells containing only part of a chromosome. This implies that there is a mechanism to prevent the septum forming too early, acting like a guillotine and splitting chromosomes in two. In other words there appears to be a link between chromosome segregation and septation. This 'nucleoid occlusion model' is clearly expounded by Woldringh et al., 1990. They suggest that the presence of a nucleoid causes localised inhibition of septation. The initiation of septation is dependent on the balance between this negative effect and another positive regulator, the 'termination signal'. They suggest that this signal need not be the synthesis of a termination protein, but could be a transient increase in the level of free replication proteins and nucleotides upon termination of chromosome replication. The observation of Donachie and Begg (1989a) working on rod shaped and spherical E. coli cells that a minimum length or diameter was required for segregation and consequent septation fits in well with this model. Interestingly Donachie and Begg (1989b) have also demonstrated that segregation of replicated chromosomes is dependent on a brief period of post-replication protein synthesis. This could perhaps explain the dependence of septation on post-replication protein synthesis. The nucleoid occlusion model implies that any region of the cell has the potential to form septa in the absence of a nucleoid. This would appear to be consistent with the finding that DNA gyrase mutants that cannot decatenate and therefore segregate their chromosomes give rise to a

range of sizes of DNA-less cells at the restrictive temperature (see Section 1.3). However this model does not explain the presence of long aseptate DNA-less regions seen in sfi mutants (D'Ari et al., 1990), or the presence of peri-septal annuli at ¼ and ¾ of the cell length (near to the nucleoids) a generation before division occurs at these positions (see below).

Instead of suggesting that the location of the nucleoid is entirely responsible for determining the position of the septa in a uniformly proficient cell wall structure Rothfield et al. (1990) expound the view that the cell wall has predetermined potential septation sites between the peri-septal annuli (PSAs). These structures are comprised of pairs of murein-membrane adhesion zones at regular intervals along the cell's length. They define the edges of plasmolysis bays revealed when cells are subjected to hypertonic solutions. These were first identified in Salmonella typhimurium (MacAlister et al., 1983). They appear to enclose periplasmic compartments that are isolated from the rest of the periplasm, and this has been demonstrated in fluorescence photobleaching experiments (Foley et al., 1989). It has been suggested therefore that these compartments might serve to contain the cell division machinery. Cook et al. (1987) investigated the presence of these plasmolysis bays throughout the cell cycle and found that in newborn cells there were peri-septal annuli only at the midpoint of the cell, which would later become the site of septation. New PSAs appear to develop from the central PSA and migrate outwards during growth of the cell, perhaps by localised membrane growth, so that in older, longer cells, the PSAs were also found at positions corresponding to ¼ and ¾ of the cell length. These

appear to define the sites of septation in the following cell-cycle. These observations led to the proposal that the septal sites are predetermined in the cell wall at least one generation before they are used. It is of interest to note that one PSA is also found at each of the cell poles, presumably as a relic of previous cell divisions that gave rise to the poles. If this is the case then it could be envisaged that these polar compartments still contain the cell division machinery that was used for the septation event giving rise to the poles. This then begs the question as to how the the cell inactivates these old polar sites and 'decides' to activate the mid-cell sites instead. DeBoer *et al.* (1988) suggest that this is due to the action of the proteins coded for by the *minB* locus (see Section 1.3).

In summary, a range of models have been proposed that attempt to describe the control of cell division. None of these appears to satisfactorily account for all the empirical observations of the *E. coli* cell-cycle, but they do provide a basis for experimentation and some sort of framework into which new observations can be fitted.

1.2 The Cell Envelope and Septum Formation

The cell envelope

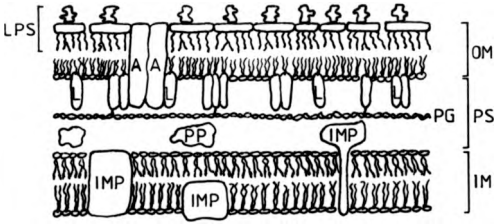
The cell envelope of Escherichia coli essentially consists of three layers: the inner or cytoplasmic membrane, a thin layer of peptidoglycan (murein), and an outer membrane. The shape of E. coli is to a large extent determined by the peptidoglycan sacculus structure. It is the combination of the turgor pressure of the cytoplasm pushing against the inner (cytoplasmic) membrane and the constraining force of the peptidoglycan sacculus holding the membrane in that results in the cells having a defined shape. The involvement of the cell envelope in morphogenesis and cell division is implicit in the importance attached to chromosome/membrane attachment and the peri-septal annuli (Section 1.1). As septation is essentially the invagination of the cell envelope and growth of the cell is only possible due to an increase in envelope surface, it should be informative when investigating cell shape and division to look at the envelope's structure.

The cell envelope is depicted in Figure 1.1. Here we can see an inner membrane that is a phospholipid bilayer containing proteins. This membrane forms the cell's major permeability barrier, with many of the proteins playing roles in the transport process. Between the inner and outer membrane lies the periplasmic space, also known, perhaps more accurately, as the periplasmic gel (Hobot et al., 1984), which contains the peptidoglycan sacculus. The outer membrane has an asymmetric bilayer

Figure 1.1 Diagram Representing the E. coli Envelope Structure

This figure shows the three layers of the cell envelope: inner membrane (IM), periplasmic space (PS), and outer membrane (OM). Other features that are shown are: inner membrane proteins (IMP), periplasmic proteins, (PP), peptidoglycan (PG), lipoprotein (L), OmpA (A) and lipopolysaccharide (LPS).

Redrawn from Lugtenberg and van Alphen (1983).



structure with an inner layer of phospholipids and an outer layer mainly composed of lipopolysaccharides, but also containing phospholipids and proteins, notably outer membrane protein A (OmpA) and Braun's lipoprotein (Lpp). The outer membrane appears to be more closely associated with the peptidoglycan, to which it is attached via Lpp and OmpA. Whilst the peptidoglycan seems to be primarily responsible for the shape of E. coli, it is worth noting that omp, lpp double mutants lose their shape and grow as spheres (Sonntag et al., 1978), implying that OmpA and Lpp, and presumably the outer membrane, also play some part in cell shape determination.

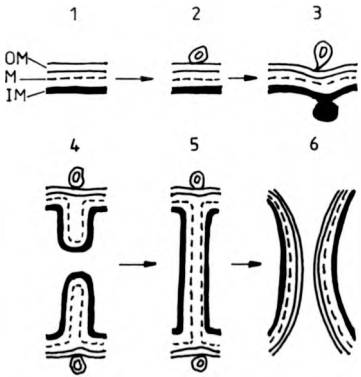
Burdett and Murray (1974) investigated the morphology of the formation of septa using electron microscopy on synchronised E. coli cultures. The stages observed are shown in Figure 1.2. It appears that a septum forms composed of cytoplasmic membrane and peptidoglycan and, when this is completed, the outer membrane invaginates during cell separation. Burdett and Murray suggest this simple four step model for septation: 1. the peptidoglycan is modified by hydrolases, 2. enzymes required for septation are assembled, 3. wall and membrane synthesis are initiated giving rise to the formation of a septum, 4. the outer membrane invaginates and the cells separate.

Burdett and Murray's identification of the formation of an outer membrane blob and an inner membrane mesosome at the centre of the septal site coincides with the observations of MacAlister et al. (1987) of a structure that they call the septal attachment site or SAS. This is placed equidistant between the peri-septal annuli and consists of an enlargement of the murein-outer membrane layer which is closely apposed

Figure 1.2 Diagram Representing the Morphology of Septation

These stages of septation were observed using electron microscopy on synchronous cultures. Stage 1: prior to septation; 2: outer membrane (OM) bleb forms; 3: inner membrane (IM) mesosome forms; 4 & 5: invagination of cytoplasmic membrane (CM) and murein (M); 6: bleb is incorporated into septum on separation.

Redrawn from Burdett and Murray (1974).



to the inner membrane. They speculated that this SAS may provide a site for the insertion of new murein during septation. Peptidoglycan appears to be the most important determinant in the septation process although it is likely that the cytoplasmic and outer membranes play some part in the assembly of enzymes and precursors.

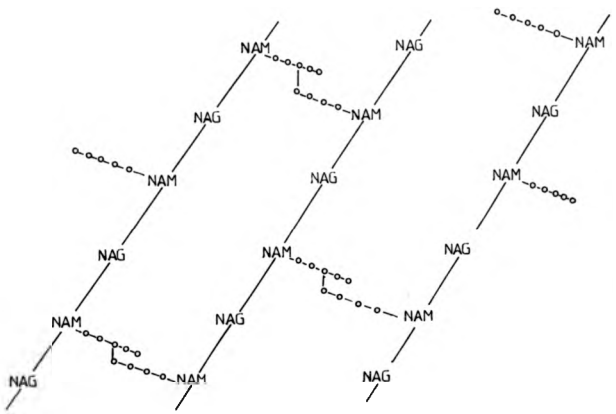
Peptidoglycan structure and synthesis

The structure of peptidoglycan consists of a repeating glycan subunit of N-acetyl glucosamine and N-acetyl muramic acid residues cross-linked to other glycan chains by a short peptide attached to the muramic acid residues. The basic subunit comprises the disaccharide with a pentapeptide moiety comprising -L-Ala-D-Glu-m-Dap-D-Ala-D-Ala. The most simple cross-linking occurs by a transpeptidation reaction linking the fourth amino acid (D-Ala) of one chain (the donor) with the m-Dap of another (the acceptor) with the concommitant loss of the donor's terminal D-Ala. The acceptor's terminal D-Ala may also be subsequently cleaved. This basic structure of cross linked peptidoglycan is shown in Figure 1.3.

The peptidoglycan had been assumed to be a monolayer from early electron microscopy results and the analysis of Dap content and sacculus size (Braun et al., 1973), however more recent work has shown the peptidoglycan to be a much more complex structure. With the advent of new slow cooling embedding techniques for electron microscopic samples Hobot et al. (1984) have demonstrated a structure that fills the region between the two membranes rather than a thin line of peptidoglycan in the middle of a periplasmic space. They have suggested that this is due to the peptidoglycan having a diffuse gel-like structure with a level of cross linking that decreases towards the cytoplasmic membrane. This

Figure 1.3 Diagrammatic Representation of Cross-linked Peptidoglycan

Chains of alternating N-acetyl muramic acid (NAM) and N-acetyl glucosamine (NAG) are covalently linked via pentapeptide sidechains attached to the NAM, the terminal D-alanine of the donor sidechain being removed in the process of crosslinking.



implies that the peptidoglycan must have several layers rather than just one.

Given that the murein sacculus has to withstand the turgor pressure of the cell, Koch (1983, 1985, 1988) has suggested in his surface stress theory that the insertion of new material into the murein of bacterial cell walls must precede the breaking of old bonds. This would seem to be essential in Gram negative organisms with their reduced levels of peptidoglycan. This means that the new peptidoglycan would already be cross-linked before it had to take any stress, which would make the cross-linking energetically easier than if old bonds were broken before new material was inserted. This also means that the peptidoglycan could not strictly be a single layer.

The peptidoglycan composition has been analysed using high performance liquid chromatography (HPLC) to separate and quantitate the muropeptides resulting from the enzymatic digestion of murein (Glauner et al., 1988). This work has revealed that there is in fact a range of different peptidyl moieties that can be attached to the n-acetyl muramic acid residue. Glauner et al. also demonstrated the existence of trimers and tetramers, a higher level of crosslinking than was previously thought to exist. This is made possible by the presence of a previously undetected cross-bridge between the Daps of two peptide chains, a so called Dap-Dap crosslinkage as opposed to the 'normal' Ala-Dap crosslinkage. The terminal D-Ala of the receptor peptide is therefore free to participate in other cross-bridges, enabling the formation of trimers and tetramers. Prats and Pedro (1989) have shown using Dap starvation that the level of murein per unit surface area can be reduced by up to 50% without any effect on cellular

morphology and murein structure. These findings are indicative of there being more than one layer of murein (Holtje and Glauner, 1990). Goodell (1985) has shown that there is a turnover of murein and a recycling process for the subunits. All this data leaves us with the picture of a much more complex, dynamic structure than was previously thought to exist.

Given the large amount of information now available about peptidoglycan structure the obvious question is whether there is any structural difference between side wall and septal murein. This has been addressed by de Jonge et al. (1989) using the HPLC methods of Glauner. They found no major differences between septal and lateral wall murein. Cross-linkage, glycan chain length and lipoprotein content were all found to be the same. The only difference consistently found was evidence of multiple strand insertion or much more rapid single strand insertion in septal murein when compared to lateral wall murein. de Jonge (1990) also analysed the murein from isogenic mutants that exhibit different cellular morphologies and found them to be identical. Holtje and Glauner (ibid) propose a simple model of murein metabolism that appears to be in agreement with all the observations above. This is that the peptidoglycan is essentially a three layered structure being synthesised in an inside to outside fashion. The peptidoglycan would have a central stress bearing layer flanked by an inner layer of newly synthesised material and an outer layer in dissolution. They speculate that septum formation could be the result of a localised halt in murein turnover giving rise to a narrow zone of multilayered murein which forms a septum. Recent work by Labischinsky et al. (1991) may result in a reworking of this model. These

workers used the technique of neutron small-angle scattering to analyse isolated murein sacculi. They found that 75 to 80% of the peptidoglycan was single layered and the rest was three (or possibly more) layers.

Mode of insertion of peptidoglycan

Higgins and Shockman (1971) first suggested that there might be separate peptidoglycan synthetic systems for elongation and septation that acted alternately in the cell cycle. If this is the case then it might be reasonable to expect there to be two zones for peptidoglycan synthesis, an elongation site and a septation site. The experimental approach adopted to investigate this possibility has been to pulse label cells with radiolabelled Dap and, using electron microscope autoradiography, look for the insertion site of nascent peptidoglycan. A primary consideration has been whether insertion is zonal or diffuse. The results of these studies have been somewhat ambiguous, doubtless confused by the turnover and recycling of murein. Koch (1988) concludes from a re-assessment of the available autoradiographic data that insertion of peptidoglycan occurs in a diffuse fashion. Wientjes and Nanninga (1989) performed a thorough assessment of the incorporation of labelled Dap into murein using pulse labelled synchronised cultures and also using electron microscope autoradiography of cells from pulse labelled steady state cultures. They found that the rate of incorporation of Dap was significantly higher in septating cells than cells that were only elongating. Incorporation during elongation appeared to be diffuse. During septation however, incorporation was greatly enhanced at the site of constriction, with a corresponding reduction (of about 40%) of incorporation in the lateral walls. This partially supports the model of Satta et al. (1979, 1980) which proposes

that in Gram negative organisms there are elongation and constriction sites for peptidoglycan synthesis which compete for peptidoglycan precursors. The consistent narrowness of the zone of increased incorporation throughout the constriction process, even in deeply invaginated cells where the new polar caps were actually wider than this zone, led Wientjes and Nanninga to speculate that the incorporation was confined to the leading edge of the nascent septum and that this might be related to the SAS structure found by MacAlister (1987).

Proteins involved in peptidoglycan synthesis

The possibility of there being two systems of peptidoglycan insertion suggested that it might be worthwhile to assess the enzymes involved in peptidoglycan assembly and look for enzymes with a specific involvement in elongation or septation. The primary enzymes responsible belong to the class of penicillin binding proteins or PBPs. There are seven of these usually found in E. coli. These are listed in Table 1.1 with their biochemical activities. The four higher molecular weight PBPs are involved in the final assembly of the peptidoglycan and have two enzymic activities. These are a transglycosylase, which acts to extend the glycan chains and a transpeptidase which acts to crosslink the glycan chains via the peptide moieties. It is the transpeptidase activity that is β-lactam sensitive. The three lower molecular weight PBPs (4, 5, and 6) have D-alanine carboxypeptidase activity.

The action of the PBPs in cell division and morphogenesis has been investigated using two major approaches: assessing the morphological effects of treatment with specific β-lactam antibiotics and finding which PBP each particular antibiotic interacts with using radiolabelled antibiotics

Table 1.1 The Penicillin-Binding Proteins

PBP	Mr	Gene	Activity	Function
1A	92000	mrcA/ponA	TPase/TGase	General synthetic/ elongation
1B	90000	mrcB/ponB	TPase/TGase	General synthetic/ elongation
2	66000	mrdA/pbpA	TPase/TGase	Elongation initiation/ shape
3	60000	ftsI/pbpB	TPase/TGase	Septation
4	49000	dacB	CPase	Unknown
5	42000	dacA	CPase	Unknown
6	40000	dacC	CPase	Unknown

TPase - transpeptidase
TGase - transglycosylase
CPase - carboxypeptidase

(a method developed by Spratt and Pardee, 1975); and isolating mutants that are defective in each of the PBPs. Correlating the morphological changes with the enzyme involved has turned out to be a powerful technique for the dissection of the elongation and septation processes.

Treatment of E. coli with mecillinam which binds exclusively to PBP 2 (Ishino et al., 1982) results in ovoid shaped cells and strains producing mutant forms of this protein grow as spheres (Spratt, 1975). This has been taken to mean that PBP 2 is involved in the control of cell shape or elongation. PBP 2 has also been shown to be involved in the turnover of peptidoglycan during stationary phase (Blasco et al., 1988) and the initiation of peptidoglycan synthesis when the cells are starting to grow following a period of stationary phase (de la Rosa et al., 1985), which may be taken to imply that it plays an important part in elongation. Antibiotic inhibition of PBP 3, for example by piperacillin or furazlocillin, or growth of a PBP 3^{ts} mutant at the restrictive temperature results in filamentation due to the cessation of cell division, and eventually the cells lyse (Spratt, 1975; Botta and Park, 1981). Therefore PBP3 appears to be required for septal murein synthesis.

Mutant PBPs 1A, 1B have been isolated that do not bind the usual βlactam antibiotics but these do not result in non-viable cells (Park, 1987), perhaps implying that these proteins are individually non-essential. However a PBP 1B, PBP $1A^{ts}$ double mutant was found to be lethal at the non-permissive temperature (Suzuki et al., 1978), suggesting that PBP 1A can normally substitute for PBP 1B, but that there is a requirement for at least one of these to be active. PBP 1B mutants produce very little cross-linked peptidoglycan (Tamaki, 1977) and purified PBP 1B has been

shown to synthesise cross-linked peptidoglycan from disaccharide pentapeptide precursors in vitro (Suzuki et al., 1980). Begg and Donachie (1985) working with double mutants that had defects in elongation and septation found that these mutants continued to grow as spheres suggesting that there is a general peptidoglycan synthetic activity with shape determination superimposed. These results suggest that PBPs 1A and 1B are the major enzymes involved in peptidoglycan growth.

Mutants have been isolated that show no detectable PBP 4, 5, or 6 activity and these appear normal, implying that these proteins are individually non-essential. Further mutants have been isolated that possess deletions in the genes coding for PBP 5 and PBP 6, proving their non-essential nature (Spratt, 1980; Broome-Smith and Spratt, 1982). A PBP 4, PBP 5 double mutant was also found to be viable (Matsuhashi, 1977). The lower molecular weight PBPs have all been shown to be capable of catalysing transpeptidation in vitro (Spratt, 1983) but whether they perform this task in vivo is not known. The role of these proteins is thus less than clear.

DePedro and Schwarz (1981) suggest that the higher molecular weight PBPs are responsible for the initial polymerisation of peptidoglycan and that PBP 4 is then responsible for further cross-linking. This is consistent with their finding that PBP 4 mutants have a poorly cross-linked murein. It has also been postulated that the D-alanine carboxypeptidases act to control the level of cross-linking, as removal of the terminal D-alanine from the peptide chains would prevent their involvement in transpeptidation reactions. Most importantly PBP 3 has been shown to catalyse transpeptidation reactions preferentially involving acceptor

molecules with tripeptide sidechains (Botta and Park, 1981; Pisabarro et al., 1986). This suggests that the role of the lower molecular weight PBPs may be in controlling the switch from elongation to septation by tailoring the peptide side-chains for particular transpeptidation enzymes.

There are however a number of other non-penicillin binding proteins that also appear to have an important role in the control of peptidoglycan synthesis. There must be several enzymes with a lytic activity to allow the insertion of new material into the sacculus. Indeed nine murein hydrolases have been described (reviewed by Holtje and Tuomanen, 1991) including the lower molecular weight PBPs. It is noteworthy that an L-D-carboxypeptidase has been isolated that presumably acts to cleave the Dap-d-Ala bond in the tetrapeptide produced by the action of the D-alanine carboxypeptidases to yield the tripeptide substrate for PBP 3. Mutants in this enzyme have not however been isolated. Matsuhashi et al. (1977) demonstrated that PBP 4 has an endopeptidase activity, but whether this activity is present in vivo and, if so, whether this function is involved in sacculus growth and division is unknown. One enzymatic step for which no enzyme has yet been isolated is the formation of Dap-Dap peptide bridges. During mutagenesis experiments a range of mutants has been isolated that carry mutations in murein metabolism related genes. One of these is the envA gene. Mutations in envA cause the splitting of the septum (which normally immediately precedes separation of the cells) to be greatly slowed giving rise to chains of cells. In envA cells a reduction is found in the levels of N-acetyl-muramyl-L-alanine amidase which may be involved in the splitting of the septal peptidoglycan (Wolf-Watz and Normark, 1976). Overexpression of

envA is lethal (Sullivan and Donachie, 1984), and it has been shown to be an essential gene (Beall and Lutkenhaus, 1987).

Other murein metabolism related mutations are found in the rodA gene. The RodA protein is, like PBP 2, involved in the determination of the cell's rod shape and hence has a role in elongation. Ishino et al. (1986) have found that the overproduction of RodA and PBP 2 in a PBP 1B mutant gives rise to easily detected murein synthetic activities, and this is ascribed to PBP 2 having both transglycosylase and transpeptidase activities. Further evidence for the involvement of PBP 2 and Rod A is that the genes coding for these proteins (pbpA and rodA) are arranged in an operon (Matsuzawa et al., 1989). Begg et al. (1988) have found a rodA mutation (sui) that phenotypically suppresses a specific pbpB mutation (ftsI23), suggesting either that RodA and PBP 3 physically interact; or that the activities of RodA and PBP 3 (the pbpB gene product) are required to be in some sort of balance, which is consistent with the idea that these proteins are active in elongation and septation respectively. Further work on these mutants (Begg et al., 1990) has revealed that the effect of the rodA(sui) mutation is to increase the levels of PBP 5 which has the effect of increasing the level of tripeptide sidechains in the murein which, by providing more of the appropriate substrate, compensates for the reduced PBP 3 activity. Other treatments that increase the level of tripeptide side chains (increased levels of PBP 6 or treatment with D-cycloserine) also had the effect of suppressing the ftsI23 mutation. These workers suggest that the cyclic variation in murein precursors with different peptide side chains may be an important part of the control mechanism of the cell cycle.

Matsuhashi et al. (1990) have made a simple proposal for the mechanism of the biosynthesis of the sacculus. PBP 2 and RodA act together to initiate peptidoglycan synthesis. The elongation of the cell is then carried out by PBP 1A and PBP 1B until such time as an unidentified signal causes the switch to PBP 3 activity and the formation of a septum. To this model we can add that at least part of the signal may be a change in the precursor pool available to the murein biosynthetic enzymes. What causes changes in the precursor pool, what other components the signal might have, and what networks control morphology and peptidoglycan synthesis are perhaps questions best addressed using the tools of genetics.

1.3 Genetic Analysis of Cell Division

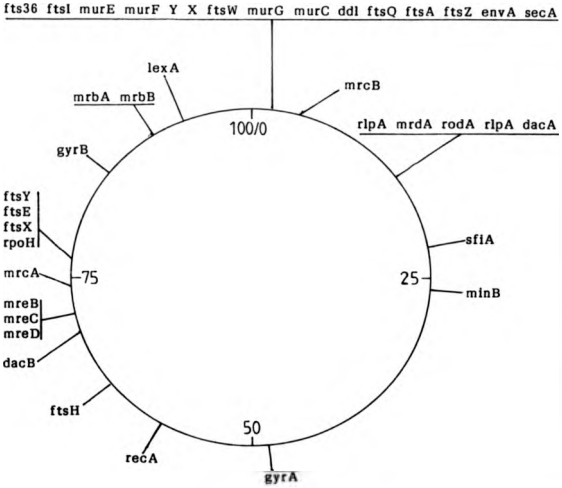
Morphogenes

The isolation and characterisation of mutants that show aberrant cell division and morphology is proving to be a useful tool for the dissection of these processes. Isolation of suppressor mutations and the construction of double mutants have given us insight into the control and regulatory networks that guide the cell cycle. Donachie et al. (1984) have coined the term 'morphogene' to describe any gene which when mutated causes altered morphology and/or division. Because cell division is the summation of all of the cell's processes the number of genes involved indirectly or directly is large. Genes coding for proteins involved in fundamental processes such as protein synthesis and DNA synthesis, disturbances in which do have morphogene-like secondary effects, have been excluded from this definition. Because most of the morphogenes are essential for normal cellular growth the vast majority of mutations isolated have had to be conditional mutations, most of them temperature-sensitive. The distribution of morphogenes around the Escherichia coli genetic map shows distinct clustering and this is shown in Figure 1.4. Clusters are seen at 2 minutes, 15 minutes, and 76 minutes.

The morphogenes isolated can be divided into groups depending on the function that appears to be disturbed in the mutants (see reviews by Matsuhashi et al., 1990; and Donachie et al., 1984; for general references to the genes listed below). One group of morphogenes is connected with murein synthesis. This includes the penicillin binding proteins discussed in Section 1.2, coded for by the genes mrcA mrcB mrdA/pbpA ftsI/pbpB/sep dacB dacA and dacC. Other genes that fall into this group are genes for

Figure 1.4 The Distribution of Morphogenes in E. coli.

The E. coli linkage map is shown as a circle divided into 100 minutes. Genes shown are involved in cell growth, cell division and peptidoglycan synthesis. Redrawn and updated from Matsuhashi et al. (1990) and Donachie et al. (1984).



proteins involved in the assembly of peptidoglycan precursors, such as murE murF mufG murC and ddl. The latter genes will not be discussed further here.

Another group of genes that are of great importance are those genes whose products are involved in septation. Most of these are filamentation temperature sensitive or fts genes. As the name implies these are genes where thermosensitive mutations have been isolated that result in an inability to form septa at the non-permissive temperature, so that the cells grow as long aseptate filaments. These include fts38 ftsI ftsW ftsQ ftsA and ftsZ in the 2 minute cluster, and ftsE and ftsX in the 76 minute cluster. These could be described as the primary cell division mutants, as the mutations are thought to cause direct loss of cell septation. No biochemical role has been ascribed to most of the protein products of these genes with the notable exception of ftsI which codes for PBP3 (see above and Section 1.2). Another gene in this group is envA which is involved in the final stages of septation (see Section 1.2). Other regulatory genes that can be added to this group include rodA mreB mreC and mreD.

Genes that are involved in chromosome decatenation/partition and choice of septal site can be considered as one group of genes that have involvement in septal placement. Appropriate genes include parD/gyrA parA/gyrB parC parE parB/dnaG and perhaps mukA and mukB, which are all involved in chromosome partition. Other genes involved in septum placement include the genes of the minB locus (minC minD minE).

Finally a group can be made out of genes whose products are involved in inhibition of the division process. Obvious examples of these include genes

involved in stress responses such as rpoH from the heat-shock response, and sfiA from the SOS response. The rest of this section will deal with observations and models arising out of research on these basic groups of morphogenes.

The genetics of septation

The largest and best studied cluster of morphogenes lies at 2 minutes on the E. coli map. This includes genes coding for peptidoglycan synthetic enzymes and a range of fts genes (fts36 ftsI ftsW ftsQ ftsA and ftsZ). Some research effort has been directed into finding an order of action of the protein products of ftsI ftsQ ftsA and ftsZ. It has been shown that ftsZ mutants do not show any sign of constrictions whereas mutations in the other three genes do result in partial invagination. This suggests that the FtsZ protein acts in the initiation of constriction (Walker et al. 1975). Begg and Donachie (1985) working on double mutants concluded that the order of action of these gene products was FtsZ for initiation of septation, followed by FtsQ and PBP 3, with the FtsA protein only required for the final stages of septation. Taschner et al. (1988) performed a detailed electron microscopic study of these mutants and concluded that the order was FtsZ, FtsQ, then FtsA and PBP3. There is only one candidate Fts protein for action earlier than FtsZ and that is the product of the ftsH gene (Santos and deAlmeida, 1975). Transferring ftsH mutants to the restrictive temperature results in a slow arrest of septation activity, reminiscent of the slow arrest of DNA replication seen in dnaA mutants (Section 1.1), in contrast to other fts mutants that exhibit an immediate halt in septation. Ferreira et al. (1987) have demonstrated that there are extremely low levels of PBP 3 in this strain

and that the overexpression of *ftsI* can suppress the *ftsH* phenotype, perhaps suggesting that FtsH protein is involved in a regulatory capacity. However more recent work of Donachie and Begg (1990), suggests that there is more than one mutation involved in the *ftsH* phenotype. This leaves us with *ftsZ* as the earliest acting cell division gene.

A possible termination protein: FtsA

Donachie *et al.* (1979) demonstrated that synthesis of the FtsA protein is required immediately prior to division or else septation will not take place, although this does not mean that FtsA protein is only synthesised at this time *in vivo*. Tormo *et al.* (1980) showed that this period of protein synthesis was in the period between DNA replication and division. This has suggested that the FtsA protein could actually be the termination or 'Ter' protein of Jones and Donachie (1973). Tormo *et al.* (1985a, 1985b) have further demonstrated that synthesis of of the FtsA protein requires a preceding period of DNA replication. Although these characteristics are consistent with a 'Ter' protein it seems unlikely from the morphology of *ftsA* mutants (which exhibit constrictions at the restrictive temperature) that this is the role of the FtsA protein. The FtsA protein has, however, been shown to participate in formation of the septum (Tormo and Vincente, 1984), as well as being found to interact with PBP 3 as measured by its inhibition of ampicillin binding to PBP 3. The native form of the FtsA protein has been found to be located in the cytoplasmic membrane (Pla *et al.*, 1990). Recent work involving overexpression of *ftsA* (Dewar *et al.*, 1989; Wang and Gayda, 1990) has shown that high levels of the FtsA protein inhibits septation. This may imply that FtsA protein is titrating out an activating factor, inhibiting

the expression of other genes, or maybe interfering with the formation of a septal protein complex. FtsA protein evidently does play an important part in septation, although its regulatory effects are still unclear.

Homologies and peptidoglycan synthesis: possible switching by mreB/ftsA

The mre cluster at 71 minutes has been found to be involved in cell shape control. This region has been cloned and sequenced and consists of at least three genes that appear to be involved in morphogenesis: mreB mreC mreD (Doi. et al., 1988; Wachi et al., 1989). Mutations in mreB result in increased sensitivity to mecillinam and formation of round cells. The product of the mreB gene is thought to negatively regulate cell division (Wachi and Matsuhashi, 1989). Doi et al. (ibid) found that the C-terminal half of the MreB protein has homology with the FtsA protein and postulated that these proteins might act as antagonists as one appears to be involved in elongation and the other in septation.

Ishino et al. (1989) isolated a mutant carrying a mutation in a new fts gene in a screen for temperature sensitive mutants. This mutation maps between murF and murG in the 2 minute cluster and they have named the gene ftsW. This mutation has subsequently been cloned and sequenced (Ikeda et al., 1989) and the deduced protein is found to have high homology with the RodA protein. RodA protein is thought to interact with PBP 2 in cell elongation (see Section 1.2). PBP 3 , which is thought to be active in cell septation is a structural analogue of PBP 2 (Asoh et al., 1986). This raises the interesting possibility that the FtsW protein might interact with PBP 3 in septation in a similar manner to the putative RodA/PBP 2 interaction in elongation (Ikeda et al., ibid). Matsuhashi et al., (1990) have boldly drawn these observations together and propose that

the elongation/division switch might be controlled by the balance between MreB and FtsA acting on PBP 2 and RodA for elongation, and PBP3 and possibly FtsW for septation.

The SOS response and division inhibition: a key role for FtsZ

Whilst the ftsA gene product may well be involved in the switch from elongation to septation at the level of peptidoglycan synthesis, the signal that causes this switch is unknown. The ftsZ gene, however, appears to be critical in the activation of cell division. Apart from the fact that it appears to be the earliest acting gene yet found in the septation process it has also been found to be pivotal in the inhibition of cell division during the SOS response to DNA damage. When DNA is damaged by mutagens the SOS response is induced (Radman, 1975). This response includes activation of DNA repair systems and temporary inhibition of cell division leading to filament formation, until such time as DNA repair is completed, at which time cell division is allowed to resume.

The current model of the SOS response is clearly reported in the review of Walker (1987). Control of the SOS response is due to a balance between the RecA and LexA proteins. LexA is a repressor of the expression of the SOS genes including recA and lexA, a range of genes involved in DNA repair and mutagenesis and the cell division inhibitor sfiA. The amount of repression exerted on the expression of each gene is variable so that there is a basal level of expression of some genes such as recA and lexA. When there is DNA damage or inhibition of DNA replication an unknown inducing signal is generated that activates the RecA protein. Interaction of activated RecA and LexA results in the cleavage of LexA, reducing the repression of the SOS genes. When the

damage is repaired or the replication inhibition is lifted the inducing signal is lost, the RecA protein returns to the non-activated form, the levels of LexA increase, and the SOS genes are repressed again.

The lon gene codes for the Lon protease. Strains carrying lon mutations were found to exhibit very slow recovery of cell division following induction of the SOS response. The tif allele of recA has been found to result in gratuitous induction of the SOS response at 42°C. The mutations sfiA and sfiB were isolated as suppressors of filamentation in a lon tif strain (George et al., 1975). Two similar suppressor mutations sulA and sulB were isolated that suppressed the filamentation of lon cells following exposure to mutagens. Both sfiA and sulA were found to map to 21 minutes and sfiB and sulB were found to map to 2 minutes on the Escherichia coli linkage map (Huisman et al., 1980; Gottesman et al., 1981) and are in fact allelic. Huisman and D'Ari (1981) using a Mud(Aplac) insertion in sfiA showed that sfiA was SOS inducible. Mizusawa and Gottesman (1983) found that the presence of the lon mutation resulted in a greatly increased half-life of SfiA protein. Therefore it was suggested that SfiA protein was produced as part of the SOS response and leads to division inhibition. In lon^+ cells the Lon protease acted to cleave SfiA thus releasing the division block during recovery from the SOS response. As sulB mutations lead to slightly temperature sensitive cell division in media with low salt concentrations, Gottesman et al. (1981) suggested that these mutations might lie in an essential cell division gene. Lutkenhaus (1983) demonstrated that the sulB mutations actually lie in ftsZ.

Evidence has now accumulated to show that the SfiA protein interacts with the FtsZ protein to cause the division block. Jones and Holland (1984) demonstrated that the presence of FtsZ stabilised SfiA protein synthesised in maxicells. This suggested an interaction between the proteins that might interfere with proteolytic cleavage of SfiA and (in vivo) prevent FtsZ acting in cell division. Ward and Lutkenhaus (1984) constructed a lacZ-ftsZ in-frame gene fusion under control of the lacZ promoter. When expression of this 'ZZ' hybrid protein was induced with IPTG it inhibited cell division. This inhibition could be suppressed by increased expression of ftsZ, or partially suppressed by the presence of either of two sulB alleles. Jones and Holland (1985) have further demonstrated that SfiA does not decrease the synthesis or stability of FtsZ. It seems from this work that FtsZ plays a crucial role in the activation of cell division, presumably interacting with other cell division gene products, this interaction being inhibited by the binding of SfiA to FtsZ, or the competition of 'ZZ' excluding FtsZ from its normal site of action. Holland and Jones, (1985) suggested that FtsZ and other cell division proteins might assemble to form a 'septalsome' complex. This idea has been given some credence by the work of Descoteaux and Drapeau (1987) on double mutants carrying a lon mutation (the effects of which are known to be mediated by FtsZ) and a mutation in one of the morphogenes ftsQ, ftsA, or ftsZ. These double mutations gave unexpected effects that Descoteaux and Drapeau ascribed to interactions between FtsQ, FtsA, and FtsZ proteins. Jones and Holland (ibid) suggested that activation of the 'septalsome' by achievement of a critical concentration of FtsZ or some other signal would result in the initiation of septation.

This suggested that the level of FtsZ protein might be limiting for division.

Teather et al. (1974) had already suggested the concept of a 'division factor' that was produced once per cell-cycle, was only sufficient for the formation of one septum and was used up in the process of septation. This idea was prompted by the phenotype of the minicell producing (minB1) mutant (Adler et al., 1967). This mutant forms a normal number of septa except that some of them are formed at the cell poles at the expense of those in the centre of the cell, giving rise to small, spherical, anucleate cells (see below), which would seem to imply that there is a strict number of division events that can take place per cell cycle. FtsZ protein would seem to be a good candidate for being the division factor of Teather et al. (ibid). If this was the case then higher levels of ftsZ expression should lead to higher numbers of division events taking place. Ward and Lutkenhaus (1985) tested this possibility by overexpressing ftsZ on a multicopy plasmid. They found that this did result in additional cell divisions taking place and the formation of minicells due to polar divisions. This clearly differentiates the overexpression of ftsZ from the action of the minB mutation and shows that FtsZ protein is indeed rate limiting for cell division. This has been confirmed by the recent work of Bi and Lutkenhaus (1990a). They found that overexpression of ftsZ completely suppressed the increased nucleated cell length seen in a minB mutant by increasing the number of septation events.

Control of the placement of septa: the minB locus

The formation of pole derived minicells in minB mutants has been taken to mean that the poles retain the 'division machinery' that was used to

form them and that it is only the action of the products of the minB locus that act to inactivate them. De Boer et al. (1988) suggested that they may act at the polar peri-septal annuli (Section 1.2). The minB locus has now been cloned, sequenced, and found to consist of three genes: minC, minD and minE (de Boer et al., 1989). The analysis of the phenotypic effects of expression of all three genes individually and in all permutations has shown that the minC and minD products act together to inhibit septation at all available sites, ie. midcell and poles. The minE gene product gives topological specificity to the action of the inhibitors, blocking inhibition of internal potential division sites (PDS) whilst allowing inhibition of polar septation. Expression of minC and minD in the absence of minE results in filamentation as all the PDS are inhibited. Overexpression of minE, which blocks all minC minD division inhibition, or deletion of the whole minB locus, results in the formation of minicells as all PDS are now available for septation. This illustrates that the minB locus is not in fact essential for cell division, only for control of septal placement. Jaffe et al., (1990) have investigated the number of nucleoids (using the nucleoid stain 6-diamidino-2-phenylindole [DAPI]) and the number of origins of replication (using flow cytometry) in nucleated minB cells, and in wild type cells under furaziocillin division inhibition and upon resumption of division following the release of this inhibition. The rationale for this was that if minB mutants had lost the ability to specify the placement of internal sites cells would be seen with odd numbers of nucleoids and replicative origins. They found that in both types of cell the majority of cells had 2^n (n=0,1,2,...) nucleoids and 2^n (n=1,2,3,...) copies of the replication origin. This shows that the minB locus acts only

to inhibit polar divisions, not to direct the placement of internal septal sites.

The target of minB division inhibition appears from the work of Bi and Lutkenhaus (1990b) to be the FtsZ protein. They have found that mutant FtsZ proteins that are resistant to SfiA inhibition are also resistant to the lethal effects of minCD overexpression. This is yet more evidence of the central importance of FtsZ in the division process.

Septum localisation and the par mutants: a link between DNA segregation and cell division

The work on the minB locus shows that there must be additional mechanisms linking the segregation of nucleoids and the placement of septal sites. Evidence for this also comes from the par mutants. These are mutants that are unable to segregate and partition their chromosomes and as a result control of septation is disturbed. The parA mutation has been found to lie in the gene gyrB coding for one subunit of the topoisomerase II, DNA gyrase (Kato et al. 1989). The parD mutation appears to lie in the gyrA gene which codes for the other DNA gyrase subunit (Hussain, 1987a, 1987b). Both of these mutations result in a similar phenotype of one condensed nucleoid (presumably as the result of an inability to decatenate the replicated chromosomes) and a range of sizes of anucleate cells from minicells up to normal size rods. Recently the parC and parE mutations have been found to map in the genes coding for the two subunits of a newly identified topoisomerase, topoisomerase IV (Kato et al., 1990). So four of the mutations resulting in a partition phenotype map in topoisomerase subunits.

There would appear to be some topological signal missing in these mutants which links segregation to correct septal placement. It is informative then to find that the placement of septa in the nucleated filaments of the DNA gyrase mutants is actually non-random. Mulder and Woldringh (1989) have investigated septal placement using a range of mutants that produce anucleate cells including gyrA and gyrB mutants. They have found that in filaments with actively replicating nucleoids constrictions were formed close to the nucleoid. In filaments with non-replicating nucleoids the placement of constrictions within the anucleate portion of the cell was nearly random. This is consistent with the nucleoid occlusion model described in Section 1.1 if the generation, or perhaps localisation, of a 'termination signal' is considered to be coupled to active DNA replication. It also suggests that the nucleoid plays an active part in determining the placement of septa, rather than there being a simple passive occlusion mechanism. In the light of this it would be interesting to investigate the placement of peri-septal annuli in these mutants to see how they correlate with the findings of Mulder and Woldringh.

Decatenation, movement of newly replicated chromosomes to effect partition, and placement of septa is a presumably a multi-stage process involving several gene products, not just the action of topoisomerases. One par mutation remains that does not map in a topoisomerase, the parB mutation. Grompe et al. (1991) have demonstrated that this mutation lies in the dnaG gene, which codes for DNA primase. Their analysis of a range of dnaG mutants that they have sequenced leads them to suggest that DnaG has two functional domains: an enzymatic domain that is

involved in the synthesis of the DNA replication primer RNA; and a structural domain that may be involved in binding the functional aggregation of DNA replicating proteins (the 'replisome'), and hence the chromosome, to other parts of the segregation machinery.

Hiraga et al. (1989) reasoned that if positioning of nucleoids at the cell quarters following replication and decatenation was an active process, then it ought to be possible to isolate other classes of mutants that could decatenate their chromosomes but were deficient in the process of actually partitioning the daughter nucleoids. This type of mutant would not be deficient in cell division but should give rise to normal size cells, some of which would either have two chromosomes or be anucleate, due to random partitioning. They have described the isolation of such mutants. One carries a mutation designated mukA1 which has been found to map in a gene (tolC) that codes for an outer membrane protein, suggesting a possible involvement in chromosome/membrane interaction. An exciting new discovery of this group of workers is a phenotypically similar mutation, mukB (Niki et al., 1991). The mukB gene has been sequenced and the predicted protein secondary structure makes MukB an intriguing protein. It appears to show homology with myosin heavy chain and other eukaryotic force generating enzymes, which is compatible with it playing a part in the physical movement of nucleoids during segregation. It is not yet known whether this is the same protein that cross reacts with a monoclonal antibody raised against Saccharomyces cerevisiae myosin heavy chain (Casaregola et al., 1990) but the molecular weight is similar (180 kD). These are the first indications of a possible mechanoenzyme involvement in E. coli division and morphogenesis.

Inhibition of division and the heat shock response

The heat shock response in E. coli resembles that in other organisms (Neidhardt and VanBogelen, 1987). Following an abrupt increase in growth temperature, or exposure to a range of stimuli such as ethanol (Travers and Mace, 1982), unfolded protein (Parsell and Sauer, 1989), or an alkaline shift (Taglicht et al., 1987), the rate of synthesis of 17 proteins is induced and there is a transient inhibition of cell division. The heat shock response is controlled by the alternative sigma factor σ^{32} which is coded for by the rpoH gene (Grossman et al., 1984) which maps at 76 minutes on the E. coli map (Neidhardt and VanBogelen, 1981). The rpoH gene is essential for growth at temperatures greater than 20°C (Zhou et al., 1988). The σ^{32} consensus recognition sequence found in the promoters of the heat shock inducible genes has been shown to be different to that of the normal σ^{70} (Cowing et al., 1985) which explains the differential expression of these genes during the transient increase in σ^{32} levels that follow heat shock (Straus et al, 1987). Interestingly induction of rpoH under the control of the lacZ promoter with IPTG resulted in the expression of almost all of the heat shock proteins but did not give rise to the thermotolerance characteristic of the induction by heat shock (VanBogelen et al., 1987). The functions of most of these heat shock proteins is unknown but they include RpoD (σ^{70}) and Lon protease which is involved in modulation of the SOS division inhibition. The GroEL and GroES and DnaK heat shock proteins appear to have a key thermally protective role (Kusukawa and Yura, 1988), probably in a structural capacity, as 'molecular chaperones' (Ellis, 1987) which aid the correct folding of other proteins. The mechanism that results in the thermally

induced increase of σ^{32} levels is unknown although there has recently been a report that three of the heat shock proteins, DnaK, DnaJ, and GrpE negatively regulate both synthesis and stability of σ^{32}, and hence provide a negative feedback control of the heat shock response (Straus et al. (1990). The cause of the transient division inhibition is unknown although temperature sensitive rpoH mutants are seen to filament at the restrictive temperature. With the growing evidence for the importance of FtsZ in division inhibition pathways it is tempting to speculate that it will prove to be involved in heat shock division inhibition as well.

Expression of morphogenes and the cell cycle

Synthesis of the majority of E. coli proteins is found throughout the cell-cycle and the cell-cycle specific synthesis of the morphogene proteins has not been demonstrated (Lutkenhaus et al., 1979), but these essential proteins may be present in very low amounts and could have escaped detection in these experiments. However overexpressing morphogenes such as ftsA, ftsZ, or envA is lethal (Wang and Gayda, 1990; Ward and Lutkenhaus, 1985; Sullivan and Donachie, 1984), whilst over expressing ftsQ has recently been shown to result in the formation of multiseptate filaments (Carson et al., 1991), suggesting that the control of expression is critical.

The 2 minute cluster shows potential for very complex transcriptional control. For example the consecutive genes ftsQ ftsA ftsZ envA all appear to have at least one functional promoter each, embedded in the coding region of the preceding gene but there are no transcriptional terminators apparent between these genes (Yi et al., 1985), although there is a strong terminator downstream of envA (Beall and Lutkenhaus, 1987).

This means that there is the potential for various polycistronic messages. It has been observed for example that expression of *ftsZ* when cloned in multicopy vectors appears to be highest when the whole region upto and including the *ftsQ* promoter is present (Yi *et al.*, 1985).

Dewar *et al.* (1989) have studied transcription from promoters within *ftsQ* and part of *ftsA* (which should reflect the promoter activity driving *ftsZ*) using a *lacZ* reporter gene. They found that promoter activity was repressed by FtsA and apparently proportional to the number of nascent septa. They suggest that as *ftsA* has to be resynthesised each cell cycle it may be inactivated or sequestered during septation which could act as a switch for the expression of morphogenes such as *ftsZ*. Interestingly Robin *et al.* (1990) have just published a study investigating *ftsZ* expression through the cell cycle. They found that expression was not just limited to the time of septation but was linear through the cycle with a doubling in expression at a time that corresponded to the initiation of DNA replication. In conclusion not much is known about the regulation of morphogene expression and it promises to be very complex, but it may well hold the key to the cell cycle.

1.4 The 76 Minute Morphogene Cluster

Ricard and Hirota (1973) isolated an fts mutant, MFT1181, that carried a mutation in a gene that they called ftsE. This was originally mapped to 73 minutes on the E. coli linkage map but is now placed at 76 minutes close to the rpoH gene (Bachmann, 1983, 1990). The mutation fam715 which indirectly causes lipoprotein defects (Torti and Park, 1978, 1980) lies in an essential gene that also maps at 76 minutes. Another mutation that maps to 76 minutes is the dnaM710 allele which again appears to lie in an essential gene although it is not involved in DNA synthesis (Glassberg et al., 1979). Salmond and Plakidou (1984) cloned the ftsE region on a lambda phage λGS22 and analysed various fts mutations that appeared to map in this region. Most lay in ftsE but one (in the strain OV22) appeared to define a new locus very close to ftsE that they called ftsS.

Gill et al., (1986) subcloned a 4.5kb HindIII fragment from λGS22 and from the DNA sequence found that this fragment carried four open reading frames. Transposon insertion experiments suggested that three of the genes were arranged in an operon-like structure and the fourth open reading frame (ORF4) was transcribed in the opposite direction. Complementation studies showed that the ftsE gene was the middle gene of the operon, and several of the non-ftsS mutants mapped by Salmond and Plakidou (ibid) to the ftsE gene were shown to actually map in the third gene of this operon which was called ftsX. The OV22 ftsS mutation has subsequently been shown to map in ftsX (Crickmore, 1987). The first gene of the operon was tentatively called ftsY although no mutations have been isolated that map in this gene. Gill et al., (ibid) also found a

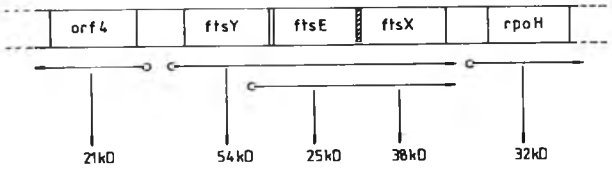
putative promoter/operator region upstream of the *ftsY* gene that showed homology with the *trp* operon promoter/operator region. The *rpoH* gene has been found to be immediately downstream of the *ftsX* gene (Gill *et al.*, 1986) and mutations *fam715* and *dnaM* were found to be alleles of *rpoH* (Crickmore and Salmond, 1986; Crickmore, 1987).

Gill and Salmond, (1990) presented data from complementation tests that indicated that there is a promoter within the *ftsY* gene that is required for expression of *ftsE* and *ftsX* in addition to the promoter upstream of *ftsY*. There is preliminary evidence of the presence of mRNA transcripts from both of these promoters, encoding *ftsE* and *ftsX*, and *ftsY*, *ftsE*, and *ftsX* respectively (Crickmore, 1987). Primer extension results (Crickmore, ibid) suggest that the large transcript may be initiated far upstream of the *ftsY* gene, just within the transcriptional unit of ORF4. This would result in overlapping divergent promoters (for ORF4 and *ftsY* etc.), which implies that some co-ordination of expression may occur. This data suggests that the transcriptional regulation of this region may prove quite complex. This information is summarised in Figure 1.5.

The molecular weights of the proteins coded for by the genes carried on the 4.5kb *HindIII* fragment has been found in maxicell experiments and these are in accord with the sizes predicted from the sequence except for the FtsY protein,which has a predicted size of 54 kD but an apparent size on SDS-PAGE of 92 kD. (Gill *et al.* ibid; Gill and Salmond, 1990). Deletion experiments suggest that this disparity reflects anomalous running on SDS-PAGE gels due to an unusually high number of acidic residues within the N-terminus of the protein (Gill and Salmond, 1990). All three proteins have been localised to the cytoplasmic side of the inner

Figure 1.5 Transcriptional Organisation of the 76 Minute Cluster

Molecular weights are given for the predicted gene products. Promoter activity is represented by a circle, and transcripts by arrows from the promoters. There is a 2bp gap between ftsY and ftsE, and an 8bp overlap between ftsE and ftsX.



membrane (Gill and Salmond, 1987). Both FtsY and FtsE proteins were found to possess a sequence motif indicative of a nucleotide binding site (Gill *et al.*, ibid). FtsE protein has been further shown to share extensive homology with a large family of ATP binding proteins, most of which are involved in periplasmic binding protein dependent transport systems (Higgins *et al.*, 1986).

1.5 Aims of this Project

The presence of three contiguous essential genes (*ftsE*, *ftsX*, and *rpoH*) mapping at 76 minutes, and the position of a gene of unknown function (*ftsY*) as the first gene in an operon whose other two genes are essential, suggested that *ftsY* might be essential and that there might be more essential genes that belong to this cluster. Only a limited range of missense mutations is available that map in *ftsE*, and only two amber mutations and one cold-sensitive missense mutation in *ftsX*. The *ftsY* and *ftsE* proteins have a potential nucleotide binding capacity, which suggests that it may be possible to assign biochemical functions to these proteins. The aim of this work was to find any more essential genes in the 76 minute cluster, and further characterise *ftsE* as follows:

1. Isolate a range of mutants carrying mutations in the 76 minute region and map them.

2. Try to identify any new essential genes in this region.

3. Characterise the *ftsE* missense mutations to yield information about the FtsE protein.

4. Investigate the nucleotide binding ability of FtsE and FtsY proteins.

Chapter 2: MATERIALS AND METHODS

2.1 Microbial Growth and Manipulation

2.11 Bacterial and Bacteriophage Strains and Plasmids

These are listed in Tables 2.1, 2.2 and 2.3. All bacterial strains used were E. coli K12 derivatives. They were routinely grown in nutrient broth (NB) and on nutrient agar (NA) at 37°C unless temperature-sensitive, in which case they were grown at 30°C or 42°C. Overnight cultures were generally grown in 5ml of NB in 25ml screwcap universal bottles in an orbital shaker at 150rpm. Cells were harvested by centrifugation at 5000g. For procedures requiring the use of 1.5ml eppendorf tubes all centrifugation steps were carried out in an MSE Micro Centaur at high speed, centrifuging for 2 mins to harvest cells. Strains were maintained on NA at 4°C for up to 2 months. For long term storage, overnight cultures grown in NB were mixed with an equal volume of 2x freezing medium and frozen at -20°C. Bacteriophage lysates were stored in the appropriate phage buffer over 50ul of chloroform at 4°C. Plasmids were stored at 4°C in TE.

2.12 Media

Media used for growth and maintenance of bacterial and bacteriophage strains are listed in Table 2.4. All media were made up in double-distilled or ultrapure water and were sterilised either by autoclaving at 121°C for 15 mins or by filtration through a 0.2u filter. Media were solidified with 1.5% w/v Bacto agar where necessary. Top agar for double layer plates contained 0.3% Bacto agar for production of lysates or 0.75% Bacto agar

Table 2.1 Bacterial Strains

Strain	Genotype	Source/Reference
OV2	F⁻ leu ilv his trp(am) thyA ara(am) galU42(am) deo nal^r galE(am) lacZ125(am) supF^{ts} tsx(am)	N.Crickmore/ Donachie et al. (1976)
OV2 zhf::Tn10	OV2 Tc^r	N. Crickmore/ Salmond & Plakidou (1984)
OV32 recA	As OV2 but ftsX32 (am) recA56 srl::Tn10	D. Gill/ Gill et al (1986)
TOE22	F⁻ thr leu proA his argE lac gal ara xyl mtl thi tsx thyA deo ftsE22 str^r	D. Gill/ Salmond & Plakidou (1984)
TC35	As TOE22 but ftsE35 instead of ftsE22	D. Gill/ Salmond & Plakidou (1984)
MFT1181	F⁻ leu-6 thr-1 argH1 his-1 trp-1 thyA lacY1 malA1 mtlZ xyl-7 tonA2 ftsE1181 supE44 str^r	D. Gill/ Ricard & Hirota (1973)
AB1157	F- thr-1 ara-14 leuB6 lacY argE3(Oc) galK2 kdgK51 rfbD1 mgl-51 hisG4(Oc) xyl-5 mtl-1 thi-1 tsx33 rpsL31 Δ(gpt-proA)62	B. Bachmann/ DeWitt & Adelberg (1962)
K10	Hfr tonA22 ompF627 relA1 pit-10 spoT1 T_2^r	B. Bachmann/ Willsky et al (1973)

CSH26ΔF6	F^- *ara* Δ(*lac-pro*) *thi* *rpsL* Δ(*recA-srl*)F6 sup^0	D. Gill/ Jones & Holland (1984)
TG1	*supE* *thi* Δ(*lac-pro*) *hsdD5* F' *traD36* $proA^+B^+$ $lacI^q$ *lacZ_M15*	J. Hinton/ Lab stock
POP2239	F^- *thi-1* *thr-1* *leuB6* *tonA21* *supE44* *lacY1* *malA510* λ^-	P. Robson/ Raibaud *et al.* (1984)
JGC127	*dnaM710* *zhg*::Tn*10* DG17 (*argG6* *metB* *his-1* *leu-6* *thyA3* *mtl-2* *xyl-7* *malA1* *gal-6* *lacY1* *rpsl104* *tonA2* *tsx* λ^r λ^- *supE44*	N. Crickmore/ Glassberg *et al.* (1979)
JCB421	AB2847 (*aroB351* *malA*) $aroB^+$ *cysG406*::Tn*5*	N. Woods/ Macdonald & Cole (1985)

Table 2.2 Bacteriophage Strains

Phage	Characteristics	Source/Reference
P1 vir	Virulent P1	K. Hussain
T4GT7	Generalised transducing T4 phage (*denB*, *alc*)	J. Hinton/ Wilson *et al* (1979)
λwt	Wild type Lambda	N. Crickmore
λpsupF	λ540 (*imm*21, *nin*R), *supF*	N. Crickmore/ Borck *et al* (1976)
λvir	virulent (λwt immunity)	N. Crickmore
λpGS22	λ540 (*imm*21, *nin*R), *ftsYEX*	G. Salmond/ Salmond & Plakidou (1984)
λS3D	As λpGS22 but extended anti-clockwise to include *rpoH* by UV induction of a λGS22 lysogen	N. Crickmore Crickmore (1987)
M13tg130	M13 cloning vector with multi-cloning site polylinker	Amersham Kieny *et al* (1983)
M13tg131	as tg130 with polylinker in inverse orientation	

Table 2.3 Plasmids and Cosmids

Plasmid	Comments	Source/Reference
pBR322	Ap^R Tc^r	Lab stock
pBR325	Ap^R Tc^r Cm^r	Lab stock
pJRD184	Ap^R Tc^r	Lab stock
pH3C	4.5kb HindIII fragment in pBR322 Carries Orf4 ftsY ftsE ftsX	N. Crickmore/ Crickmore (1987)
pH3CdA	2.4kb AatII deletion from pH3C	ibid
pH3CdAdP	2.8kb PvuII deletion from pH3CdA	ibid
pH3CdB	85bp BstE2 deletion from pH3C	ibid
pH3CdS	1.7kb SphI deletion from pH3C	ibid
pH3CdR	0.4kb EcoRV deletion from pH3C	ibid
pH3CdSR	3.3kb StuI-EcoRV deletion from pH3C	ibid
pDB1	identical to pH3C	D. Gill/ Gill et al. (1988)
pDG1	identical insert to pDB1/pH3C but in pBR325 vector	D. Gill/ Gill et al. (1988)
pDBS8	3.4 kb AatII-EcoRV fragment from λS3D in pBR322. Carries ftsE ftsX rpoH	D. Gill
pR2	rpoH gene cloned in EcoRV site of pBR322	ibid
pTW4.5	4.5kb HindIII fragment from OV2 in pBR325	This study/ Chapter 4
pTW1	4.5kb HindIII fragment from SG1 in pBR325	This study/ Chapter 4
pTW10	4.5kb HindIII fragment from SG10 in pBR325	This study/ Chapter 4
pTW11	4.5kb HindIII fragment from SG11 in pBR325	This study/ Chapter 4
pTW19	4.5kb HindIII fragment from SG19 in pBR325	This study/ Chapter 4
pTW20	4.5kb HindIII fragment from SG20 in pBR325	This study/ Chapter 4

pTW21	4.5kb HindIII fragment from SG21 in pBR325	This study/ Chapter 4
pTW23	4.5kb HindIII fragment from SG23 in pBR325	This study/ Chapter 4
pTW35	4.5kb HindIII fragment from SG35 in pBR325	This study/ Chapter 4
pTWTOE22	4.5kb HindIII fragment from TOE22 in pBR325	This study/ Chapter 4
pTWTC35	4.5kb HindIII fragment from TC35 in pBR325	This study/ Chapter 4
pDMW140	ss Ori and lacZ from M13mp19 in pJRD184 based vector	D. Whitcombe
pTWG1	3.5kb EcoRI fragment from cTOM1 in pDMW140	This study/ Chapter 6
pTWG2	3.5kb EcoRI fragment from cTOM2 in pDMW140	This study/ Chapter 6
pTWG3	3.5kb EcoRI fragment from cTOM3 in pDMW140	This study/ Chapter 6
pTWG4	3.5kb EcoRI fragment from cTOM4 in pDMW140	This study/ Chapter 6
pTWG5	3.5kb EcoRI fragment from cTOM5 in pDMW140	This study/ Chapter 6
pTWG6	3.5kb EcoRI fragment from cTOM6 in pDMW140	This study/ Chapter 6
pTWG9	3.5kb EcoRI fragment from cTOM9 in pDMW140	This study/ Chapter 6
pTWG10	3.5kb EcoRI fragment from cTOM10 in pDMW140	This study/ Chapter 6
pTWG11	3.5kb EcoRI fragment from cTOM11 in pDMW140	This study/ Chapter 6
pTWG12	3.5kb EcoRI fragment from cTOM12 in pDMW140	This study/ Chapter 6
pHC79	Ap^r Tc^r cosmid cloning vector	Lab stock/ Hohn & Collins (1980)
cTOM1	SG33 complementing cosmid	This study/ Chapter 6

cTOM2	SG33 complementing cosmid	This study/ Chapter 6
cTOM3	SG33 complementing cosmid	This study/ Chapter 6
cTOM4	SG33 complementing cosmid	This study/ Chapter 6
cTOM5	SG33 complementing cosmid	This study/ Chapter 6
cTOM6	SG33 complementing cosmid	This study/ Chapter 6
cTOM9	SG33 complementing cosmid	This study/ Chapter 6
cTOM10	SG33 complementing cosmid	This study/ Chapter 6
cTOM11	SG33 complementing cosmid	This study/ Chapter 6
cTOM12	SG33 complementing cosmid	This study/ Chapter 6

Table 2.4 Media

Medium	Constituents	Per litre
Nutrient Broth (NB)	Nutrient broth	13g
Nutrient Agar (NA)	Nutrient agar	24g
Luria Broth (LB)	Bacto tryptone	10g
	Yeast extract	5g
	NaCl	10g
	pH 7.2	
Double Difco (DD)	Bacto tryptone	20g
	NaCl	8g
2x YT	Bacto tryptone	10g
	Yeast extract	10g
	NaCl	5g
SOB	Bacto tryptone	20g
	Yeast extract	5g
	1M NaCl	10ml
	1M KCl	2.5ml
After autoclaving add:	1M $MgSO_4$	10ml
	1M $MgCl_2$	10ml
M9 Minimal (M9)	Na_2HPO_4	6g
	KH_2PO_4	3g
	NH_4Cl	1g
	NaCl	0.5g
	pH 7.4	
After autoclaving add:	1M $MgSO_4$	2ml
	1M $CaCl_2$	0.1ml
Terrific Broth (TB) (per 900 ml)	Bacto tryptone	12g
	Yeast extract	24g
	Glycerol	4ml
After autoclaving add:	10xTB salts	100ml

(10xTB salts: KH_2PO_4 23.1g/l, K_2HPO_4 125.4g/l)

Hershey Salts	NH_4Cl	1.1g
	$CaCl_2.2H_2O$	15mg
	$MgCl_2.6H_2O$	0.2g
	$FeCl_3.6H_2O$	0.2mg
	KH_2PO_4	87mg
	Trizma base	121.1g
	pH 7.4	

H Medium	20% Glucose	2ml
(per 100ml Hershey salts)	2% Proline	1ml
	0.1% Thiamine	0.1ml

K Medium — as M9 plus 1% w/v casamino acids, 0.2% w/v glucose, and 0.1ug/ml B1

XGAL Agar — 100ml LB agar plus 250ul of 2% w/v XGAL and 250ul of 2.5% w/v IPTG

XGAL — 2% w/v 5-bromo-4-chloro-3-indolyl-β-galactoside dissolved in DMF

IPTG — 2.5% w/v (isopropyl-β-d-thiogalactopyranoside)

Tryptone Broth	Bacto tryptone	10g
	NaCl	8g

P1 Buffer	Tryptone broth	10ml
(per100ml H_2O)	1M $CaCl_2$	1ml

T4 Buffer	Tryptone broth	10ml
(per100ml H_2O)	2mg/ml Tryptophan	1ml

Lambda Buffer	1M Tris pH7.4	10ml
	1M $MgSO_4$	10ml
	Gelatine	100mg

Phosphate-EDTA Buffer — Add 1M K_2HPO_4 to 1M KH_2PO_4 to give pH 6.0. Add equal volume 10mM EDTA.

for production of well defined plaques. All rich media were supplemented with thymine at 2.5ug/ml. Minimal medium was supplemented where appropriate with amino acids at 20ug/ml, thymine at 2.5ug/ml, thiamine at 1ug/ml, and sugars at 0.2% w/v (final concentrations). Ampicillin was used at 50ug/ml and tetracycline at 10ug/ml final concentrations.

2.13 Preparing Bacteriophage Lysates

Bacteriophage lysates were prepared using a plate lysate technique. Several tubes were set up containing 200ul of an overnight culture with 2.5ml of molten Double Difco (DD) top-agar containing the appropriate additives (10mM Mg^{2+} for Lambda phage, 20ug/ml tryptophan for T4GT7, and 10mM Ca^{2+} for P1). To all but one of these were added varying amounts of appropriately diluted phage lysate to give approximately 10^5 pfu per tube. The contents of the tubes were mixed gently and poured on top of thick, wet, fresh DD plates containing the appropriate additives. These were incubated overnight, without inverting the plates, at 37°C or, if the bacterial strain was temperature sensitive, at 30°C. The plates were inspected for plaques and compared with the "no phage" control. Plates with near-confluent and just-confluent lysis were harvested. The soft top-agar layer was scraped into a universal bottle using a plate spreader or spatula. The DD plate was washed with 2ml of the appropriate phage buffer in order to elute any remaining phage and this was poured into the same universal bottle. 0.5ml of chloroform was added and the mixture vortexed for about 15 mins. Following centrifugation at 5000g for 10 mins to pellet the agar and cellular debris, the resulting clear supernatant was decanted into a bijoux bottle, a few drops of chloroform added and this lysate stored at 4°C. Lysates prepared in this

manner were titrated and if none was of a sufficiently high titre the whole procedure was repeated adjusting the initial phage inoculum where appropriate.

2.14 Titration of Bacteriophage Lysates

A dilution series of the lysate was constructed using the appropriate phage buffer and dilution steps of 10fold or 100fold as necessary. 10ul drops of each dilution of the phage were spotted onto a lawn of phage-sensitive bacteria in a DD top-agar layer on a dried DD plate. Up to 2 full titrations could be accommodated per plate. After the spots had dried in, the plates were incubated inverted for 6 or more hours until plaques became visible and the titre could be ascertained.

2.15 Hydroxylamine Mutagenesis of P1 Lysates

0.5ml of a high titre P1 lysate was mixed with 1ml of phosphate-EDTA buffer, 1.5ml of sterile H_2O and 2ml of fresh hydroxylamine solution (0.35g hydroxylamine plus 560ul of 4M NaOH made up to 5ml with sterile H_2O). Following incubation for an appropriate length of time at 37°C, phage were harvested at 36,000g for 2½ hours at 4°C. Phage were eluted from the pellet by covering the pellet with 0.5ml LBSE (LB, 1M NaCl, 1mM EDTA) and standing at 4°C overnight. This phage suspension was then titrated before using it to transduce a suitable recipient E.coli strain.

2.16 Transduction of E.coli

P1 transduction

A 10ml overnight culture of the strain to be transduced was centrifuged

at 5000g for 10 mins, the supernatant decanted, and the pellet resuspended in 1ml of P1 buffer. Phage were added to give a multiplicity of infection (m.o.i.) of between 0.01 and 1, overnight cultures generally being taken as containing approximately 10^9 cfu per ml. This was incubated without shaking at 37°C (30°C for temperature-sensitive mutants) for 25 mins. The cells were harvested, washed with 10ml of NB plus 0.05% citrate and the pellet resuspended in 10ml of NB plus 0.05% citrate. The suspension was incubated with shaking for one hour at 37°C (30°C for temperature-sensitive mutants) to allow for gene expression, before harvesting the cells and resuspending in 800ul of NB. Finally 200ul aliquots of this were spread on the appropriate selective media plates and incubated to isolate transductants.

T4GT7 transduction

This was essentially the same as P1 transduction except that the transduction was performed in T4 buffer using an m.o.i. of between 0.1 and 0.25. Phage were allowed to adsorb at room temperature for 15 mins and citrate was omitted when washing and expressing. For large numbers of transductions all volumes were reduced by a factor of ten and the transductions were performed in 1.5ml eppendorf tubes.

2.17 Construction of Lambda Lysogens

200ul of an overnight culture of the strain to be lysogenised was added to 2.5ml of 0.75% top agar in a sterile phage tube. This was poured on top of an NA plate and allowed to set. A 10ul drop of the appropriate λlysate was spotted onto this top lawn. Following overnight incubation the turbid centre of the spot was streaked out to single colonies on an NA plate. Colonies were assessed for lysogeny by testing for the release of

phage. Colonies were patched onto NA plates and top agar lawns of LE392 and these were incubated at the appropriate temperature overnight. Patches surrounded by zones of lysis on the LE392 lawn were treated as putative lysogens. Overnight cultures of such putative lysogens were used to seed top agar lawns and these were spotted with 10ul spots of a homoimmune phage lysate and with λvir. Strains that showed no lysis with the homoimmune phage but were still sensitive to λvir were considered to be lysogens.

2.18 Transformation of E.coli

Routine transformation

Sigma grade 1 $CaCl_2$ was used for all transformation procedures. 20ml of NB was inoculated with 1ml of an overnight culture of the strain to be transformed. This was grown for 3 hours and subsequently chilled on ice for 20 mins. The cells were harvested at 5000g for 10 mins and resuspended in 10ml of ice-cold 0.1M $CaCl_2$. This was chilled on ice for a further 20 mins, the cells harvested and the pellet resuspended in 1ml of 0.1M $CaCl_2$. The cells were then considered to be competent.

DNA was added to 100ul of these competent cells in a sterile 1.5ml appendorf tube and the contents mixed by vortexing. Following storage on ice for 30 mins the cells were heat-shocked at 42°C for 2 mins. Where expression time was required 1ml of NB was added and the cell suspension incubated for 1 hour, the cells subsequently being harvested and resuspended in 100ul of NB. The transformation mixture was plated out on selective media and incubated at 37°C overnight, or in the case of temperature-sensitive strains, 30°C for up to 72 hours.

Efficient transformation

Where a particularly high transformation efficiency was desirable, such as when isolating clones, the above method was altered slightly as follows: 50ml of cells grown in SOB in a 250ml flask to A_{600}=0.35 were used instead of 20 ml of NB grown cells. All harvesting of cells was at 1300g instead of 5000g, and the competent cells were stored with DNA on ice for 2 hours instead of 30 mins. Where required, cells were incubated for expression of antibiotic resistance genes in SOB. Pipettes were cooled to 4°C before use and centrifugation steps took place at 4°C.
Competent cells were stored at -70°C following the addition of sterile glycerol to 15% final concentration.

2.19 Assessment of Temperature-Sensitivity

Temperature-sensitivity was assessed in a variety of ways. Where large numbers of colonies were to be tested they were patched out using sterile toothpicks on the desired medium (generally NA) in a grid of 50 per plate in duplicate. One of these plates was incubated at 30°C and the other at 42°C. The growth was compared after overnight incubation and in some cases after a further 24 hours incubation. For smaller numbers of colonies, or to confirm the results of patching out, colonies or patches were streaked out to single colonies in duplicate (up to 8 per plate) and the plates incubated as above. In order to obtain quantitative results for temperature-sensitivity viable counts were assessed as follows: A dilution series was performed on an overnight culture of the strain to be tested giving 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7} dilutions. 10ul of neat overnight culture and of each of these dilutions was spotted onto dried NA plates in duplicate. One of these plates was incubated at 30°C and the other at

42°C and the growth compared after overnight incubation. Strains were considered temperature-sensitive where the viability at 42°C was 10^5 fold less than that at 30°C. In all cases assessment was confirmed by microscopic observation of the bacteria from representative colonies or patches grown at the two temperatures.

2.2 Preparation, Manipulation and Analysis of DNA

The solutions used are listed in Table 2.5. All solutions were prepared using double-distilled or ultrapure (reverse osmosis purified) water. DNA concentrations were measured at 260nm (1 A_{260}=0.05 mg/ml), and the purity checked by comparing A_{260} with A_{280}, ratios between 1.6:1 to 2.0:1 being considered adequately pure. For small amounts of DNA the concentration was assessed by running a sample on an agarose gel with a standard of known concentration.

2.21 Preparation of DNA

Large scale preparation of plasmid DNA

A strain carrying the plasmid was grown overnight in terrific broth (TB). 5ml of this was used to inoculate 150ml of TB containing the relevant antibiotics. This was grown with shaking to A_{600} 0.5. Spectinomycin was added to 300ug/ml and the culture grown overnight. Cells were harvested by centrifugation (10,000g for 10min), resuspended in 5ml of tris-sucrose, and transferred to a 50ml "Oakridge" tube. 1.5ml of lysozyme mix was added and the tube stored on ice for 5 mins. 5.4ml of lysis mix was added and the tube was alternately inverted and warmed to 42°C until the contents lysed, visible as a clearing of the mixture. The lysate was

Table 2.5 Solutions for DNA Preparation and Manipulation

Solution	Constituents	Per litre
1M Tris	Trizma base	121.1g
	Add HCl to desired pH	
0.5M EDTA	EDTA	186.1g
	Add NaOH to pH 8.0	
100x TE	Trizma base	121.1g
	EDTA	37.22g
	pH 8.0	
Tris sucrose	Trizma base	6.06g
	Sucrose	250g
	pH 8.0	
Lysozyme mix (per 10ml)	Lysozyme	50mg
	1M Tris pH 8.0	2.5ml
Lysis mix	Trizma base	6.06g
	EDTA	23.28g
	BRIJ 58	20g
	Na deoxycholate	4g
	pH 8.0	
Phenol/Chloroform (per 250ml)	Phenol	125g
	Chloroform	120ml
	Iso-amyl alcohol	5ml
	8 hydroxyquinoline	250mg
Equilibrate with 2 changes of 50ml 1M Tris pH 8.0 and 2 changes of 50ml TE, store under TE		
Chloroform/IAA (per 250ml)	Chloroform	240ml
	Iso-amyl alcohol	10ml
GTE (per 250ml)	Glucose	2.25g
	0.5M EDTA	5ml
	1M Tris pH 8.0	6.25ml

KAc pH 4.8 (per 100ml)	5M potassium acetate	60ml
	Glacial acetic acid	11.5ml
50x TAE (per litre)	Trizma base	242g
	Glacial acetic acid	57.1ml
	0.5M EDTA	100ml
10x Loading Buffer (per 10ml)	Ficoll 4000	1.5g
	Bromophenol Blue	25mg
STE (per 10ml)	1M Tris pH 8.0	1ml
	5M NaCl	2ml
	0.5M EDTA	200ul
10x NT buffer (per 10ml)	1M Tris pH7.2	5ml
	1M $MgSO_4$	1ml
	1M Dithiothreitol	10ul
	Bovine serum albumin	5mg
5x Ligation buffer (per 10ml)	1M Tris pH 7.6	2.5ml
	1M $MgCl_2$	0.5ml
	PEG 8000	2.5g
	1M ATP	50ul
	1M Dithiothreitol	50ul

centrifuged at 40,000g for 30 mins at 4°C and the clear supernatant decanted. This cleared lysate was used to make 5ml CsCl gradients (5ml lysate, 4.6g CsCl, 200ul 10mg/ml ethidium bromide) which were centrifuged at 300,000g for 16 hours at 15°C. The plasmid band was removed through the side of the centrifuge tube using a 21 gauge hypodermic needle. The ethidium bromide was extracted by vortexing with an equal volume of propan-2-ol (equilibrated with NaCl saturated TE), centrifuging in a microfuge to separate the phases and removing the supernatant. This was repeated until all colour was removed. The CsCl was removed by dialysing the DNA against a large volume of TE overnight at 4°C.

Preparation of chromosomal DNA

Chromosomal DNA was prepared in a similar manner to plasmid DNA except that 100ml of culture grown in NB was used, and instead of the clearing spin the lysate was phenol extracted using an equal volume of phenol/chloroform (centrifuging at 5000g for 10mins to separate the phases). This was repeated until no white precipitate was visible at the phenol/aqueous interface. The lysate was vortexed with an equal volume of chloroform/IAA, centrifuged (5000g 10mins) and the supernatant put into a plastic Petri dish to remove the last traces of chloroform. This cleared lysate was used to make 5ml CsCl gradients as above.

Small scale preparation of plasmid DNA ("miniprep")

This is essentially the alkaline lysis method of Maniatis et al. (1982). 1ml of an overnight culture of a strain carrying the plasmid grown in NB was transferred into a 1.5ml Eppendorf tube. The cells were harvested, resuspended in 150ul of ice-cold GTE and left for 5 mins at room temperature. 200ul of freshly prepared 0.2N NaOH/1% SDS was added, the

tube inverted several times and stored on ice for 5 mins. 150ul of ice-cold potassium acetate was added, the tube inverted 5X and stored on ice for 5 mins. The tube was centrifuged for 5 mins and the supernatant transferred to a new tube. An equal volume of phenol/chloroform was added, the tube vortexed, centrifuged for 2 mins and the supernatant transferred to a new tube. This was repeated using an equal volume of chloroform/IAA. Two volumes of ethanol were added, the tube allowed to stand at room temperature for 2 mins and centrifuged for 10 mins. The supernatant was discarded and 2ml of 70% ethanol gently poured down the side of the tube. This was re-centrifuged for 5 mins and all the supernatant removed. The pellet was dried in a vacuum desiccator for 15 mins and resuspended in 50ul of TE. 5ul aliquots of this were used for restriction analysis. Where necessary RNase was added to 20ug/ml final concentration and the preparation heated to 37°C for 30 mins.

2.22 Agarose Gels

DNA fragments were resolved by electrophoresis through agarose gels. 0.6% agarose gels (0.6% agarose, 1x TAE, 0.5ug/ml ethidium bromide) were run routinely in TAE buffer and 0.5ug/ml ethidium bromide. These were run at up to 8V/cm. DNA was visualised by placing the gel on a long-wave UV trans-illuminator.

2.23 Phenol Extraction and Ethanol Precipitation of DNA

DNA was phenol extracted by vortexing in a 1.5ml Eppendorf tube with an equal volume of phenol/chloroform, centrifuging for 2 mins to separate the phases, and carefully transferring the supernatant to a new tube. This process was repeated using chloroform/IAA. DNA treated in this manner

was generally ethanol precipitated: 3 volumes of ethanol, 1 volume of NH_4Ac and 2ul of glycogen were added and the mixture vortexed. The tube was allowed to stand at room temperature for at least 20 mins before centrifuging for 10 mins. The supernatant was removed and the pellet dried in a vacuum desiccator for 15 mins. The DNA pellet was resuspended in the desired volume of TE.

2.24 Endonuclease Restriction of DNA

Restriction endonucleases were supplied by Amersham and BRL and used in the buffers provided. A typical restriction contained 100ng of DNA, 1 unit of restriction enzyme, and 1ul of the supplied 10X buffer in a total volume of 10ul. This was incubated at 37°C for 90 mins (25°C in the case of SmaI), 1ul of loading buffer was added and the whole restriction digest run on an agarose gel.

2.25 Removal of 5' Phosphate Groups ("phosphatasing")

DNA was phosphatased using calf intestinal phosphatase (CIP). 1 unit of CIP was added to the restriction digest mix (enough to phosphatase 100ug of 4kb DNA fragments) and it was incubated at 37°C for 30 mins. 10ul of STE and 5ul of 10% SDS was added and the solution was made up to 100ul with H_2O. The mixture was heated to 65°C for 15 mins, phenol extracted and ethanol precipitated.

2.26 Filling in 5' Overhanging Ends

5' overhangs were filled in using the Klenow (large) fragment of DNA polymerase 1. A typical reaction contained 2n moles each of dATP, dCTP, dGTP, & dTTP, 1ug of DNA, 1 unit of Klenow fragment, and 2ul of 10X

NT buffer, made up to 10ul with H_2O. Following incubation at room temperature for 30 mins, 0.5ul EDTA was added and the DNA phenol extracted and ethanol precipitated.

2.27 Isolation of DNA Fragments

DNA fragments were separated by electrophoresis through agarose gels, the desired fragment being isolated using an IBI electroeluter or by eluting into troughs cut into the agarose gel. In order to use the electroeluter a slice containing the band of interest was cut out of the gel and placed in the electroeluter. The DNA was eluted into 7.5M NH_4Ac. typically (for a 4.5kb fragment) being run at 100V for 15 mins. Progress of the elution was followed using a hand-held long-wave UV lamp. When elution was complete the DNA in the NH_4Ac was precipitated by adding 2 volumes of ethanol (or 1 volume of isopropanol) and 2ul of glycogen.

Alternatively the level of buffer in the electrophoresis tank was reduced so that the agarose gel was no longer submerged and a trough was cut in the gel just below the DNA band of interest. This trough was filled with 100ul of TAE and the DNA eluted into it in bursts, typically (for a 830bp fragment) 4 bursts of 100V for 45 seconds, putting a fresh aliquot of TAE in the trough each time. The progress of elution was followed by placing the gel on a long wave UV trans-illuminator. When all the fragment had been eluted the aliquots of TAE were pooled and the DNA ethanol precipitated.

2.28 Ligations

Ligations were performed using a vector to insert ratio of approximately 1:2 (2:1 when the vector was phosphatased) and a DNA concentration of between 20 and 100ug/ml in a volume of up to 20ul. For ligations involving 'sticky' ends the DNA was mixed and heated to 65°C for 5 mins and cooled on ice to anneal the ends. The appropriate amount of 5x ligation buffer and 1 unit of T4 DNA ligase was added and the ligation incubated at 15°C for 16 hours. The ligation was diluted 1:4 with TE before being used to transform competent cells, adding no more than 20ul of ligation per 200ul of cells.

2.29 Preparation of an OV2 Cosmid Library

Aliquots of total OV2 DNA were digested for 30 minutes at 37°C with a range of dilutions of the restriction enzyme Sau3A in Eppendorf tubes. All the reactions were placed on ice and then the digestion products were analysed on agarose gels. A dilution was chosen that gave only partial digestion leaving large fragments. A bulk digest was set up using Sau3A at the chosen concentration. The fragments from this digest were run on an agarose gel. A trough was cut in the gel corresponding to a position just below the 23kb λ HindIII marker. The large fragments from the Sau3A digest were run into aliquots of TAE in this trough in short bursts. These aliquots were pooled and the nucleic acids were precipitated and resuspended in a small volume.

The cosmid vector pHC79 was digested to completion with the restriction enzyme BamHI. The digestion was checked on an agarose gel and then the BamHI cut vector was treated with Calf Intestinal Phosphatase. The DNA

was then phenol extracted, ethanol precipitated and resuspended in a small volume. Samples of vector and fragment DNA were run on an agarose gel to compare their concentration. Vector and fragment DNA were then mixed at an estimated 2:1 ratio and ligated. This ligation mix was *in vitro* packaged using a Stratagene 'Gigapack Gold' kit. The resulting 'lysate' was used to transduce DH1 cells, transductants being selected on NA plates containing 50ug/ml ampicillin. Following overnight incubation at 37°C approximately 1000 colonies had grown on each plate. 480 of these colonies were each transferred into 100ul of LB in a well of a microtitre plate. These were incubated overnight at 37°C, 100ul of 2 x freezing medium added to each well and the plates frozen at -20°C as a library. The original transduction plates were incubated at 37°C overnight to grow up the remains of the colonies that had been picked. The colonies were then washed off the plates with NB and pooled. Some of this pooled library was frozen at -20°C. A plasmid preparation was performed on the remainder.

2.3 DNA Sequencing

DNA sequencing was performed using the dideoxy chain termination method of Sanger *et al.* (1977).

2.31 Isolation of Recombinant M13 clones

Competent TG1 cells were made using a $CaCl_2$ procedure based on that of Miller (1972). 100ml of LB was inoculated with 1ml of a TG1 overnight and grown with shaking at 37°C to A_{550}=0.2. The culture was chilled on ice for 10 mins and centrifuged at 4000g for 5 mins at 4°C. The pellet was resuspended in 50ml of freshly prepared ice-cold 50mM $CaCl_2$ and

10mM MOPS (pH 6.8), and left on ice for 15 mins. The cells were again harvested at 4000g for 5 mins at 4°C, and the pellet resuspended in 6ml of the $CaCl_2$/MOPS solution. The cells were then ready for use.

200ul of competent TG1 cells and 20ul of fragment/M13 ligation were mixed and incubated on ice for 45 mins. The transformation was heat shocked for 4 mins at 42°C then added to 3ml of molten M13 top agar (tryptone broth plus 0.8% bacto agar) plus 25ul of 2% XGAL (5-bromo-4-chloro-3-indolyl-β-galactoside) in DMF and 25ul of 2.5% IPTG (isopropyl-β-d-thiogalactopyranoside) in H_2O. This was mixed and poured on top of a 2x YT plate. When the top layer had set the plate was incubated inverted at 37°C overnight. Recombinant M13 clones gave colourless plaques with a low level background of (non-recombinant) blue plaques.

2.32 Preparation of Template

Single stranded DNA template was prepared from recombinant M13 clones as follows: An overnight culture of TG1 in 2x YT was diluted 1 in 100 in 2x YT. This was pipetted into sterile phage tubes in 1.5ml aliquots and a single white plaque toothpicked into each tube. These were grown at 37°C with shaking at 440rpm for 5 hours. Each culture was transferred to a 1.5ml eppendorf tube and centrifuged for 15 mins. The supernatant was poured into a clean tube and 200ul of PEG solution (Table 2.8) added. The tube was inverted 5x and left at room temperature for 10 mins. It was subsequently centrifuged for 15 mins and most of the PEG removed. The tube was re-centrifuged for 30 secs and all the residual PEG removed. The phage pellet was resuspended in 100ul of TE and an equal volume of buffer saturated phenol added. The tube was vortexed, allowed to stand for 5 mins, vortexed again and centrifuged for 2 mins. The

aqueous layer was transferred to a new tube, 3 volumes of ethanol and ¼ volume of 7.5M NH_4Ac were added, and the DNA was precipitated at -20°C for at least 30 mins. The tube was centrifuged for 5 mins and the ethanol poured off. 1ml of 95% ethanol was added, the tube centrifuged for a further 5 mins and the ethanol again poured off. The pellet was dried in a vacuum desiccator, resuspended in 20ul of TE, and stored at -20°C.

2.33 Sequencing Reactions

Materials

Oligonucleotide primers were synthesised on an Applied Biosystems 380B DNA Synthesiser. Deoxy- and dideoxy-NTPs were supplied by Pharmacia. Klenow fragment of DNA polymerase I was supplied by BRL. ^{35}S-dATP was supplied by Amersham. Materials are listed in Table 2.6. Table 2.7 shows the composition of the termination mixes.

Method

An annealing mix was made by mixing 1ul TM, 1ul primer, and 4ul ultrapure sterile water, per clone to be sequenced. This was dispensed in 6ul aliquots to a series of Eppendorf tubes each containing 4ul of template to be sequenced. The resulting solutions were each dispensed into a microtitre dish in 2ul aliquots to four wells per clone marked A, C, G, and T. The wells were sealed with Nescofilm and the dish placed in a 55°C oven for 20 mins to allow annealing.

2ul of 1x extension mix (made up in H_2O), 1ul of 0.1M DTT, 0.5ul of ^{35}S dATP (10uCi/ul), 5.2ul of H_2O, and 0.3ul of Klenow fragment (5u/ul), per clone were mixed in an Eppendorf tube. 2ul of this was dispensed to the

Table 2.6 DNA Sequencing Solutions

Solution	Constituents
20% PEG	20% w/v Polyethylene glycol (6000 mw) 2.5M NaCl
TM	100mM Tris pH 8.5 50mM $MgCl_2$
5x E mix	7.5uM dCTP 7.5uM dGTP 7.5uM dTTP in TE
Formamide dye mix	100ml Deionised formamide 0.1g Xylene cyanol F.F. 0.1g Bromophenol blue 2ml 0.5M EDTA

Table 2.7 Termination Mixes

(Volumes in microlitres)

Termination Mix:	A	C	G	T
100mM dATP 1mM stock:	12.5	1.25	1.25	1mM stock:12.5
100mM dCTP	1.25	1mM stock:12.5	1.25	1.25
100mM dGTP	1.25	1.25	1mM stock:12.5	1.25
100mM dTTP	1.25	1.25	1.25	1mM stock:12.5
5mM ddATP	30.0			
5mM ddCTP		15		
5mM ddGTP			10	
5mM ddTTP				50
TE	450	485	470	455

side of each well containing annealed template and primer. The solutions were mixed and the extension reaction started by briefly centrifuging in a microtitre plate centrifuge. The extension reaction was carried out at room temperature for 4 mins and 30 secs.

During this extension time 2ul of the A, C, G or T termination mix was dispensed to the side of each well as appropriate. When the extension time had elapsed the microtitre plate was centrifuged briefly to mix in the termination mixes and thus start the termination reactions, and was then transferred to a 37°C incubator for 15 mins.

2ul of formamide dye mix was dispensed to the side of each well and mixed in by centrifuging the plate briefly. The samples could be stored at this point at -20°C for several days. The plate was then placed (after thawing at room temperature if necessary), with the lid removed, in an 80°C oven for 15 mins to denature the DNA and to reduce the volume. The samples were then loaded onto a sequencing gel.

2.34 Pouring Buffer Gradient Sequencing Gels

Buffer gradient acrylamide gels were routinely used for sequencing. Recipes are given in Table 2.8. Bio Rad 38x50cm sequencing rigs with 0.4mm spacers were used for casting and running the gels. The glass plates, comb and spacers were thoroughly cleaned with soap and water, rinsed with water and wiped over with ethanol. One of the plates was silanised by spreading 3ml of Repelcote (Sigma) over its surface and allowing the solvent to evaporate in a fume hood. This plate was again rinsed with water and ethanol and the rig was assembled ready for pouring.

Table 2.8 Sequencing Gel Solutions

Solution	Constituents	Per litre
10x TBE	Trizma base	108g
	Boric acid	55g
	EDTA	9.3g
40% Acrylamide	Acrylamide	380g
	NN-methylene-bisacrylamide	20g
Deionised by storing with 20g Amberlite MB1		
0.5x TBE gel mix	40% Acrylamide	150ml
	10x TBE	50ml
	Urea	480g
5.0x TBE gel mix (per 200ml)	40% Acrylamide	30ml
	10x TBE	100ml
	Urea	92g
	Bromophenol blue	10mg
25% AMPS	25% w/v Ammonium persulphate	

10ul each of 25% AMPS and TEMED was added to 5ml of 5xTBE gel mix which was pipetted in-between the gel plates at the bottom to form a plug. The comb was wiped with a paper towel soaked in 25% AMPS and allowed to dry. When the plug had set 110ul each of 25% AMPS and TEMED were added to 75ml of 0.5xTBE gel mix and 30ul each of 25% AMPS and TEMED were added to 15ml of 5xTBE gel mix. 12ml of the 0.5xTBE gel mix and 12ml of the 5xTBE gel mix were taken up in the same 25ml pipette and a few bubbles introduced, causing partial mixing as they rose through the interface. This mixture was gently pipetted in-between the plates keeping the plates less than 45° from horizontal. The remaining 0.5xTBE gel mix was poured in using a syringe, taking care not to disrupt the gradient or introduce bubbles. When the acrylamide was up to the top of the plates they were lowered to about 5° from horizontal and the comb inserted. They were left at this angle until the gel had set.

2.35 Running Sequencing Gels

Top and bottom tanks were filled with 1xTBE, the comb was removed from the gel and the slots were rinsed using TBE from the top tank. 2ul samples were loaded using a Drummond sequencing pipette, and the gel was run at 85W until the lower dye front (bromophenol blue) had reached the bottom of the gel, about 3½ hours. The gel was then taken off as follows: The silanised plate was levered up and removed and a large piece of Whatman 3MM paper placed over the gel. This was smoothed down and then lifted by the corner, peeling the gel off the glass plate on the 3MM backing. The gel was covered with cling-film and dried down under vacuum at 80°C for 40 mins. An autoradiograph was made by exposing X-ray film (Fuji RX) to the dried down gel overnight.

2.4 Protein Analysis

2.41 Radio-Labelling of Proteins Using the Maxi-cell System of Sancar et al. (1979)

10ml of CSH26ΔF6 carrying the plasmid of interest was grown at 37°C in K-medium with antibiotics to A_{600}=0.5 and the culture chilled on ice. The culture was transferred to a plastic petri dish and swirled as it was irradiated with 50 J/m^2 shortwave UV. The culture was transferred to a new universal bottle and cycloserine was added to 200ug/ml. The culture was incubated overnight at 37°C, then washed 2x in Hershey salts, resuspended in 1ml Hershey medium with 200ug/ml cycloserine and transferred to an eppendorf tube. After 1 hour incubation at 37°C with shaking, 30uCi of ^{35}S methionine was added. The culture was incubated for a further hour, the cells harvested, washed 2x with 10mM Tris pH 7.4, and finally resuspended in 1ml of 10mM Tris pH 7.4. These could be stored at -20°C.

2.42 Preparing Membrane and Soluble Fractions

8ml of maxi-cells (40 A_{600} units) were sonicated on ice using an amplitude of 8um with a ½ inch probe in a 150 watt MSE ultrasonic disintegrator. Sonication was for 3x 30 secs with 30 sec breaks to allow cooling. If the cells had not lysed, ie. the suspension had not cleared, this procedure was repeated. The lysate was centrifuged at 4000g for 5 mins at 4°C to remove any remaining whole cells, the supernatant was transferred to fresh tubes and $MgSO_4$ added to 10mM final concentration. This was centrifuged at 85,000g for 30 mins at 4°C. The soluble fraction (supernatant) was removed and the membrane pellet was resuspended in

10mM phosphate buffer pH 7.2 containing 10mM $MgSO_4$. The membranes were washed 2x in this buffer (centrifuging at 85,000g for 30 mins at 4°C each time) and the pellet resuspended in 8ml of 1% Triton-x-100 in 10mM Tris pH 7.4. In order to prepare soluble proteins and peripheral membrane proteins, sonicated maxi-cells were centrifuged at 85,000g for 30 mins at 4°C in the absence of Mg^{2+} to remove whole cells and membranes.

2.43 SDS-PAGE Gels

A discontinuous denaturing acrylamide gel system based on that of Laemmli et al. (1970) was routinely used to resolve proteins using gels 160x180x2mm. The composition of 10% linear gels is shown in Table 2.8. All components of stacking and resolving gels except catalysts (10% AMPS and TEMED) were mixed, and degassed in a vacuum desiccator for 10 mins. Catalysts were added to the resolving gel and it was poured and overlayed with water saturated butan-1-ol. When the gel had set, as shown by an accumulation of water at the butan-1-ol/gel interface, the butan-1-ol was poured off, the top of the gel rinsed with water and residual water removed with a paper towel. Catalysts were added to the stacking gel and it was poured on top of the resolving gel. The comb was inserted and the gel allowed to set. Immediately prior to loading, the comb was removed and the wells rinsed with running buffer. An equal volume of 2x sample buffer was added to samples and they were boiled for 5 mins before loading onto the gel. Gels were run at 30mA continuous current until the dye front reached the bottom of the gel (approximately 3½ hours).

Table 2.8 SDS-PAGE Gel Solutions

Solution	Constituents
Resolving Gel Buffer	3M Tris pH 8.8
Stacking Gel Buffer	0.05M Tris pH 6.8
Resolving Gel Acrylamide	60% w/v Acrylamide 1.6% w/v NN-methylenebisacrylamide
Stacking Gel Acrylamide	10% w/v Acrylamide 0.5% w/v NN-methylenebisacrylamide
10x Running Buffer	0.25M Tris 1.92M Glycine 1% w/v SDS
10% AMPS	10% w/v Ammonium Persulphate
2x Sample Buffer	20mM Tris pH8.0 2mM EDTA 5% w/v SDS 10% v/v β-mercaptoethanol 0.05 % w/v Bromophenol Blue

10ml Stacking Gel		
	Stacking buffer	2.4ml
	Stacking acrylamide	3.0ml
	10% SDS	100ul
	H_2O	4.4ml
	10% AMPS	100ul
	TEMED	5ul

30ml Resolving Gel (10%)		
	Resolving buffer	3.78ml
	Resolving acrylamide	4.8ml
	10% SDS	300ul
	H_2O	20.9ml
	10% AMPS	60ul
	TEMED	6ul

2.44 Coomassie Staining of Gels

In order to visualise protein bands gels were stained overnight in IPA/H_2O/GAA (25% isopropanol : 65% H_2O : 10% glacial acetic acid) containing 0.1% Coomassie Blue R250. To destain the background, gels were soaked in 2 changes of IPA/H_2O/GAA in the presence of a foam bung to absorb excess stain.

2.45 Fluorography

For each gel 40g of 2,5-diphenyloxazole (PPO) was dissolved in 180ml of glacial acetic acid (GAA). The gel was immersed for 5 mins in GAA, then for 90mins in the PPO/GAA solution. The gel was soaked in H_2O for 30 mins, then dried down under vacuum for 90 mins, 60 mins at 80°C and 30 mins with the heater turned off and the drier cooling down.

Alternatively the gel was fixed in IPA/H_2O/GAA for 30 mins then soaked in Amplify (Amersham) for 30 mins before drying down as above. An autoradiograph was then made of the gel, exposing the film (Fuji RX) for between 16 hours and several weeks at -70°C.

Chapter 3: LOCALISED MUTAGENESIS OF THE 76 MINUTE REGION

3.1 Introduction

Cloning and complementation studies have shown that three essential genes, *ftsE*, *ftsX*, and *rpoH*, lie adjacent to two genes of unknown function, *ORF4* and *ftsY*, at 76 minutes on the *E.coli* chromosome (Crickmore and Salmond, 1986). *ftsY* is the first gene in a 3 gene operon with *ftsE* and *ftsX*. As such there is a distinct possibility that *ftsY* is an essential gene. The promoter of *ORF4* appears to overlap that of the operon, suggesting that expression from these divergent promoters may be interdependent in some manner, and raising the possibility that *ORF4* may also be involved in essential processes. However no mutations have been isolated in either of these genes to date. By analogy with the large cluster of essential genes at 2 minutes on the chromosome (Donachie *et al*, 1984) there is also a chance that there may be more as yet undiscovered essential genes at 76 minutes.

In order to find out whether *ftsY* is essential a site-directed mutagenesis procedure could be used to construct a mutation which could then be tested for conditional lethality. This should be quite feasible as the DNA sequence of *ftsY* is known, therefore an amber mutation could be put into a *supF*^{ts} (temperature-sensitive amber suppressor) background resulting in a temperature-sensitive FtsY phenotype. However this might not produce a viable temperature-sensitive strain because of the low suppression rate (40% of *supF* at 30^{o}C, Smith *et al*, 1970) and frequent mischarging (50% of *supF*^{ts} tRNAs charge with glutamine rather than tyrosine) of *supF*^{ts}. A second strategy would be to attempt to force an *in-vitro* constructed

deletion of *ftsY* into the chromosome. If this succeeded then it would be proof that *ftsY* was non-essential. If it failed, however, it would not be proof that *ftsY* was essential.

A third strategy would be to conduct a localised mutagenesis on the 76 minute region and screen for mutants that were temperature-sensitive or cold-sensitive for growth. If any of these mapped to *ftsY* it would be proof that *ftsY* was essential. This strategy has several advantages over the other two. Firstly mutations isolated in this way would be known to come from bona fide mutants, overcoming the uncertainties of attempting to site-direct a conditional mutation. Secondly there is the possibility of isolating mutations mapping in new essential genes in this region or in *ORF4*. Finally such a procedure should result in the isolation of a range of alleles of the known essential genes in this region ie. *ftsE*, *ftsX*, and *rpoH*. These might be useful in a variety of ways, such as looking for clustering of mutations or finding interacting proteins by looking for extragenic suppressor mutations, which are often allele specific. Taking account of all these advantages this third approach was chosen.

The method used was that of Hong and Ames (1974). A high titre transducing lysate is made on a strain that carries a selectable marker next to the genes of interest. This lysate is mutagenised with hydroxylamine *in vitro* then used to transduce into a recipient strain of *E.coli*, selecting for the marker. This selects for transduction of the region of interest which in a proportion of cases will be carrying hydroxylamine induced mutations. Transductants are finally screened for the desired mutant phenotype.

3.2 Mutagenesis and Isolation of Mutants

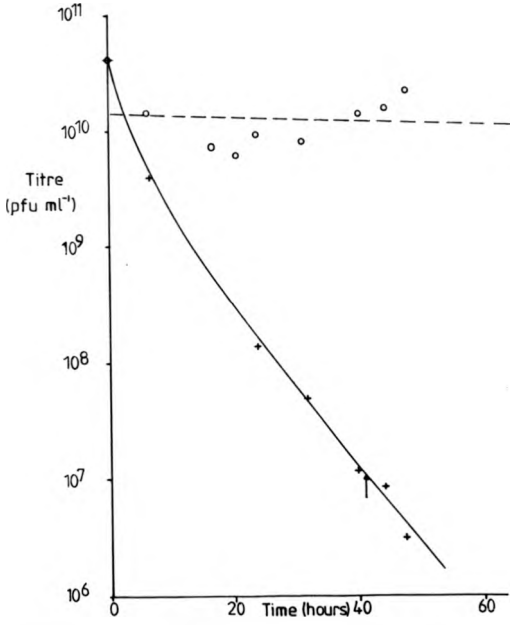
3.21 Localised mutagenesis procedure

A P1 lysate was made on OV2 zhf::Tn10 with a titre of 3×10^{11} pfu/ml. In order to establish an appropriate level of mutagenesis this was mutagenised with hydroxylamine for up to 64 hours (see chapter 2). A control was also set up with H_2O replacing the hydroxylamine solution. 100ul samples were taken at intervals throughout the mutagenesis. These samples were diluted in 10 ml of LBSE (LB plus 1M NaCl and 1mM EDTA) at 4^oC and allowed to stand for 1 hour at 4^oC. The phage in the mutagenised and control samples were then titrated on a lawn of C600 in order to establish the level of mutagenesis. The $\log_{10}$ of the titre was plotted against the time of incubation for control and mutagenised lysates. Figure 3.1 shows one such graph. The control titres were found to be extremely variable. A simple linear regression was performed on the control data and the point found where control and mutagenised titres differed by a factor of 10^3. This point was after approximately 40 hours of exposure to hydroxylamine. This level of mutagenesis was chosen in order to give a reasonably high frequency of mutations without giving rise to double mutations.

Larger volumes of lysate were then mutagenised for 40 hours. Following mutagenesis the lysates were titrated in order to check that the titre had dropped by a factor of 10^3 relative to a control lysate incubated in H_2O. The supernatant from harvesting the mutagenised phage was also titrated to check recovery efficiency. Typical figures are given below:

Figure 3.1 Inactivation Curve for Hydroxylamine Mutagenesis of P1

A high titre P1 lysate was incubated in the presence (+, 'mutagenised') and absence (o, 'control') of hydroxylamine. Samples were taken and titrated at various time points. The titre vs. time of exposure to hydroxylamine is plotted below. A regression line was drawn through the control points as they are very scattered. Using this line it is seen that the mutagenised titre was 0.1% of the control titre at ca. 40 hours. (↑)



Lysate	Titre(pfu/ml)
Initial	1.3×10^{11}
Mutagenised	2.0×10^{7}
Control	1.3×10^{10}
Supernatant	$<10^{4}$

It is clear from this that little phage was lost in the harvesting step. The lytic phage survival following mutagenesis was approximately 0.15%, which is almost exactly the desired level (0.1%).

3.22 Isolation of temperature-sensitive mutants

Mutagenised lysates were used to transduce OV2 to tetracycline resistance, plating out the transductions on NA containing tetracycline and incubating the plates at 30°C. Tetracycline resistant transductants were then patched onto OV2 minimal medium and two NA plates. One of the NA plates was incubated at 42°C and the other two plates were incubated at 30°C. Any transductants that grew at 30°C and not at 42°C were streaked out in duplicate on NA, one plate being incubated at 30°C and the other at 42°C to check the temperature-sensitivity. No transductants were found that did not grow on OV2 minimal medium. 10,000 transductants were screened in this manner and 28 temperature-sensitive mutants were isolated. These mutants were stored in frozen culture as initial isolates. In order to improve the chances of working on single mutations, T4GT7 transducing lysates were made on all of these temperature-sensitive mutants and used to transduce the mutations into OV2, selecting for tetracycline resistance and screening for temperature-

sensitive transductants. All subsequent experiments were performed using these transductants.

3.23 Screening for cold-sensitive mutants

OV2 was transduced with mutagenised lysates as above using a temperature of 37°C for all steps of the transduction. Selection for transductants was on NA plates containing tetracycline, incubated at 37°C. Transductants were then patched out on OV2 minimal medium, and NA in duplicate, one of the NA plates being incubated at 25°C and the other two plates being incubated at 37°C. Patching of transductants was performed in a warm room (30°C) onto prewarmed plates in order to minimise exposure of the transductants to low temperatures. Out of 2000 colonies screened no cold-sensitive mutants (that grew at 37°C and not at 25°C) or transductants carrying additional auxotrophies were isolated.

3.24 Assessing the level of localised mutagenesis

In order to further assess the level of localised mutagenesis the level of mutagenesis of adjacent non-essential genes was investigated. The *glpRD* locus maps at approximately 75.3 minutes on the *E.coli* linkage map (Bachmann, 1983) and is therefore close to the transposon in OV2 *zbf*::Tn*10*. A number of the tetracycline resistant transductants being screened for temperature-sensitivity were also patched onto OV2 minimal glycerol (0.5%) plates and incubated at 30°C. Those transductants that grew on OV2 minimal (glucose) and not on OV2 minimal glycerol were streaked out on these media and NA and incubated at 30°C. Any

transductants that still did not grow using glycerol as a carbon source were considered to be glpR or glpD mutants. Of 800 tetracycline resistant transductants tested, 5 were found to be Glp$^-$. This gives a level of localised mutagenesis of 0.62% which is low enough to make isolation of double mutants unlikely. The localised mutagenesis data is summarised in Table 3.1.

3.3 Characterisation of the Mutants

3.31 Cellular morphology of mutants

The appearance of temperature-sensitive mutants grown on NA plates at 30°C and 42°C was examined under phase contrast. The appearance of the mutants at the restrictive temperature fell into three classes: filamentous (fts-like); short filaments with inclusions (rpoH-like); and pleomorphic. Typical mutants are shown in Figure 3.2, and the classification of each of the mutants is given below:

Filamentous (fts-like)
SG1, SG9, SG10, SG11, SG12, SG14, SG18, SG19, SG20
SG21, SG23, SG27, SG29, SG31, SG35

Short Filaments (rpoH-like)
SG7, SG8, SG13, SG15, SG17, SG25, SG26, SG30, SG32

Pleomorphic
SG24, SG28, SG33, SG34

3.32 Complementation of mutations with pH3C and λGS22

All the mutants were initially transformed with pH3C (Figure 3.3) which carries ORF4, ftsY, ftsE, and ftsX. Transformants were patched out in

Table 3.1 Level of Localised Mutagenesis

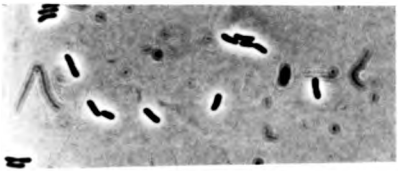
(Glp^- = unable to grow on OV2 minimal glycerol plates, Ts^- = unable to grow at 42°C on NA plates.)

Lytic phage survival	0.15%
Number of transductants screened for Glp^-	800
Number of Glp^- transductants isolated	5
Percentage of transductants Glp^-	0.62%
Number of transductants screened for Ts^-	10000
Number of Ts^- transductants isolated	28
Percentage of transductants Ts^-	0.28%

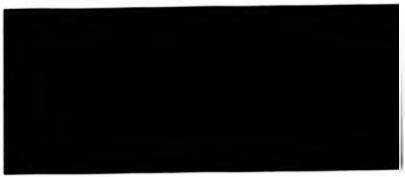
Figure 3.2 Microscopic Appearance of the Temperature-Sensitive Mutants at the Restrictive Temperature (42°C)

Photomicrographs were taken using a Leitz Dialux 22 camera system. All photographs are shown at the same magnification.

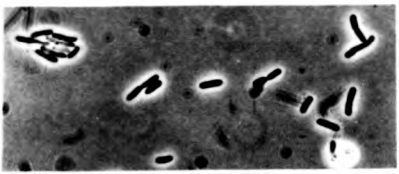
Wild type (OV2)



Filamentous mutant



rpoH-like mutant



Pleomorphic mutant

duplicate on NA plates and assessed for temperature-sensitivity. Viable counts were performed at permissive and restrictive temperatures. Strains with a viability at 42°C that was more than 10% of their viability at 30°C were considered to be no longer temperature-sensitive and hence show complementation. Using these criteria 15 of the mutants were complemented by this plasmid as shown by the viable counts in Table 3.2. Three of the mutants: SG17, SG23, and SG26 had similar viability at 30°C and 42°C on NA without pH3C, although the colony formation was weaker and microscopy showed abnormal cell morphology at the restrictive temperature. To improve the distinction between complemented and mutant phenotypes viable counts were performed on these strains on LB0 (LB agar containing no NaCl), which generally increases the temperature-sensitivity of these mutants. In order to check that pH3C gave true complementation and not a multi-copy suppression effect λGS22 lysogens of each of the complemented mutants were constructed. These lysogens were also found to be temperature-resistant suggesting true complementation by pH3C.

3.33 Mapping of mutations complemented by pH3C

The 15 mutants carrying pH3C complementable mutations were transformed with each of the plasmids in the series shown in Figure 3.3. Complementation was analysed by assessing temperature-sensitivity of plasmid bearing strains, initially by streaking out, and then by performing viable counts at permissive and restrictive temperatures. These results are shown in Table 3.3. As in the case of complementation with pH3C, clearer results were obtained for a few strains (SG23 and SG27) by

Table 3.2 Complementation with pH3C

Viability at 42°C expressed as a percentage of viability at 30°C on NA plates. Strains marked (*), viability measured on LBO plates (see text).

Strain	No Plasmid	pH3C	Strain	No Plasmid	pH3C
OV2	106				
SG1	<0.001	72	SG21	<0.001	58
SG7	<0.001	<0.001	SG23*	0.033	62
SG8	<0.001	<0.001	SG24	0.029	0.005
SG9	<0.001	70	SG25	<0.001	<0.001
SG10	<0.001	90	SG26*	<0.001	<0.001
SG11	<0.001	74	SG27	<0.001	59
SG12	<0.001	142	SG28	<0.001	<0.001
SG13	<0.001	<0.001	SG29	<0.001	50
SG14	<0.001	84	SG30	<0.001	<0.001
SG15	<0.001	<0.001	SG31	<0.001	96
SG17*	<0.001	<0.001	SG32	0.002	0.005
SG18	<0.001	64	SG33	<0.001	<0.001
SG19	<0.001	16	SG34	<0.001	0.037
SG20	<0.001	74	SG35	<0.001	117

Figure 3.3 The pH3C plasmid series

This is a plasmid deletion series based on pH3C, which carries the 4.5kb HindIII fragment from λ S3d in pBR322. Only the insert DNA is shown. Key to enzymes: A2=AatII; P2=PvuII; B2=BstEII; S1=SphI; RV=EcoRV; Stu=StuI, H3=HindIII. ▲ = 65bp deletion between two BstEII sites.

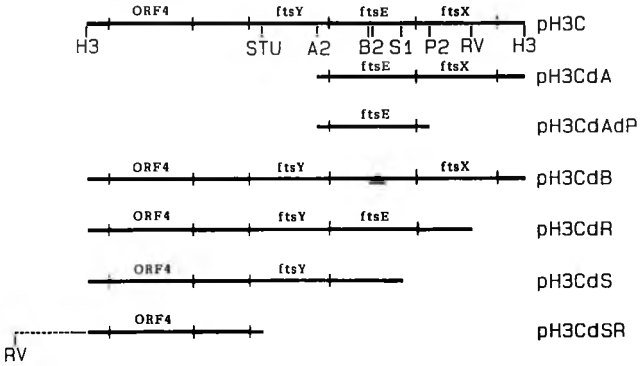


Table 3.3 Complementation with the pH3C Plasmid Series

Viability at 42°C expressed as a percentage of viability at 30°C on NA plates. Strains marked (*), viability measured on LBO plates (see text).

STRAIN	PLASMID					
	pH3CdA	pH3CdAdP	pH3CdB	pH3CdR	pH3CdS	pH3CdSR
SG1	124	105	0.178	101	0.065	<0.001
SG9	105	0.223	0.091	0.030	0.034	<0.001
SG10	112	119	0.006	85	0.051	<0.001
SG11	69	108	0.027	127	0.053	<0.001
SG12	104	0.706	114	0.078	<0.001	<0.001
SG14	100	0.563	84	0.341	<0.001	<0.001
SG18	96	0.022	1.168	0.011	0.040	<0.001
SG19	46	40	0.006	59	0.045	<0.001
SG20	116	115	0.005	77	0.015	<0.001
SG21	194	106	0.004	235	0.015	<0.001
SG23*	95	93	0.005	89	0.016	0.008
SG27*	95	<0.001	50	0.025	<0.001	<0.001
SG29	94	0.282	84	0.351	<0.001	<0.001
SG31	108	79	0.746	77	0.128	<0.001
SG35	91	82	0.167	120	0.050	<0.001

measuring viability on LBO.

3.34 Complementation of mutations with pDBS9, pR2, and λS3D

Mutants that were not complemented by pH3C were transformed with pDBS9 (which carries *ftsE*, *ftsX*, and *rpoH*), and pR2 (which carries the *rpoH* gene expressed from the *tet* promoter of pBR322) and assessed for temperature-sensitivity by streaking out and measuring viability at permissive and restrictive temperatures. As this data was ambiguous for some of the strains they were all lysogenised with λS3D. Complementation with λS3D was assessed by streaking out lysogens and non-lysogens on NA in duplicate, incubating the plates at 30°C and 42°C, and comparing growth. Nine out of the thirteen mutants tested in this manner were assessed as *rpoH* mutants. Viable counts and λS3D complementation data are given in Table 3.4.

3.35 Construction of λpsupF lysogens

In order to distinguish between temperature-sensitive mutants due to missense and amber mutations, strains carrying a temperature-stable amber suppressor were constructed. If these strains carry an amber mutation in an essential gene they will no longer be temperature-sensitive as the mutation will now be suppressed at all temperatures. To this end λ*psupF* lysogens were constructed using all the mutants isolated and the lysogens tested for temperature-sensitivity. The results of this are shown in Table 3.5.

Table 3.4 Complementation with pDBS9, pR2, and λS3D

Viability at 42°C expressed as a percentage of viability at 30°C on NA plates, except λS3D: (+) indicates strong growth at 42°C of λS3D lysogen relative to non-lysogen when streaked out on NA plates, (-) indicates growth of lysogen and non-lysogen similar at 42°C, (nd) indicates not determined.

Strain	No Plasmid	pDBS9	pR2	λS3D
SG7	<0.001	<0.001	96	+
SG8	<0.001	<0.001	96	+
SG13	<0.001	9.23	2.86	+
SG15	<0.001	7.06	5.83	+
SG17	<0.001	0.447	65	nd
SG24	0.029	<0.001	<0.001	+
SG25	<0.001	17.3	0.157	+
SG26	<0.001	12.5	105	nd
SG28	<0.001	<0.001	<0.001	-
SG30	<0.001	1.83	0.072	+
SG32	<0.001	20	63	+
SG33	<0.001	<0.001	<0.001	-
SG34	<0.001	0.227	0.006	+

Table 3.5 Growth of λpsupF Lysogens at 42°C

(+) indicates growth of λpsupF lysogen at 42°C much stronger than non-lysogen, ie supF suppression of amber mutation. (-) indicates growth of λpsupF lysogen at 42°C the same as non-lysogen, ie. non-suppressed temperature-sensitive missense mutation.

Strain	Growth	Strain	Growth
SG1	-	SG21	-
SG7	-	SG23	-
SG8	+	SG24	-
SG9	+	SG25	-
SG10	-	SG26	nd
SG11	-	SG27	-
SG12	+	SG28	-
SG13	+	SG29	+
SG14	+	SG30	+
SG15	-	SG31	+
SG17	+	SG32	-
SG18	-	SG33	-
SG19	-	SG34	+
SG20	-	SG35	-

3.4 Discussion

Viable count data for plasmid complementation (Table 3.3) shows three distinct types of growth at 42°C. The first is non-complementation, giving a viability at 42°C which is similar to the mutant without the plasmid, generally less than 0.001% of the viability at 30°C. This is seen for SG1(pH3CdSR). The second type of growth is trans-complementation, giving growth at 42°C greater than 10% of that at 30°C, as seen for SG1(pH3CdAdP). The third type of growth gives a percentage viability at 42°C between these values, such as SG1(pH3CdS). The explanation for this intermediate level of growth would appear to be marker rescue. If, for example, we have an *ftsE* mutant we could expect full trans-complementation with pH3CdA, pH3CdAdP, and pH3CdR, non-complementation with pH3CdSR, and possibly marker rescue with pH3CdB and pH3CdS if the portion of *ftsE* carried on these plasmids happens to overlap the mutation and the host recombination systems are functional. Similarly, for *ftsX*, pH3CdA and pH3CdB ought to fully complement, pH3CdS and pH3CdSR should be non-complementing, and pH3CdR and possibly pH3CdAdP may show marker rescue. All but two of the mutants (SG9 and SG18) fit these patterns. SG9 and SG18 are only fully complemented by pH3CdA, which carries both *ftsE* and *ftsX*, therefore it is reasonable to assume that they possess mutations in both of these genes. Interestingly both show a low level of growth with pH3CdS, which should only be able to rescue *ftsE* mutations. The reason for this is not clear. One of these double mutants, SG9, appears to be suppressible by λ*psupF*, which raises the possibility that this mutant may have an amber mutation in *ftsE* that is causing loss of *ftsX* as a polar effect. The fact

that SG18 is not λ*psupF* suppressible does not necessarily indicate that both of its mutations are missense, there could be one amber and one missense. All these mutants appeared filamentous at the restrictive temperature and a small percentage of filaments was usually present at the permissive temperature.

The remaining temperature-sensitive mutants were mapped with pDBS9, pR2, and λS3D. (Table 3.4). These results are not as clear-cut as those for *ftsE* or *ftsX* mutants. There are only 3 mutants that are clearly not complemented by either pDBS9 or pR2 (SG24, SG28, and SG33). Seven of the remaining mutants appear to be complemented by pR2, having a reasonable recovery of viability at 42°C when carrying this plasmid (SG7, SG8, SG13, SG15, SG17, SG26, and SG32), and therefore the mutations can be assigned to *rpoH*. SG25 and SG30 have the appearance of *rpoH* mutants at the restrictive temperature, forming small filaments with inclusions, and show some complementation with pDBS9, so they probably are also *rpoH* mutants. Although the recovery of viability with pR2 is very limited this may be an allele specific effect, perhaps due to the absence of the normal *rpoH* promoter region in pR2. Alternatively these strains might carry double mutations, one in *rpoH* and one in either *ftsE* or *ftsX*, which would explain the difference in complementation between pDBS9 and pR2. The locus of the mutation in SG34 is not clear. Viability at the restrictive temperature is increased in the presence of pDBS9 and, to a lesser extent, pR2, but this does not approach wild-type levels. This may mean that it is a more dominant allele of *rpoH*, or SG34 may carry multiple mutations. When λS3D lysogens were constructed using all these mutants, only SG28 and SG33 remained temperature-sensitive. SG24

appeared to be complemented by λS3D implying that this strain is mutant in an essential gene flanking the region ORF4 to rpoH but present on λS3D, or carries multiple mutations at least one of which lies outside the region cloned in pDBS9 (ftsE to rpoH). Thus all but two of the mutants isolated can be complemented by existing clones. These two remaining mutants (SG28 and SG33) may define a new essential gene in the 76 minute region (see Chapter 6). All the mutants that are thought to map to rpoH have the typical appearance of short filaments at the restrictive temperature. All four of the mutants that do not map to rpoH (SG24, SG34, SG28, and SG33) have a pleomorphic morphology at the restrictive temperature with cells shaped like spheres, plump rods and normal shaped rods. The mutations isolated and mapped in this study are summarised in Table 3.6.

It is clear from Table 3.6 that no ftsY mutants were isolated. This could be for a variety of reasons. The simplest explanation would be that ftsY is non-essential, therefore it would not be possible to isolate temperature-sensitive lethal mutations in this gene. This is unlikely because of ftsY's position in an complex operon of essential genes, and as attempts to introduce ftsY mutations constructed in vitro back into the chromosome have failed, whereas introduction of similar non-mutagenic constructs has been successful (Gill & Salmond, 1990). This suggests that ftsY is essential. It might be that ftsY is not as susceptible to temperature-sensitive missense mutations as the other essential genes in this region and that the low suppression levels of $supF^{ts}$ even at the permissive temperature (Smith et al, 1970) are insufficient to allow amber nonsense mutants to remain viable. This would seem to imply that

Table 3.6 Summary of Mutations Isolated

Strain numbers and nature of mutations listed for each locus. N/D indicates not determined.

ftsE	
SG1	Missense
SG10	Missense
SG11	Missense
SG19	Missense
SG20	Missense
SG21	Missense
SG23	Missense
SG31	Amber
SG35	Missense

ftsX	
SG12	Amber
SG14	Amber
SG27	Missense
SG29	Amber

ftsE ftsX	
SG9	'Amber'
SG18	'Missense'

rpoH	
SG7	Missense
SG8	Amber
SG13	Amber
SG15	Missense
SG17	Amber
SG25	Missense
SG26	N/D
SG30	Amber
SG32	Missense

Complemented, Not Fully Mapped	
SG24	Missense
SG34	Amber

Not Complemented	
SG28	Missense
SG33	Missense

the structure or level of FtsY protein is very important, although the presence of *ftsY* on multicopy plasmids does not seem to have deleterious effects. Alternatively it could be purely bad luck that out of 28 temperature-sensitive mutations isolated in the 76 minute region none happened to lie in *ftsY*. An uneven distribution of mutations is by no means unprecedented, for example after screening for reversible, temperature-sensitive mutants blocked in cell division over 50% of mutations are found to lie in *ftsQ*, *ftsA*, and *ftsI* (Donachie *et al*, 1984). Lutkenhaus (1988) suggests that the fact that *ftsA* mutants have been isolated in every hunt for mutants may be due to *ftsA* mutants retaining their viability longer and hence surviving the selection process better. I believe that *ftsY* is probably very sensitive to mutations, which makes the occurrence of viable mutants extremely rare and that this precluded the isolation of a mutant in this screening experiment. No ORF4 mutations were isolated, and similar arguments can be raised for this. However there is perhaps less reason to suspect that ORF4 might be an essential gene, therefore screening for a temperature-sensitive lethal phenotype might not be expected to detect any ORF4 mutations.

In summary, a range of temperature-sensitive mutants has been isolated in a localised mutagenesis of the 76 minute region. Most of these have been mapped to *ftsE*, *ftsX*, and *rpoH*. No mutants were isolated that mapped in ORF4 or *ftsY*, which raises doubts about whether these genes are essential or not. Two temperature-sensitive missense mutations were not complemented by any clones available, and these were tentatively ascribed to a new essential gene.

Chapter 4: ANALYSIS OF ftsE MUTANTS

4.1 Introduction

With a range of mutants carrying mutations in the same gene it is possible to compare phenotypes and find ways to classify them into sub-groups. This type of approach may yield insights into the number and nature of functions or interactions of the mutant protein. Mutations in proteins that have several functions may result in diverse phenotypes, and the correlation betwen mutation and phenotype may give information about the multiple activities of the protein. For example, if the protein naturally interacts with a number of proteins, mutants carrying one of the alleles may lose one of the interactions whereas other alleles may result in the loss of other interactions. Alternatively certain alleles may be suppressed by the interaction of the mutant protein with certain alleles of other proteins (extragenic suppression) and if this can be demonstrated it is good evidence for there being an interaction between the proteins in the wild type situation. This can again be dissected by analysis of the phenotype. A range of ftsE temperature-sensitive missense alleles was generated in this study and as little is known about the function of the FtsE protein comparison of the phenotypes seemed to be an appropriate strategy to take. It was envisaged that in addition to physiological assessment of the mutants the corresponding mutations might be analysed at the DNA level. As was shown in Chapter 1 the ftsE gene exhibits extensive homology with the genes of a superfamily of ATP-binding cassette (ABC) proteins. In addition to the nucleotide-binding consensus sequences there are other regions of strong homology whose function has

yet to be determined. There was the possibility that the mutants would fall into groups phenotypically and that these could be related to clustering of the mutations into important domains of the protein. It might then be expected that these domains would correlate with the regions of homology within the group of ABC proteins. This might give some information as to the functional significance of the different regions of the FtsE protein.

4.2 Physiological Characterization of Mutants

4.21 Temperature-sensitivity and viable counts

The temperature-sensitivity of the *ftsE* strains was most simply assessed by streaking out in duplicate and then incubating the plates at the permissive and restrictive temperatures. SG1, SG10, SG11, SG19, SG20, SG21, and SG35 were all extremely temperature-sensitive in terms of growth on NA plates. There was virtually no growth at all on the plates incubated at 42°C whereas these strains grew strongly on the plates incubated at 30°C. SG23, TC35, and TOE22 appeared to grow quite strongly on NA plates at 42°C. However closer inspection revealed a heterogeneity in colony size with TC35 and TOE22 at 42°C that was not evident at 30°C. SG23 grew slightly more weakly at 42°C than at 30°C. SG23 was streaked out in duplicate on LB0 plates and was found to be quite temperature-sensitive for growth on this medium.

As seen in Chapter 3 temperature-sensitivity in these strains may also be conveniently assessed by measuring the viable count when the strains are plated out at the permissive and restrictive temperatures. The differences

in growth on plates at 30 and 42°C are reflected in the viable count data shown in Table 3.2. To summarise: SG1, SG10, SG11, SG19, SG20, SG21, and SG35 all show a viability at 42°C that is <0.001% of that at 30°C when grown on NA. SG23 did not appear to exhibit temperature-sensitivity as regards viable counts when grown on NA. When SG23 was plated out on LBO plates, however, the temperature-sensitivity was easily demonstrated. SG23 gave a viability on LBO at 42°C that was 0.033% of that at 30°C.

In conclusion there would appear to be some grounds for distinguishing between the ftsE mutants on the basis of temperature-sensitivity. That is SG23, TC35, and TOE22 do not have a very clear-cut temperature-sensitive phenotype as far as growth on NA plates is concerned whereas all the other ftsE mutants are very 'clean'. It must be noted however that TC35 and TOE22 are not isogenic with the SG series of mutants, and this could have an effect on the observed phenotype.

4.22 Microscopic examination

Microscopically there was very little to distinguish between any of the ftsE mutants (SG1, SG10, SG11, SG19, SG20, SG21, SG23, SG35, TC35 and TOE22). Strains were examined after overnight growth on NA plates incubated at 30°C and 42°C. All strains grew predominantly as normal short rods at the permissive temperature. Some filaments were present at 30°C in all cases. At 42°C all strains demonstrated extensive filamentation and very few normal rods were visible. SG35 filaments were

notably longer than any of the other strains when grown on NA plates at 42°C.

Liquid cultures were also examined microscopically. Cultures were grown at 30°C to mid-log phase growth (approximately OD_{600}= 0.3). Half the culture was then transferred to a prewarmed flask and incubated at 42°C for between two and four hours. Cultures were examined under the light microscope. Again there was little difference between the various strains. In liquid culture at the permissive temperature there were virtually no filaments visible, almost all the cells appearing to be short rods. At the restrictive temperature all strains examined except SG23 showed a high percentage of filamentation. Where cultures were incubated at the restrictive temperature for longer the length of the filaments was correspondingly greater. SG23 proved to be less filamentous in terms of both length of filaments and percentage of filamentation than the other 'SG' strains. The ftsE mutants TC35 and TOE22 were also examined under the microscope from liquid culture. These were found to have a higher number of normal length cells at the restrictive temperature when compared with the 'SG' mutants. These observations for SG23, TC35 and TOE22 are in accord with the 'leakiness' of these strains' phenotype as regards temperature-sensitive growth on plates.

4.23 Salt-reversibility of phenotype

There have been reports in the literature that high levels of NaCl in the medium can enable ftsE strains to grow at the restrictive temperature. In order to test this LB agar plates were made with a range of salt concentrations (0, 0.1, 0.25, 0.5, 0.75, 1.0, 2.5, 5.0, 7.5, and 10.0 g/l).

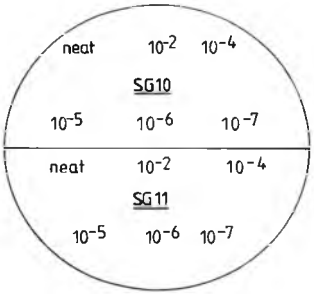
SG1, SG10, SG11, and OV2 were all streaked out in duplicate on the LB plates and the plates incubated at 30°C and 42°C.

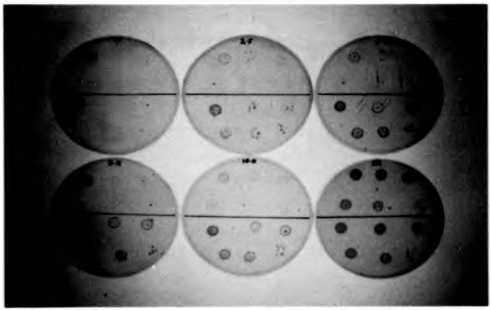
The plates were assessed for growth after overnight incubation. OV2 grew normally at all NaCl concentrations tested at 30 or 42°C. The ftsE mutants grew on all the plates at 30°C but did not grow at all on the plates containing less than 0.5g/l NaCl at 42°C. At higher concentrations there was a gradual improvement in growth of all three strains, but it was very difficult to assign a concentration at which growth became normal. At 10g/l NaCl all three ftsE strains appeared to grow well at the restrictive temperature.

From previous experience when mapping the mutations (Chapter 3) it appeared that performing viable counts at permissive and restrictive temperatures on media containing different NaCl concentrations might be a more useful way of assessing the growth response of the ftsE strains. Consequently viable counts were performed with a number of the mutants on LB plates with 0, 2.5, 5.0, 7.5, and 10 g/l NaCl (LB0, LB2.5, LB5.0, LB7.5, and LB10 respectively). The results obtained were difficult to interpret because the variation in growth response of these strains to media containing different NaCl concentrations did not generally manifest itself in different viable counts. This is shown in Figure 4.1 for strains SG10 and SG11, which exhibited the weakest and strongest response to increasing NaCl concentrations respectively. Similar numbers of colonies were seen at high dilutions on LB2.5 and media containing NaCl at higher concentrations but the confluent growth at low dilutions was always obviously weaker on the media containing less NaCl and the colonies were generally smaller on media containing very low NaCl concentrations.

Figure 4.1 NaCl Reversibility of SG10 and SG11 at 42°C

10ul of dilutions of overnight SG10 and SG11 cultures were spotted onto each plate according to the template below. The spots were allowed to dry in and the plates were incubated at 42°C overnight, except for the NA plate. Codes on plates correspond to media as follows: 0=LB0; 2.5=LB2.5; 5.0=LB5.0; 7.5=LB7.5; 10.0=LB10; NA=Nutrient agar plate incubated at 30°C for reference.





Therefore the viable count remained the same across a range of NaCl concentrations and, as was the case when the strains were streaked out, subjective assessment of the level of growth had to be made. In terms of viable counts both SG10 and SG11 appeared normal on all the media except LB0, however it is readily apparent from Figure 4.1 that the response of the strains to the different media in terms of density of growth at confluent dilutions was quite different. On LB10 at 42°C SG11 growth was nearly as strong as on NA at 30°C, whereas SG10 growth was clearly much weaker.

In order to simplify assessment of salt-reversibility all the *ftsE* strains generated in this study were tested twice for viability at 30°C and 42°C on LB0 and LB10. These concentrations were chosen with the expectation that salt-reversibility would be clearly discernible on the LB10 plates when compared with the LB0. As far as viable counts were concerned all the strains were salt-reversible, exhibiting very low viability on LB0 and high viability on LB10 when incubated at the restrictive temperature. SG10 was again the weakest growing strain on LB10 at 42°C and SG20 was the strongest. Under the microscope both strains showed extensive filamentation on LB10 at 42°C, whether bacteria from colonies or confluent spots were examined. The only detectable difference between the two strains was that SG10 filaments more frequently showed signs of lysis than SG20 filaments.

The common observation that strong growth occurred at high dilutions and weak growth at lower dilutions was investigated using several of the *ftsE* strains. It was thought that this might be due to carry-over of an inhibitory substance from the initial cultures used to set up the salt-

reversibility studies. It was also thought that a component of the NB used as diluent for the viable counts such as NaCl might be causing a general increase in growth that was masking real differences in response to the media. In order to test this, overnight cultures of OV2 (control), SG11 (strong growing on LB10) and SG10 (weak growing on LB10) were grown in NB. These cultures were split in two, half being washed with phage buffer the other half not being treated (control). Both control and washed cultures were then assessed for viable counts on LB0, LB5, and LB10 at permissive and restrictive temperatures, the dilution series for the washed culture being performed using phage buffer as diluent, the control culture being diluted in NB. Identical results were obtained for both washed and control samples for all three strains. These were consistent with previous observations of these strains, SG11 showing strong growth and SG10 showing weaker growth on LB10 at the restrictive temperature, but both exhibiting normal viable counts on LB10 and increasingly strong growth at higher dilutions. These results suggested that carry-over was not responsible for the dilution effect, and that the use of NB as diluent was not a factor affecting the apparent salt-reversibility of phenotype.

The degree of salt-reversibility of each mutant was very difficult to quantify. A few generalisations can however be made. All strains tested were phenotypically reversible by LB containing 10 g/l NaCl as far as viable counts were concerned. Strains SG11, SG20, and SG23 all appeared to be highly reversible on LB10, growing strongly on this medium at 42°C, whereas SG10 was found to grow especially weakly at the restrictive temperature on LB10. TC35 and TOE22 grew sufficiently strongly on LB0

at 42°C to make it impossible to detect any improvement in their growth on LB agar with added NaCl.

4.24 Protein inactivation and the requirement for protein synthesis

One way of classifying temperature-sensitive missense mutants is to assess the thermo-reversibility of the mutant phenotype. That is to ascertain whether the strains regain a wild-type phenotype if they are returned to the permissive temperature following incubation at the restrictive temperature. If they do regain a wild-type phenotype it can be due to one of two reasons. The missense protein that was denatured at the restrictive temperature may re-fold into its permissive-temperature tertiary structure and regain wild-type activity. Alternatively new protein may need to be synthesised at the permissive-temperature if existing thermolabile missense proteins that have been exposed to the restrictive-temperature have been permanently inactivated. These two possibilities can be distinguished by the strain's thermo-reversibility in the presence of an inhibitor of protein synthesis such as chloramphenicol. If the strain requires de novo protein synthesis at the permissive temperature for thermo-reversibility of phenotype it would not be expected to recover wild-type phenotype in the presence of chloramphenicol. If the strain does recover wild-type phenotype in the presence of chloramphenicol it can be assumed that the existing proteins have not been permanently denatured by exposure to the restrictive temperature.

This experiment was performed on strains OV2, SG1, SG10, SG19, SG20, SG21, SG23, SG35, MFT1181, and TOE22. Liquid cultures were grown to mid-logarithmic phase in NB shake flasks at 30°C (OD_{600} 0.3 to 0.4).

The cultures were transferred to a 42°C incubator for 60 to 90 minutes to allow approximately two cell divisions to occur. A 10ul sample was taken at this point (time 0) and immediately diluted in 10ml of 0.2 micron filtered isoton which is bacteriostatic. The cultures were then split into three aliquots. Two aliquots were transferred to prewarmed flasks at 30°C, one of which contained sufficient chloramphenicol to give a final concentration of 200ug/ml. The third aliquot remained in the original flask at 42°C. 10ul samples were taken at 15, 30, 60, 90, 150, and 180 minutes from all three flasks. These samples were also immediately diluted in 10ml of 0.2 micron filtered isoton. All samples were assessed before the next sampling time for total particle number and particle size distribution using a Coulter Counter and channelyser with a 30um by 80um orifice.

Idealised results for a thermo-reversible cell-division mutant in this experiment would be as follows: In the sample that was retained at 42°C particle numbers could be expected to remain constant as the cells would be unable to divide at this temperature. Mean particle size however could be expected to gradually increase as the filaments grew longer. In the sample transferred to 30°C the particle numbers would increase and the particle size decrease as the filaments septated and the daughter cells began growing and dividing normally. Following transfer to 30°C in the presence of chloramphenicol if the mutant protein was thermo-reversible one could expect the particle numbers to increase and then level out and the particle size to decrease and then level out, as the filaments divided using the existing proteins which had regained wild-type activity due to the reduced temperature. Further growth and division would then be inhibited by the inability to synthesise more protein. If the mutant protein

was not thermo-reversible the particle numbers and size could be expected to remain at or near to their time 0 values as the cells would be unable to synthesize new protein to enable the filaments to divide or grow.

The particle numbers obtained from this experiment showed a large amount of scatter. The cultures kept at 42°C demonstrated fairly constant particle numbers with the exception of OV2 and SG23. These two strains showed a gradual increase in particle number with time. When the 30°C results are compared with the 42°C results it is clear that all of the ftsE mutants tested were phenotypically thermo-reversible. That is all the cultures demonstrated rapidly increasing particle numbers following transfer from 42°C to 30°C. This was paralleled by a decrease in particle size. This result means that none of these mutants lose their viability upon exposure to the restrictive temperature for short periods.

The cultures transferred to 30°C in the presence of chloramphenicol exhibited a constant level of particle numbers that was generally slightly less than that for the culture kept at 42°C. This would suggest that none of these strains possessed thermo-reversible FtsE proteins. The exception was MFT1181 which gave an increase in particle numbers following transfer to 30°C in the presence of chloramphenicol which levelled out at a value greater than the 42°C control culture. This is the expected result if the temperature-sensitive protein is thermo-reversible. Superficially then the results in terms of particle numbers suggest that all of these strains are thermo-reversible but that in all cases except MFT1181 de novo protein synthesis is required for recovery of the phenotype following the return of the strain to the permissive temperature.

However the channelyser results which give particle size distributions indicate a different situation with respect to the requirement for protein synthesis following a temperature shift-down. These results are shown in Figure 4.2a to 4.2j. For each strain the six graphs show initial and final particle size distributions under the three experimental conditions, 42°C (i, ii), shifted down to 30°C (iii, iv), and shifted down to 30°C in the presence of chloramphenicol (v, vi). It should be remembered that the initial 42°C sample (time 0) was taken immediately before the cultures were split and hence represents the initial distribution of particle size before the shift-down to 30°C took effect.

The expected distribution for a wild-type strain is shown by OV2 (Figure 4.2a). Here the size distribution was essentially constant irrespective of the conditions or length of incubation. The only difference was that the cells were slightly smaller after prolonged incubation at 30°C (iv and vi). The expected size distribution for a thermo-reversible mutant with a thermo-reversible FtsE protein was demonstrated by MFT1181 (Figure 4.2i). At time 0 there was a broad distribution of cell sizes (i). After 30 minutes at 30°C the particle sizes were all decreasing (iii) and by 180 minutes after the shift to 30°C the particle size distribution was a very narrow single peak at approximately the same size as OV2 in the same conditions (iv). In the presence of chloramphenicol after 15 minutes at 30°C the particle sizes had started to decrease (v) and by 180 minutes there was a large peak at about the same size as that seen in the absence of chloramphenicol (vi). Some larger particles remained and the distribution of these formed a peak that was at twice the size of the large one. The portion of the culture that was retained at 42°C showed a

Figure 4.2 Thermoreversibility of ftsE Mutants

Graphs show particle size distributions (determined using a Coulter Counter channelyser) under a variety of conditions following growth at 42°C for 60 to 90 minutes. For each strain the six graphs show initial and final particle size distributions under three experimental conditions: continued incubation at 42°C ('42°C'; i, ii); shifted down to 30°C ('30°C'; iii, iv); and shifted down to 30°C in the presence of chloramphenicol ('30°C+Cm'; v, vi). The sample time after the cultures were split is given on each graph.

Scales

The maximum value on the horizontal scale (128) is equivalent to a particle volume of 4.16fl, except on graphs marked with 'Size scale: 8' (4.2 d,e,f graph ii., and all graphs on 4.2 h) where the scale range is doubled (128 = 8.3fl). The maximum value on the vertical scale (100) is equivalent to the number of particle counts given as the 'Count range' on each graph.

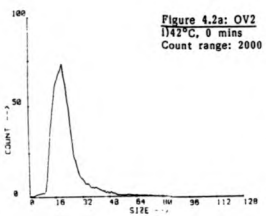
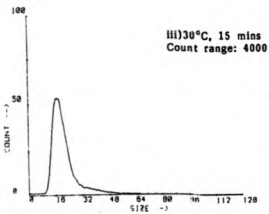
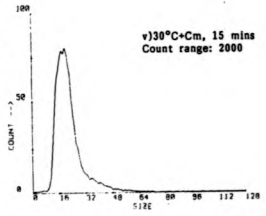


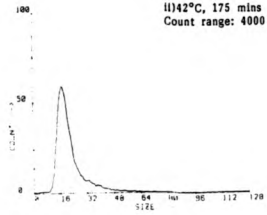
Figure 4.2a: OV2
i)42°C, 0 mins
Count range: 2000



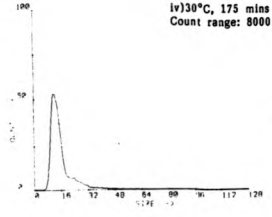
iii)30°C, 15 mins
Count range: 4000



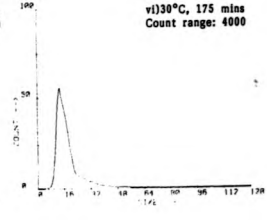
v)30°C+Cm, 15 mins
Count range: 2000



ii)42°C, 175 mins
Count range: 4000



iv)30°C, 175 mins
Count range: 8000



vi)30°C, 175 mins
Count range: 4000

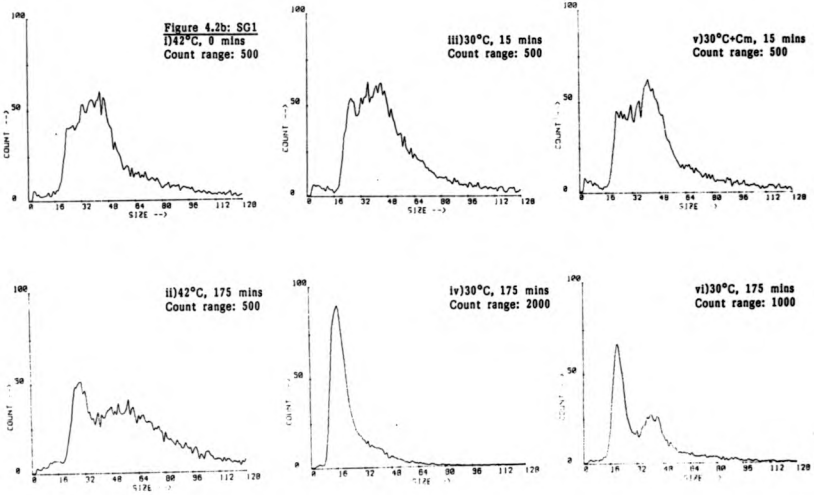


Figure 4.2b: SG1
i)42°C, 0 mins
Count range: 500
iii)30°C, 15 mins
Count range: 500
v)30°C+Cm, 15 mins
Count range: 500
ii)42°C, 175 mins
Count range: 500
iv)30°C, 175 mins
Count range: 2000
vi)30°C, 175 mins
Count range: 1000
COUNT -->
SIZE -->

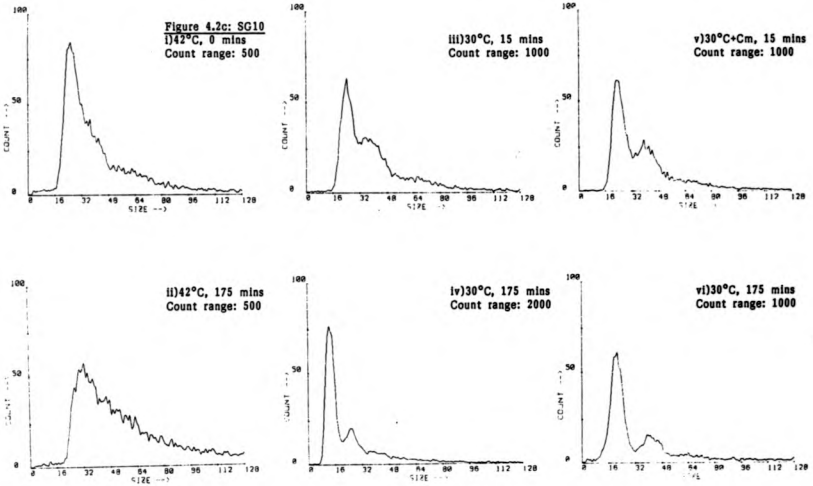


Figure 4.2c: SG10
i)42°C, 0 mins
Count range: 500
iii)30°C, 15 mins
Count range: 1000
v)30°C+Cm, 15 mins
Count range: 1000
ii)42°C, 175 mins
Count range: 500
iv)30°C, 175 mins
Count range: 2000
vi)30°C, 175 mins
Count range: 1000
COUNT -->
SIZE -->

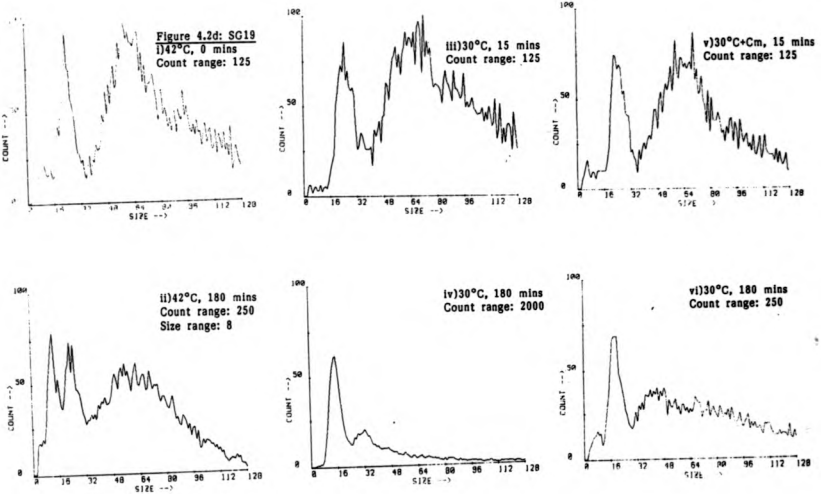


Figure 4.2d: SG18
i)42°C, 0 mins
Count range: 125
iii)30°C, 15 mins
Count range: 125
v)30°C+Cm, 15 mins
Count range: 125
ii)42°C, 180 mins
Count range: 250
Size range: 8
iv)30°C, 180 mins
Count range: 2000
vi)30°C, 180 mins
Count range: 250
COUNT -->
SIZE -->

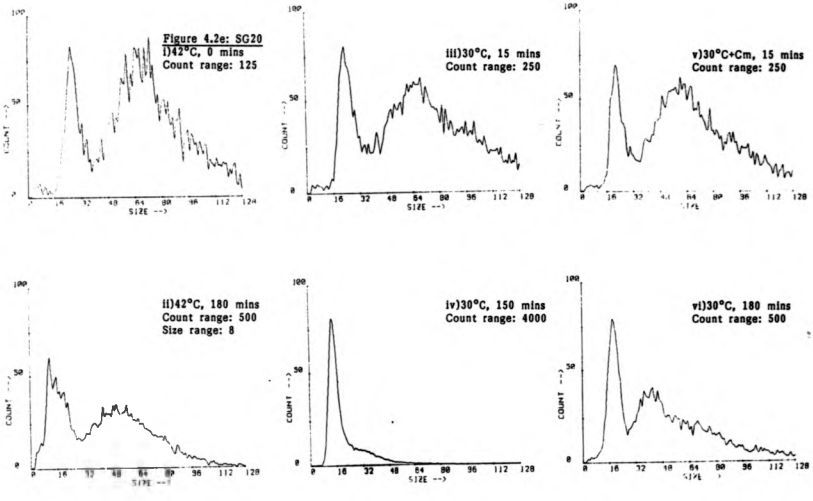


Figure 4.2e: SG20
i)42°C, 0 mins
Count range: 125
iii)30°C, 15 mins
Count range: 250
v)30°C+Cm, 15 mins
Count range: 250
ii)42°C, 180 mins
Count range: 500
Size range: 8
iv)30°C, 150 mins
Count range: 4000
vi)30°C, 180 mins
Count range: 500
COUNT -->
SIZE -->

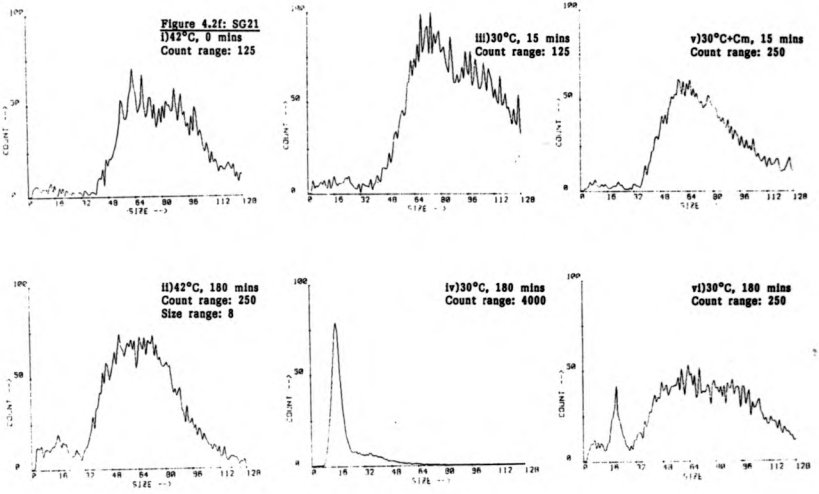


Figure 4.2f: SG21
i)42°C, 0 mins
Count range: 125
ii)30°C, 15 mins
Count range: 125
v)30°C+Cm, 15 mins
Count range: 250
ii)42°C, 180 mins
Count range: 250
Size range: 8
iv)30°C, 180 mins
Count range: 4000
vi)30°C, 180 mins
Count range: 250
COUNT -->
SIZE -->

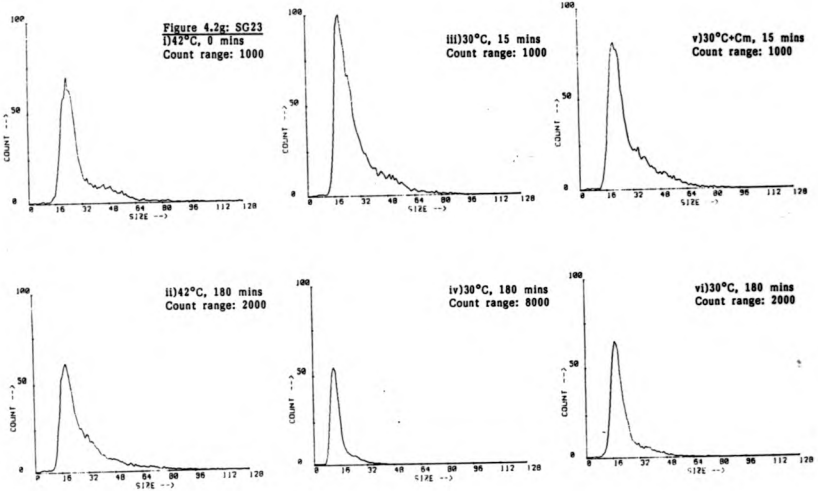


Figure 4.2g: SG23
i)42°C, 0 mins
Count range: 1000
iii)30°C, 15 mins
Count range: 1000
v)30°C+Cm, 15 mins
Count range: 1000
ii)42°C, 180 mins
Count range: 2000
iv)30°C, 180 mins
Count range: 8000
vi)30°C, 180 mins
Count range: 2000
COUNT -->
SIZE -->

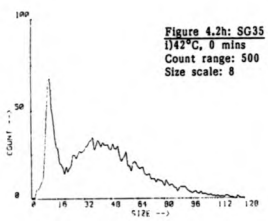
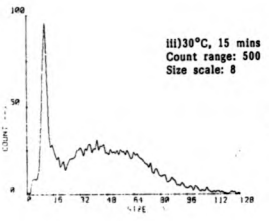
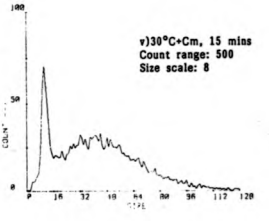


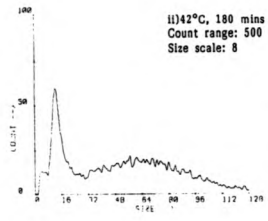
Figure 4.2h: SG35
i)42°C, 0 mins
Count range: 500
Size scale: 8



iii)30°C, 15 mins
Count range: 500
Size scale: 8



v)30°C+Cm, 15 mins
Count range: 500
Size scale: 8



ii)42°C, 180 mins
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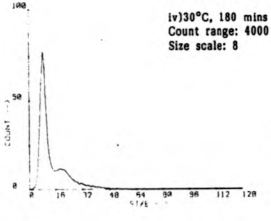
Size scale: 8



iv)30°C, 180 mins
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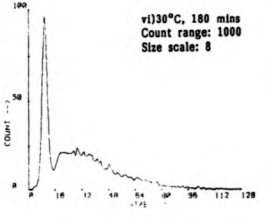
Size scale: 8



vi)30°C, 180 mins
Count range: 1000
Size scale: 8

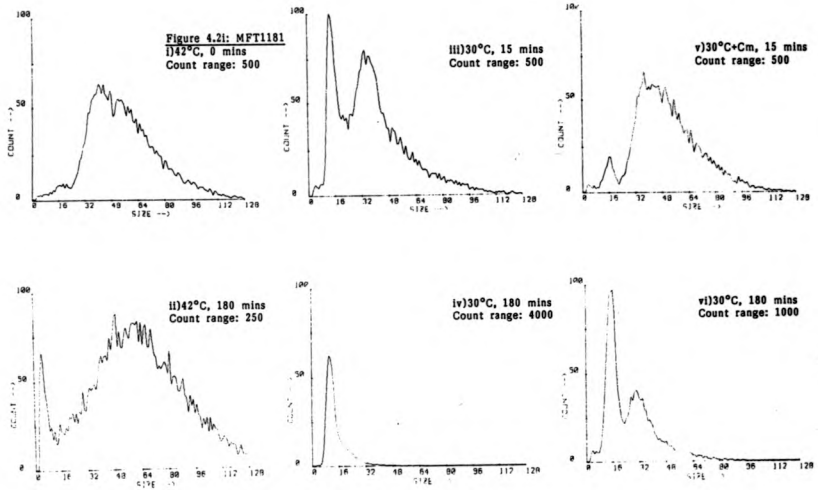


Figure 4.21: MFT1181
i)42°C, 0 mins
Count range: 500
iii)30°C, 15 mins
Count range: 500
v)30°C+Cm, 15 mins
Count range: 500
ii)42°C, 180 mins
Count range: 250
iv)30°C, 180 mins
Count range: 4000
vi)30°C, 180 mins
Count range: 1000
COUNT -->
SIZE -->

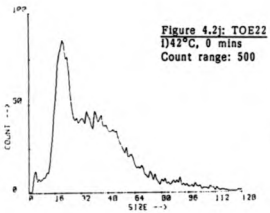
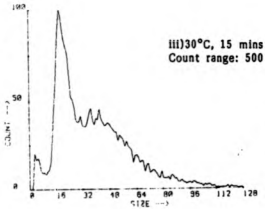
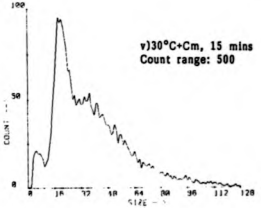


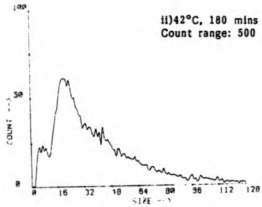
Figure 4.2j: TOE22
i)42°C, 0 mins
Count range: 500



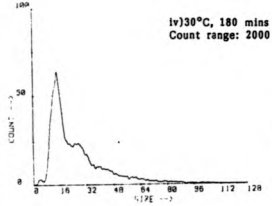
iii)30°C, 15 mins
Count range: 500



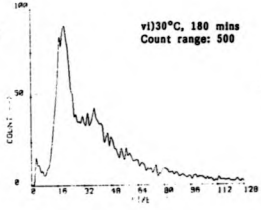
v)30°C+Cm, 15 mins
Count range: 500



ii)42°C, 180 mins
Count range: 500



iv)30°C, 180 mins
Count range: 2000



vi)30°C, 180 mins
Count range: 500

further broadening of size distribution by 180 minutes (ii) presumably reflecting the continued linear growth of filaments. TOE22 (Figure 4.2j) exhibited size distributions that would be expected for a thermo-reversible strain that does require de novo protein synthesis following a return to the permissive temperature. At 30°C in the absence of chloramphenicol the size distribution became less broad with time, the peak size becoming smaller (iii, iv) showing that the strain is thermo-reversible. After 180 minutes at 30°C (iv) there were still a few large particles. This is probably a reflection of the general 'leakiness' of this mutant as observed under the microscope, normal short rods and filaments being present at both permissive and restrictive temperatures. When the strain was returned to 30°C in the presence of chloramphenicol the size distribution at 15 minutes (v) and even at 180 minutes (vi) was very similar to the initial size distribution at 42°C (i) which strongly suggests that new proteins must be synthesised for the cells to resume septation following exposure to the restrictive temperature.

The particle size distributions for the 'SG' series of mutants did not fit in neatly with these three prototypes. All of these strains exhibited a phenotypic recovery following transfer to 30°C as shown by an overall decrease in particle sizes and the emergence of, or increase in relative magnitude of, a peak corresponding to that seen in OV2 (iii and iv in Figure 4.2b to h). Thus all of these strains are thermo-reversible. SG1, SG10, SG18, and SG20 all displayed a general decrease in particle sizes when returned to 30°C in the presence of chloramphenicol although all the distributions at 180 minutes showed that there was still a relatively high number of large particles (iii and iv, Figure 4.2 b, c, d, e,

respectively). Strains SG21 and SG35 appeared to conform to the same pattern of particle size distributions (Figure 4.2 f, h), but the changes in distribution following the shift to 30°C in the presence of chloramphenicol were not as great. In the case of SG35 (and to a lesser extent SG19 and SG20) the ambiguity of the results is partly due to the presence of a low particle size peak at 42°C at time 0 that may be due to the presence of revertants. SG23 exhibited very low numbers of large particles at 42°C at time 0, suggesting only weak filamentation (i, Figure 4.2g). Despite this it is possible to see some downward shift in particle size after 180 minutes at 30°C (iii), suggesting that the strain is thermo-reversible, however it is very hard to see whether there is any meaningful decrease in the presence of chloramphenicol (v, vi). Therefore one cannot conclude whether or not the FtsE protein in SG23 is thermo-reversible.

To summarise these results all FtsE mutants tested were phenotypically thermo-reversible following a brief exposure to the restrictive temperature. TOE22 appeared to require de novo protein synthesis for this phenotypic reversibility, whereas all the other strains tested exhibited a degree of reversibility in the presence of the protein synthesis inhibitor chloramphenicol. SG23 did not filament sufficiently well enough to allow assessment of it's requirement for de novo protein synthesis.

4.3 Cloning of the Mutant ftsE Alleles

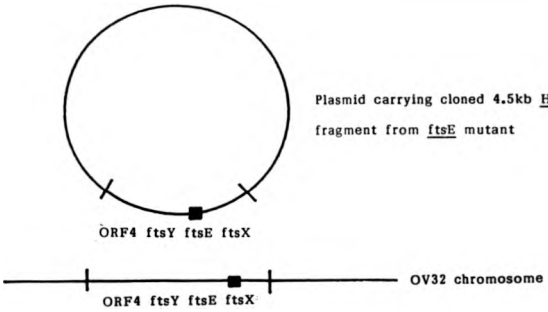
4.31 Cloning the mutant 4.5 kb HindIII fragments by biological complementation

Cloning of the 4.5 kb HindIII fragments carrying the mutant ftsE alleles was greatly facilitated by the presence on these fragments of a wild-type ftsX allele. Isolation of the desired fragment could thus be selected for by cloning into an ftsX recA mutant and selecting for growth at the restrictive temperature. Transformants that grew at the restrictive temperature should be carrying a cloned trans-complementing wild-type ftsX gene and therefore the associated ftsE mutant allele. This is shown in Figure 4.3.

Chromosomal DNA was prepared from the following ftsE mutants: SG1, SG10, SG11, SG19, SG20, SG21, SG23, SG35, TOE22, and TC35, and the wild-type strain OV2. This DNA was digested to completion with HindIII, and fragments in the range 4.0 to 5.0 kb isolated. These fragments were ligated into pBR325 that had been digested with HindIII and treated with calf intestinal phosphatase. This ligation mix was used to transform competent OV32 recA (relevant markers: ftsX, recA) and the desired transformants were selected by plating out on NA plus ampicillin, and incubating at 42°C. Any colonies that grew at 42°C were streaked out in duplicate and the plates incubated at 30°C and 42°C. All transformants tested were found to grow at both temperatures so plasmid DNA was prepared from representative transformants on a small scale.

Figure 4.3 Cloning of ftsE Alleles by Biological Complementation

4.0 to 5.0kb HindIII fragments from the mutant chromosomal DNA were ligated into pBR325. This mixture was used to transform OV32 recA which is an ftsX mutant. The cloning of ftsE alleles was selected for by incubating the selection plates at 42°C. OV32 transformants can only grow at 42°C if they carry a wild type ftsX gene, which is included on the same 4.5kb HindIII fragment as the ftsE mutant allele. This is depicted below, with ■ representing a temperature-sensitive mutation.



This miniprep DNA was used to transform OV32 recA to ampicillin resistance and these transformants were again tested for temperature-sensitivity. All transformants were found to be temperature-insensitive although they only grew weakly at 42°C. Microscopy showed a combination of lysed cells and normal length rods at 42°C. This contrasted with OV32 recA transformed with the control plasmid pBR325 which did not grow at 42°C and was found to consist of lysed cells and filaments at the non-permissive temperature. This strongly suggested that the correct fragments had been cloned so the plasmids were tentatively named pTW1, pTW10, pTW11, pTW19, pTW20, pTW21, pTW23, pTW35 where derived from the respective SG strains, and pTWTOE22, pTWTC35, and pTW4.5 where derived from TOE22, TC35 and OV2 respectively (Table 4.1). Large scale (CsCl purified) plasmid preparations were then performed on the OV32 recA strains carrying these plasmids. This DNA was digested with the restriction enzymes AvaI and HindIII and the resulting fragments analysed on agarose gels. All the plasmids except pTW10 showed the expected fragment sizes of 6.0 kb (vector) and 4.5 kb (insert) when digested with HindIII. As well as confirming the cloning of the desired 4.5kb fragment the results of the AvaI digestions allowed the orientation of the insert to be determined. The expected fragment sizes for the insert in the same orientation as pDG1 (that is with ftsX being transcribed towards the pBR325 tet gene) are 7.2 and 3.2 kb. The opposite orientation should generate fragments of 6.4 and 4.0 kb. All of the clones except pTW10 gave rise to one of these pairs of fragments. There appeared to be no bias in the orientation of cloned fragments, 4 being in the same orientation as pDG1 and 6 in the opposite orientation.

Table 4.1 Plasmids Carrying ftsE Alleles

Name of Plasmid	Source of 4.5kb HindIII fragment
pTW4.5	OV2
pTW1	SG1
pTW10	SG10
pTW11	SG11
pTW19	SG19
pTW20	SG20
pTW21	SG21
pTW23	SG23
pTW35	SG35
pTWTOE22	TOE22
pTWTC35	TC35

pTW10 gave rise to an extra fragment in each digestion suggesting that there were two inserts in this plasmid. In order to eliminate the extra insert the plasmid was cut with HindIII and religated, and this ligation mix used to transform OV32 recA to ampicillin resistance, selecting for the desired clone as before at 42°C. No colonies grew at 42°C but 75 colonies grew on the control plate (NA plus ampicillin) that was incubated at 30°C. These 75 colonies were tested for temperature-sensitivity by patching out and 5 were found to be temperature-insensitive. These 5 (clones #1 to #5) were retested for temperature-sensitivity by streaking out as above and all were found to grow, albeit weakly, at 42°C. Microscopy confirmed that clones #3 and #5 appeared normal at 30 and 42°C whereas the other three clones appeared filamentous at 42°C. Small scale preparations of plasmid were performed on these 5 clones and this miniprep DNA was restricted with HindIII and AvaI as before. Only clone #3 gave two fragments of the expected size in each digest. This clone was named pTW10#3 and was used to retransform OV32 recA to ampicillin resistance. Once again this resulted in only a modest level of complementation, but was clearly less temperature-sensitive than OV32 recA(pBR325). A large scale plasmid preparation was therefore performed on OV32 recA(pTW10#3), and the plasmid rechecked by restriction analysis and retransformation of OV32 recA. These tests all gave the expected results so this clone was considered to be the equivalent of the other plasmids in the pTW series. The insert was seen to be in the same orientation as pDG1.

As a final test of all these plasmids prior to subcloning and DNA sequencing they were used to transform their respective parental strains

to check for non-complementation, for example pTW1, pTW10#3 and pTWTOE22 were used to transform SG1, SG10 and TOE22 respectively. These strains were tested for temperature-sensitivity by streaking out in duplicate on NA plates and incubating the plates at 30 and 42°C. SG1(pTW1), SG19(pTW19), SG20(pTW20), SG21(pTW21), SG23(pTW23), and SG35(pTW35) all gave the expected result of no growth at 42°C. SG10(pTW10) and SG11(pTW11) both gave weak growth at 42°C but the cells proved to be very filamentous which was taken as evidence of non-complementation. TOE22(pTWTOE22) and TC35(pTWTC35) both gave quite strong growth at the non-permissive temperature. This however is also true of the plasmid-less strains, these being only weakly temperature-sensitive with respect to growth on plates. Microscopic examination revealed that the plasmid bearing strains were in fact highly filamentous, strongly suggesting non-complementation.

In conclusion the 4.5 kb HindIII fragment carrying a wild-type ftsX gene and, by implication, an ftsE allele was cloned into pBR325 from 10 ftsE mutants and one wild-type strain. This was verified by restriction analysis and complementation studies.

4.32 Subcloning the 830 bp ftsE fragment

From the DNA sequence of the ftsE gene and flanking regions it was apparent that there were no appropriate sites for the subcloning of the entire gene into a DNA sequencing vector. However from the complementation and mapping studies of Chapter 2 it appeared that all the ftsE mutants isolated in this study (the SG series) exhibited marker rescue when transformed with pH3CdS. This would imply that the mutations in these strains are not in the 40 bp between the SphI site in

the ftsE gene and the ftsE stop codon. Consequently it appeared that all these mutations would lie in the 630 bp region between the AvaI site (13 bp upstream of the ftsE start codon) and the SphI site. Therefore this AvaI-SphI fragment was cloned from each of the pTW plasmids into the M13 sequencing vectors tg130 and tg131. This subcloning is summarised in Figure 4.4.

Preparation of the 630 bp fragments

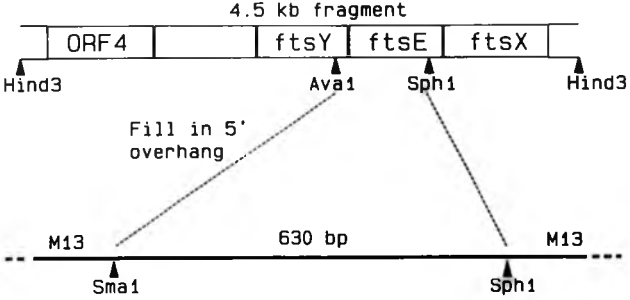
The pTW plasmids constructed above were cut with the restriction enzyme AvaI. Residual enzyme was removed by phenol/$CHCl_3$ extraction of the digest followed by ethanol precipitation of the nucleic acids. As the M13 cloning vectors used did not have an AvaI site in the polylinker the 5' overhanging ends of the AvaI cut DNA were filled in so that they could be cloned into the SmaI (blunt ended) site in the vector. The AvaI cut and filled DNA was then cut with the restriction enzyme SphI. This resulted in the production of four fragments per digest, the smallest of which was the desired 630 bp AvaI-SphI fragment carrying the majority of the ftsE gene. These 630 bp fragments were prepared from agarose gels.

Preparation of M13 vector

M13 tg130 and M13 tg131 DNA was cut with SmaI. The restriction digest was phenol extracted and ethanol precipitated. The SmaI cut DNA was then cut with SphI. A small quantity of previously uncut M13 tg131 was used as a cutting control as the SphI digest does not alter the size of the SmaI cut M13 enough to be detected on agarose gels. Ligations were set up in duplicate (one for M13 tg130, and one for M13 tg131) of the M13 DNA and the 630 bp fragments, using an insert to vector ratio of

Figure 4.4 Subcloning of the ftsE Alleles

The 630bp AvaI, SphI fragment was subcloned into M13 tg130 and M13 tg131 as shown below. The use of the two M13 vectors allows both strands to be recovered as single-stranded DNA template.



approximately 2:1. Recombinant M13 clones were isolated and single stranded template was prepared.

4.4 Sequencing the Cloned ftsE Alleles

The strategy used for sequencing the 630 bp *ftsE* fragments required use of the M13 universal primer and two internal primers for each strand. Internal sequencing primers (Figure 4.5) were designed to be homologous to unique sequences within the 630 bp fragments. Primers for opposite strands were non-overlapping to ensure that sequence for the whole 630 bp fragment could be obtained even if there was a mutation lying within one of the priming sites.

Clones for all the alleles were sequenced simultaneously using the same primer. Contrary to normal practice the sequencing gels were loaded with the termination reactions for each base for all the clones in adjacent tracks, ie. all eleven A terminating reactions for wild-type and ten mutants were loaded adjacent to each other, then all eleven C terminating reactions were loaded adjacent to each other and likewise for the G and T terminating reactions. A normal sequencing ladder for the wild-type clone was also loaded on the same gel. This obviated the need to read the DNA sequence of each clone, mutations being immediately identified by the presence or absence in any lane of a band absent or present respectively in the other ten lanes. The position of this mutation in the sequence could be quickly established by comparing it with the wild-type sequencing ladder on the same gel. The success of this method is clearly demonstrated in Figure 4.6. This shows a portion of a sequencing gel which has all the A termination reactions for the 11

Figure 4.5 Internal Sequencing Primers for ftsE

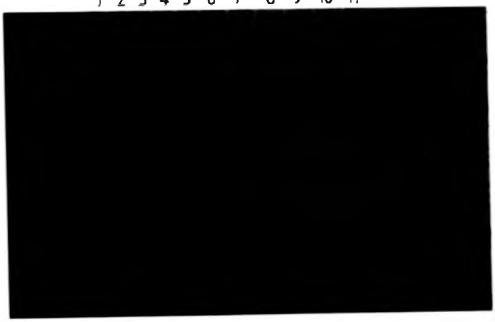
The DNA coding sequence of ftsE is shown below with coding strand uppermost. Primers are shown adjacent to their priming site. Primers 1c and 2c anneal to coding strand template and 3n and 4n to non-coding strand template. The translation initiation (ATG) and termination (TAA) codons are boxed. The Sph1 and Aval sites used for subcloning are also shown.

```
                                    Aval
                          TTGCCCGAGAGGATTAACAATGATTCGCTTTGAACATGTCAGCAAGGCTTATCTCGGTGGGAGAC
                          AACGGGCTCTCCTAATTGTTACTAAGCGAAACTTGTACAGTCGTTCCGAATAGAGCCACCCTCTG

AGGCGCTGCAGGGCGTTACGTTCCATATGCAGCCGGGTGAGATGGCGTTTCTGACCGGTCATTCCGGCGCAGGGAAAAGTACCCTCCTGA
TCCGCGACGTCCCGCAATGCAAGGTATACGTCGGCCCACTCTACCGCAAAGACTGGCCCGTAAGGCCGCGTCCCTTTTCATGGGAGGACT

                                                    1c: TACTGTAGTGCGCAGACTTT
AGCTGATCTGTGGGATTGAGCGGCCCAGCGCCGGGAAAATCTGGTTTAGCGGCCATGACATCACGCGTCTGAAAAACCGTGAAGTTCCGT
TCGACTAGACACCCTAACTCGAAGGGTCGCGGCCCTTTTAGACCAAATCGCCGGTACTGTAGTGCGCAGACTTTTTGGCACTTCAAGGCA
                  3n: CCAGCGCCGGGAAAATCTG

TTCTGCGCCGCCAGATTGGCATGATTTTCCAGGATCACCATCTACTGATGGACCGTACTGTCTACGATAACGTGGCGATCCCGCTGATTA
AAGACGCGGCGGTCTAACCGTACTAAAAGGTCCTAGTGGTAGATGACTACCTGGCATGACAGATGCTATTGCACCGCTAGGGCGACTAAT

TCGCCGGTGCCAGCGGTGACGATATTCGTCGCCGGGTGTCGGCGGCGCTGGATAAAGTCGGGCTACTGGACAAAGCGAAGAACTTCCCTA
AGCGGCCACGGTCGCCACTGCTATAAGCAGCGGCCCACAGCCGCCGCGACCTATTTCAGCCCGATGACCTGTTTCGCTTCTTGAAGGGAT
                                                              4n: CTGGACAAAGCGAAGAACTT
   2c: AAGCCCGCCACTTGTTGTCG
TTCAGCTTTCGGGCGGTGAACAACAGCGTGTTGGCATTGCCCGCGCCGGTGGTGAACAAGCCCGCGGTACTGCTGGCGGACGAACCGACTG
AAGTCGAAAGCCCGCCACTTGTTGTCGCACAACCGTAACGGGCGCGCCACCACTTGTTCGGGCGCCATGACGACCGCCTGCTTGGCTGAC

GTAACCTGGACGACGCGCTGTCGGAAGGCATTTTACGTCTGTTTGAAGAGTTTAACCGCGTTGGGGTAACCGTATTGATGGCAACGCACG
CATTGGACCTGCTGCGCGACAGCCTTCCGTAAAATGCAGACAAACTTCTCAAATTGGCGCAACCCCATTGGCATAACTACCGTTGCGTGC

                          Sph1
ACATCAACCTGATCTCGCGGCGTTCCTATCGCATGCTCACCCTGAGCGATGGTCACTTGCATGGAGGCGTGGGCCATGAATAA
TGTAGTTGGACTAGAGCGCCGCAAGGATAGCGTACGAGTGGGACTCGCTACCAGTCAACGTACCTCCGCACCCGGTACTTATT
```

Figure 4.6 Autoradiograph of ftsE Allele Sequencing Gel

This autoradiograph shows part of the A termination tracks for all of the ftsE missense alleles run in adjacent lanes, 1 to 11. Mutations are clearly visible as extra bands in lanes 5 to 9.

1 2 3 4 5 6 7 8 9 10 11



alleles sequenced. There are five mutations clearly visible on this portion of this gel as shown by extra bands in tracks 5 to 9. One band in an identical position was missing in each of the tracks carrying the corresponding G termination reactions.

Using this method point mutations (or two point mutations) were found in each of the mutants except SG10. This initially appeared to have a promoter-proximal frameshift mutation. This would lead to termination of translation within a few codons and, given that SG10 is a temperature-sensitive missense mutant, this seemed likely to be a cloning artefact rather than the mutation responsible for the phenotype. The 630bp *ftsE* fragment from SG10 was therefore re-isolated from SG10 chromosomal DNA and re-sequenced. This time a point mutation was found and upon close inspection of the original sequencing gels its presence in the first SG10 clone was confirmed.

The results of the DNA sequencing are shown in Figure 4.7 which displays the wild type *ftsE* sequence with deviations from it for each of the mutants in boxes. The effect of the mutations on the amino acid specified by the relevant codons is summarised in Table 4.1. It is immediately apparent that SG1 and SG35 share the same two point mutations. This was thought to be an unlikely coincidence and probably due to one of the strains being mis-labelled. To address this possibility SG1 and SG35 were recovered from the earliest frozen stocks available and the *ftsE* genes cloned and re-sequenced from these. The results of this DNA sequencing were identical to the original results, suggesting that if the strains were mixed up it was very soon after the original isolation of them.

Figure 4.7 Location of the ftsE Missense Mutations

DNA mutations are marked below the coding strand and boxed. The strains carrying the mutations are also marked. Predicted amino acid changes are shown above the amino acid sequence. The putative nucleotide binding site is boxed.

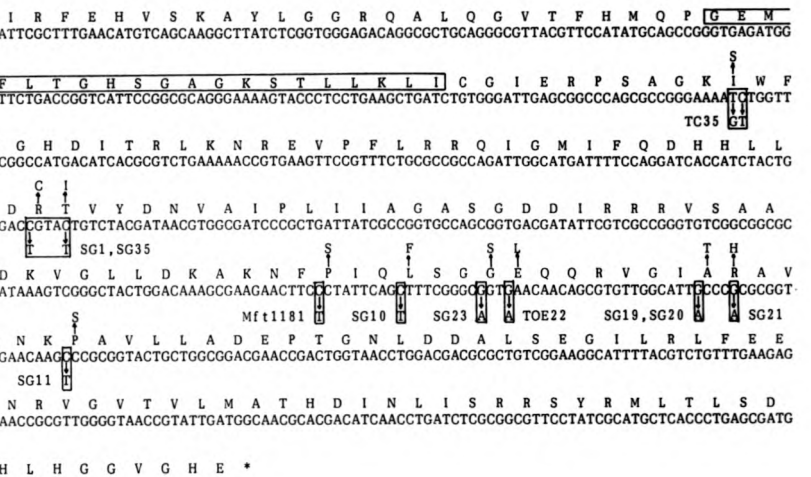


Table 4.2 Codon and Amino Acid Changes in ftsE Mutants

The MFT1181 ftsE allele was cloned and sequenced by D. Gill.

Strain	Codon Change	Amino Acid Change
SG10	CTT -> TTT	LEU -> PHE
SG11	CCC -> TCC	PRO -> SER
SG18, SG20	GCC -> ACC	ALA -> THR
SG21	CGC -> CAC	ARG -> HIS
SG23	GGT -> AGT	GLY -> SER
TOE22	GAA -> AAA	GLU -> LEU
TC35	ATC -> AGT	ILE -> SER
MFT1181	CCT -> TCT	PRO -> SER
SG1, SG35	CGTACT -> TGTATT	ARG THR -> CYS ILE

4.5 Discussion

The *ftsE* mutants analysed did not appear to fall at all neatly into simple groups as regards phenotypic characteristics. Only three strains stood out when temperature-sensitive growth was assessed. These were SG23, TOE22 and TC35 which were all poorly temperature-sensitive for growth. This is not so surprising for TOE22 and TC35 as a) they were isolated using a temperature oscillation enrichment protocol which selects for filamentation at the restrictive temperature and phenotypic thermo-reversibility and b) they are in a different genetic background. However it is somewhat surprising for SG23 to be so poorly temperature-sensitive for growth because the screening procedure used to isolate it involved looking for temperature-sensitive growth of patches on NA plates. Microscopic observation also showed very little in the way of sub-groups. SG23 again stood out as being a poor filament former. SG35 stood out as being a long filament former. NaCl reversibility studies confirmed that SG23, TOE22 and TC35 were particularly weakly temperature-sensitive, whereas SG10 was found to be only poorly salt reversible. All the *ftsE* mutants tested were found to be phenotypically thermo-reversible and only one of these (TOE22) was found to require *de novo* protein synthesis for this. SG11 and TC35 were not included in this experiment. In summary these *ftsE* mutants can be loosely put into two groups on the physiological assessment above: SG23, TOE22 and TC35 which were all weakly temperature-sensitive in terms of growth and as a corollary filamentation; and SG1, SG10, SG11, SG18, SG20, SG21, SG35 and MFT1181 which were all strongly temperature-sensitive and extensive filament formers. Noteworthy exceptions to this grouping were SG35 that

was especially filamentous on solid media, SG10 that was only very weakly salt-reversible, and TOE22 which possessed an FtsE protein that was apparently not thermo-reversible.

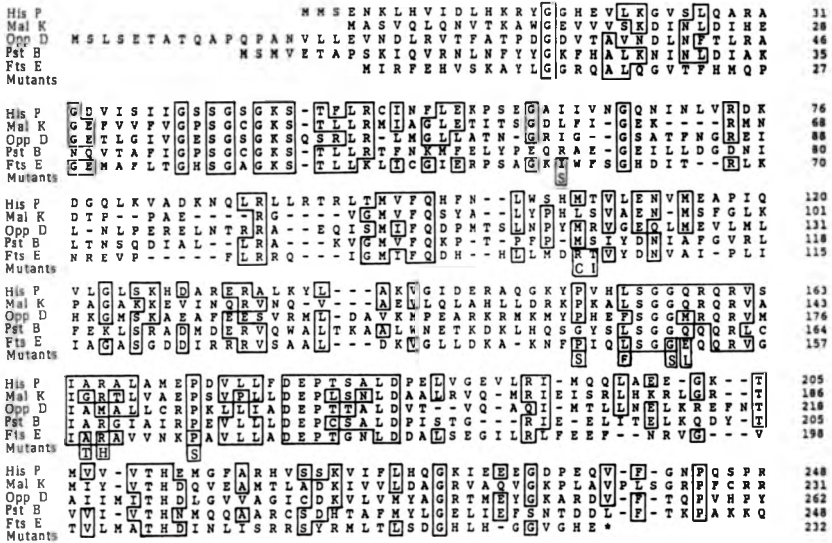
Three questions arise with regard to the DNA sequence of the ftsE mutations: Firstly are the mutations clustered in any way? Secondly how does the location of the mutations relate to the physiological observations? Thirdly how does the location of the mutations relate to the regions of extended homology with the superfamily of ATP binding proteins and does this tell us anything about the function of these regions? The answer to the first question is that the mutations do appear to cluster. Eight out of the eleven sequenced ftsE alleles lie within 63 nucleotides of each other in a structural gene that is 885 base pairs long. This equates to 70% of the mutations lying within 10% of the sequence, an obviously non-random distribution. Of the remaining three mutants two carry an identical two base pair change which results in a two amino acid change in the protein. This appears at first sight to be an unlikely coincidence but it must be borne in mind that the selection pressures on the type of mutation isolated were very stringent as a consequence of the screening procedure and the essential nature of the gene. Any mutation isolated had to result in a temperature-sensitive conditional phenotype which in the case of missense mutations means a temperature-sensitive protein. This presumably restricts the potential location of the mutations and the amino acid substitutions that can be tolerated. Therefore it may be that if one of the amino acids is altered as in the SG1 and SG35 mutant FtsE proteins then the adjacent amino acid must also be changed or the mutation would either be cryptic or lethal.

The answer to the second question is that the clustering does not appear to relate to the phenotypic observations in a simple manner. From Figure 4.7 one can see that the mutations in SG10, and SG23 lie within 9 nucleotides in the larger cluster of mutations. SG23 is one of the mutants that stood out as being particularly weakly temperature-sensitive and very highly salt-reversible whereas SG10 was very temperature-sensitive and only barely salt-reversible at 10g/l NaCl. Similarly SG1 and SG35 share the same double mutation yet SG35 was noted as being a particularly long filament former. This observation is backed up by the Coulter counter particle size distributions after 180 minutes further incubation at 42°C. The SG35 graph (4.2hii) has a horizontal scale that represents twice the scale on the SG1 graph (4.2bii) and taking this into consideration SG35 is clearly shown to produce longer filaments than SG1. This could be due to an increased growth rate of SG35 or a quicker response to the temperature shift up. This result suggests that these mutants really were independent isolates of the same mutation at the DNA level, and that one of these mutants carries an additional lesion that does not lie within the portion of the *ftsE* gene that has been sequenced. This is despite the fact that all the 'SG' series of mutations were re-transduced into OV2, in order to diminish the chances of there being extragenic mutations in the strains that might play some part in modifying the *ftsE* phenotype. The complementation and mapping studies of Chapter 3 suggest that if there is a second mutation present in SG1 or SG35 then it is ineffective if the primary *ftsE* mutation is complemented. SG19 and SG20 also share an identical mutation at the DNA level. The Coulter counter data for these, as might be expected, look almost identical (Figure 4.2d and e).

Figure 4.8 gives the answer to the third question as to how these mutations relate to the regions of homology with the ABC proteins. The ftsE mutations are mainly clustered in a region of extended homology with the ABC proteins. The function of this region has not yet been established. However Hyde et al. (1990) recently published a proposed structural model for the superfamily of ftsE homologues. They compared the consensus secondary structure of this superfamily with a range of ATP binding proteins of known tertiary structure. The protein that the superfamily most closely matched on secondary structure was adenylate kinase (ADK). Therefore the proposed structure for the superfamily was based on the three-dimensional structure of ADK. There were four regions where the superfamily consensus had no obvious counterpart in ADK. These regions were termed loops 1 to 4. Hyde et al. state that loops 2 and 3 were the most important deviation from the ADK structure. Neither loop is likely to be involved in ATP binding or hydrolysis; loop 2 because it is inserted on the surface of the molecule according to the Hyde model and loop 3 because it is so highly conserved amongst the superfamily that it seems very likely that it serves a function specific to these proteins. The equivalent regions in ADK are found to undergo the greatest conformational change on the binding of ATP. Therefore it was proposed that these loops serve to transmit ATP dependent conformational changes to the membrane associated domains of the proteins. The importance of these regions is confirmed by the fact that the majority of Caucasian cystic fibrosis (CF) sufferers have an amino acid deletion in loop 2 of the CF protein (CFTR) (Riordan et al., 1989). Cutting et al. (1990) recently reported a cluster of mutations in CF sufferers that lie in

Figure 4.8 Location of Amino Acid Changes in FtsE Protein

The alignment of FtsE protein with four ABC proteins is shown from Gill et al. (1986). MalK and PstB proteins are the maltose and phosphate permeases respectively from Escherichia coli, and HisP and OppD are the histidine and oligopeptide permeases respectively from Salmonella typhimurium. The full length protein is only shown for FtsE. Regions of conservation are boxed. Amino acid changes predicted for the FtsE mutants are shown below the aligned sequence. The double mutation changing amino acids 104 and 105 was found in two mutants, as was the mutation changing amino acid 159.


His P
Mal K
Opp D
Pst B
Fts E
Mutants

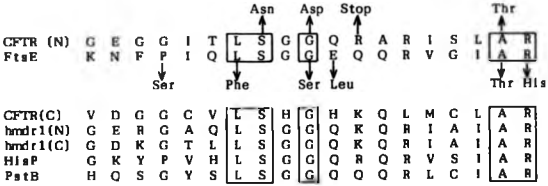
loop 3 of the CFTR protein. These mutations and the ftsE mutations are shown in Figure 4.9. It is highly significant that of the ftsE mutations sequenced in this study the SG1 and SG35 mutations lie in loop 2 of the proposed structure. In addition the region of extended homology where the majority of the ftsE mutations are clustered encompasses loop 3. The MFT1181, SG10, SG23, and TOE22 mutations would be in loop 3, and the SG19, SG20, and SG21 mutations would be immediately adjacent to loop 3.

The possibility that these ftsE mutations cause changes in regions of the protein that couple ATP hydrolysis to the cell division functions is very exciting. That these regions are proposed to couple ATP hydrolysis to another protein function by means of conformational changes seems highly consistent with the nature of the ftsE alleles. The ftsE alleles are temperature-sensitive missense mutations which give rise to proteins that are presumably rendered inactive at the restrictive temperature due to altered conformation. This raises the possibility that the other protein function may be permanently switched on (or off) due to the thermally induced conformational change overriding any change due to ATP binding or hydrolysis. One obvious implication is that ATP binding and hydrolysis may be unaffected by the ftsE mutations and it would be interesting to be able to test this.

Thus the results of the DNA sequencing of the ftsE alleles not only supports the model of Hyde et al. but also confirms the clustering of mutations found in the CFTR protein. It is truly remarkable that there is a high frequency of mutation in the same region (and in some cases the same amino acid) in an essential cell division gene and an evolutionary

Figure 4.8 Clustered Mutations in CFTR and FtsE Proteins

The cystic fibrosis transmembrane conductance regulator (CFTR) mutations and local amino acid sequence are taken from Cutting et al. (1990). This sequence is aligned with the homologous region from FtsE. Mutations in CFTR are shown above the CFTR (N) sequence. Mutations in FtsE are shown below the FtsE sequence. Homologous sequences from human multiple drug resistance (hmdr1) protein (Cutting et al., 1990) and HisP and PstB proteins (Gill et al., 1986) are aligned below for reference. Regions of conserved amino acids are boxed. C: C terminal, N: N terminal.



distinct human protein. This is made all the more remarkable by the fact that the ftsE mutations were selected for temperature-sensitivity whereas the CFTR analogues are null mutations. Gill et al. (1986) originally suggested that the FtsE protein might be involved in some sort of transport function, and it is intriguing to see that as the ABC superfamily grows, transport of specific substrates varying in size from sugars to proteins becomes an ever more dominant unifying theme. In the light of the homology between the FtsY protein and eukaryotic docking protein (Romisch et al., 1989; Bernstein et al., 1989) is it possible that in the 76 minute cell division genes we have the basis of a transport system, perhaps for transporting septation specific proteins (or septalsome components) into the periplasm, the energy required being supplied by the hydrolysis of ATP by the FtsE protein?

Chapter 5: NUCLEOTIDE BINDING OF THE FTSE PROTEIN

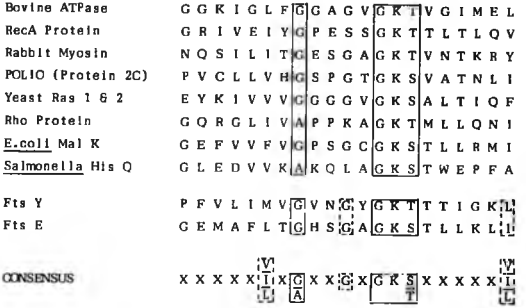
5.1 Introduction

The FtsE protein appears by amino-acid sequence homology to be a member of the ATP-binding cassette (ABC) superfamily of proteins (Gill et al., 1986; Higgins et al., 1986). Part of the sequence that is conserved between these proteins is, as the name implies, a putative ATP binding site or 'Walker motif', with the amino acid consensus G----GKS/T (Figure 5.1). All the proteins in this family that have been investigated have been shown to bind ATP (Higgins et al., 1986) and more recently hydrolysis of ATP by some of these proteins, many of which are components of binding-protein-dependent transport systems, has been demonstrated to be linked to substrate accumulation (Hyde et al., 1990). It was therefore apparent that FtsE might well bind ATP and that this was a potentially fruitful avenue for research. Interestingly FtsY protein also possesses a putative nucleotide binding site, so there is a clear possibility that one of these proteins may bind and hydrolyse ATP to provide energy for a cell division related process.

Previous approaches to the study of nucleotide binding by proteins have been varied. Potentially useful techniques include binding the protein to a radiolabelled ATP analogue such as 5'-p-fluorosulphonylbenzoyl adenosine (used by Higgins et al. (1985) with the oligopeptide permease protein OppD, and Knight and McEntee (1985) with the RecA protein) or the photoaffinity label, 8-azido-ATP (used by Hobson et al. (1984) with the histidine permease protein HisP, and Knight et al. (1988) with the RecA

Figure 5.1 Putative Nucleotide Binding Sites in a Range of Proteins

Adapted from Gill *et al.* (1986). Conserved amino acids are boxed.



protein). With both of these reagents the protein is run on a PAGE gel following the labelling reaction and co-migration of the radiolabel and nucleotide binding proteins is observed. Another possible technique is to study the binding of ^{32}P labelled ATP and here again the binding is demonstrated by co-migration of protein and label on a PAGE gel. Finally the binding of the protein to affinity media such as Cibacron Blue Sepharose and its subsequent elution by specific nucleotides can be studied (used by Higgins et al. (1985) with OppD).

With the 76 minute cell division proteins there is a problem in that they are in very low abundance. No purified protein is available and there are no specific antibodies for detection of the proteins. Currently the only way of visualising these proteins is by radiolabelling them using a method such as the maxi-cell system (Sancar et al, 1979). Even when these proteins are produced in maxi-cells they are not distinguishable from the general background of other proteins on Coomassie or silver-stained acrylamide gels. Acrylamide gels need to be fluorographed and long autoradiograph exposures made in most cases to get unambiguous results. Because of this detection problem and the presence of large quantities of non-labelled contaminating proteins it was felt that techniques relying on demonstrating the binding of radiolabelled substrates were likely to give ambiguous results. Therefore affinity chromatography was the technique chosen for a preliminary investigation of the nucleotide binding properties of the 76 minute cell-division proteins.

5.2 Use of Dye-Ligand Affinity Chromatography

Dye-ligand chromatography media were used to assess nucleotide binding by the 76 minute cell-division proteins. These chromatography media exhibit a broad group specificity. The original dye-ligand medium was the triazinyl textile dye Cibacron Blue F3GA (Blue A) which is now commonly linked to a support such as agarose. This was found to exhibit a group specificity for nucleotide binding proteins. Following the discovery of the utility of Blue A for protein purification there was a systematic search for other related dyes that would possess similar group selective properties. This has resulted in the availability of a range of dye-ligand chromatography media with subtly different binding specificities. Red A has generally similar binding properties to Blue A but with different selectivity, Orange A and Blue B bind only a few proteins but bind these with great specificity, Green A binds many proteins and is generally useful if the protein of interest will not bind to any of the other media.

The principle behind using these media to investigate nucleotide binding is that the protein of interest will bind onto the matrix linked dye through the protein's nucleotide-binding site, irrespective of the site's specificity for a particular nucleotide. It should then be possible to elute the protein using the corresponding nucleotide at moderate concentrations due to the nucleotide displacing the dye from the nucleotide binding site. Inappropriate nucleotides should either be required at considerably higher concentrations for elution or should not elute the protein off the dye matrix at all. Thus using an affinity medium that does not presuppose a particular nucleotide-binding specificity (as opposed to using a matrix bound nucleotide as an affinity medium) enables a protein of uncertain

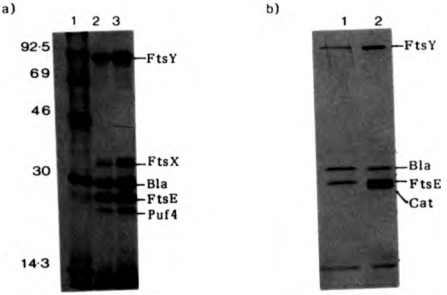
nucleotide-binding specificity to be tested against a variety of nucleotide and non-nucleotide eluants using only one chromatography medium.

5.3 General Method for Analysis of the FtsY and FtsE Proteins Using Dye-Ligand Chromatography

The protocol followed in all experiments was similar. ^{35}S radiolabelled proteins were made using the maxi-cell protocol on strain CSH26ΔF6 (pDG1) or CSH26ΔF6 (pDB1). These produce the following labelled proteins in order of decreasing molecular weight: FtsY, FtsX, Tet (the tetracycline resistance protein, which is rarely detectable), Bla (β lactamase), FtsE, Cat (the chloramphenicol resistance protein, only in pDG1 maxi-cell preparations) and the ORF4 protein (Figure 5.2). The maxi-cells were sonicated on ice for 3 X 30 seconds with 30 seconds rest between each period of sonication. The lysates were then given a clearing spin at 4000g for 5 minutes at 4°C to remove any remaining whole cells. These lysates were either used directly or membrane and soluble fractions were separated before use. The buffers used for washing, loading, and eluting off the columns were based on 10mM Tris.HCl pH7.4. Small volumes of lysate were loaded onto one or more equilibrated affinity columns packed with one of the following dye-matrices: Blue A, Red A, Blue B, Green A, Orange A or a control agarose matrix with no dye-ligand bound. The sample was generally washed in with a small volume of buffer. Following an equilibration period the columns were washed with several bed volumes of the loading buffer to remove unbound proteins. Elution buffer containing a nucleotide (such as 10mM ATP) or other eluant was then passed through the columns. Any remaining proteins

Figure 5.2 Radiolabelled Proteins from pDB1 and pDG1 Maxicells

a.) Lane 1: Molecular weight markers (sizes in kD). Lanes 2 & 3: Overloaded pDB1 maxicell lysate. Puf4 (Protein of Unidentified Function 4) is the ORF4 protein product. b) Comparison between pDB1 and pDG1 maxicell lysates, reduced loading to resolve Cat and FtsE. Lane 1: pDB1. Lane 2: pDG1.



were removed and the columns regenerated by passing several bed volumes of either 8M urea or 6M guanidine hydrochloride through them. The whole of each wash, elution, and regeneration was collected and the samples desalted where appropriate by dialysis against double-distilled water or using PD-10 desalting columns. Samples were finally reduced in volume by precipitation with 70% acetone at -20°C or freeze drying, before running them on 10% linear SDS-PAGE gels. These gels were fluorographed, dried under vacuum, and autoradiographs made.

5.4 Results

The first experiment used a 5ml Blue A column run at room temperature and followed the protocol in Table 5.1. The column was hand packed and did not have a frit on the top of the matrix bed. To prevent the column drying out it was left with a small amount of liquid lying over the top of the bed at all times. This column was loaded with one bed volume of labelled CSH26ΔF6 (pDG1) lysate. This was estimated to contain approximately 0.3mg of protein. The sample loading was followed by a two bed volume wash. One bed volume of 10mM ATP was then passed through the column, and two more washes were performed. Figure 5.3 shows an autoradiograph of the polyacrylamide gel run with these samples. FtsY, FtsE and Bla proteins were seen in the flow-through sample and the following two washes. FtsY and FtsE proteins appeared to be enriched relative to Bla in the second wash, which immediately preceded the ATP elution. This was thought to reflect elution of these proteins by ATP, their appearance in the preceding wash being due to mixing of the ATP with the layer of liquid lying on the top of the column matrix as the

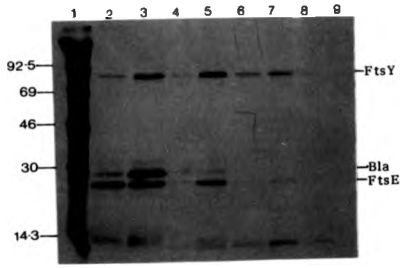
Table 5.1 Chromatography Protocol 1

All washes and elutions were one bed volume unless otherwise stated. All solutions except NaCl were made up in equilibration buffer.

Temperature	Room Temperature
Matrix	Blue A
Bed Volume	5ml
Equilibration	10mM Tris.HCl pH7.4
Load	5ml, approximately 300ug of protein
Wash	2 X 5ml
Elution	10mM ATP
Wash	2 X 5ml
Elution	10mM GTP
Wash	2 X 5ml
Elution	10mM NaCl
Elution	100mM NaCl
Elution	1M NaCl
Wash	5ml

Figure 5.3 Autoradiogram of Samples from Blue A Column

Lane 1: molecular weight markers (sizes in kD); lane 2: equilibration; lane 3: sample flow-through; lanes 4 & 5: wash; lane 6: ATP elution; lanes 7 & 8: wash; lane 9: GTP elution.



ATP eluant was applied to the column. This liquid then formed part of the wash as the bulk of the ATP eluant was run into the column. This result appeared to be very promising. No further radiolabelled protein was eluted with GTP or up to 1M NaCl.

It was felt that the mixing problem could be averted by loading the sample in a smaller volume (0.5ml) and by washing with a greater number of bed volumes of wash, taking each bed volume as an individual sample. Three 5ml Blue A columns were run separately at 4°C following the protocol in Table 5.2, one each using one bed volume of 10mM ATP, 10mM GTP, or up to 1M NaCl, as eluant. No radiolabelled proteins were seen in any of the elution samples except for a trace of FtsY and Bla proteins in the 1M NaCl elution. Large amounts of proteins were seen however in the guanidine hydrochloride regeneration samples. Therefore there appeared to be three possibilities: 1) most of the FtsY and FtsE proteins were membrane bound in this maxi-cell preparation so that they remained at the top of the columns until they were released from the membrane fragments by the guanidine hydrochloride; 2) because the maxi-cell lysate was precipitated and resuspended in a small volume it was possible that the proteins had not been resuspended properly and hence were stuck in aggregates at the top of the column until they were released by the action of the guanidine hydrochloride; 3) the low temperature could have caused pH and affinity changes which made the proteins more difficult to elute off the columns.

In order to address these possibilities two Blue A columns were run to essentially the same protocol as in Table 5.2 but at room temperature, one being loaded with 5ml of standard maxi-cell lysate (one bed volume)

Table 5.2 Chromatography Protocol 2

All washes and elutions were one bed volume unless otherwise stated. All solutions except NaCl and guanidine hydrocloride were made up in equilibration buffer.

Temperature	4°C
Matrix	Blue A
Bed Volume	5ml
Equilibration	10mM Tris.HCl pH7.4
Load	0.5ml, plus 2ml equilibration buffer 'wash in'
Wash	40ml
Elution	10mM ATP or 10mM GTP or 10mM NaCl then 100mM NaCl then 1M NaCl
Wash	40ml
Regeneration	20ml of 6M guanidine hydrochloride

and the other with 0.5ml of correspondingly concentrated lysate. ATP was used as eluant in both cases. More radiolabelled protein was visible in the washes of the column loaded with 0.5ml of sample than in the washes of the previous column that was run at 4°C. Even more protein was detected in the washes from the column loaded with 5ml of sample and FtsY and FtsE were seen in small quantities in the ATP eluate. However the vast majority of radiolabelled protein from both columns was found in the guanidine hydrochloride washes. This experiment suggested that loading the labelled proteins in a larger volume and running the columns at room temperature were better and resulted in a greater probability of eluting the proteins back off the column. The results also suggested that the labelled proteins of interest might well be stuck in membrane fragments at the top of the column. This explanation is consistent with the demonstration by Gill and Salmond (1987) that a large proportion of FtsY and FtsE proteins are inner membrane associated.

A fresh batch of maxi-cell labelled proteins was made and this was fractionated into soluble and membrane proteins, the membrane proteins being solubilised from envelope preparations in 1% Triton-X-100, 10mM Tris.HCl pH7.4. These samples were run on two identical 5ml Blue A columns using a 1 bed volume sample loading and the same protocol as in the previous experiment. The control loading on the PAGE gels showed that the fractionation had been incomplete as the markers β-lactamase (a periplasmic protein) and the tetracycline resistance protein (membrane bound) were found in both fractions. There was an enrichment for the proteins in the appropriate fraction but this was not complete. FtsY and FtsE were seen in the ATP elution from the column loaded with soluble

proteins. Virtually no protein was visible in the ATP elution from the column loaded with the membrane fraction although a small amount of FtsY could be seen in this sample. It is conceivable that this observation might be the result of a difference between the binding of soluble and membrane bound forms of the Fts proteins. This would suggest that separate experiments should be performed with membrane and soluble fraction derived proteins, however for simplicity it was decided that future experiments should use soluble fractions prepared in the absence of Mg^{2+}. This procedure results in the recovery of peripheral membrane proteins such as FtsE and FtsY in the soluble fraction. Very little of these proteins is lost to the membrane fraction in such a preparation.

Due to the ambiguity of the results with the Blue A columns using ATP as eluant two experiments were then performed running Blue A, Red A and Green A columns in parallel. These and all subsequent columns were of 2 ml bed volume and had a correspondingly reduced sample loading. The protocol is given in Table 5.3. 5 bed volumes of 10mM ATP were tested as an eluant on all three columns in the first experiment and 5 bed volumes of 10mM NADH in the second. Figures 5.4 and 5.5 show autoradiographs of PAGE gels of the samples from these columns using ATP and NADH as eluant respectively. On the Blue A column a small amount of FtsY was seen in the sample flow-through and wash, none in the ATP elution, and a large amount in the 8M urea regeneration fraction. FtsE was found in the flow-through and wash, and to a small extent in the regeneration. When NADH was used as the eluant the results were similar except that FtsY and FtsE were not found in the 8M urea fraction. On the Red A column with ATP or NADH as eluant FtsE

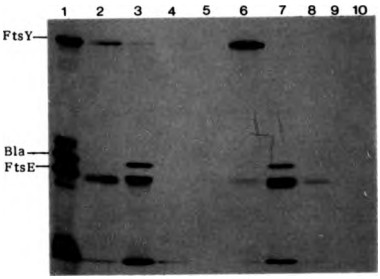
Table 5.3 Chromatography Protocol 3

All washes and elutions were one bed volume unless otherwise stated. All solutions except 8M urea were made up in equilibration buffer.

Temperature	Room Temperature
Matrix	Blue A/Red A/Green A
Bed Volume	2ml
Equilibration	10mM Tris.HCl pH7.4
Load	1.5ml, plus 100ul equilibration buffer 'wash in'
Wash	10ml
Second Wash	10ml
Elution	10ml of 10mM ATP or 10ml of 10mM NADH
Regeneration	10ml of 8M urea

Figure 5.4 Autoradiogram of Samples from Blue A, Red A, and Green A columns Using ATP as Eluant

a) Samples from Blue A and Red A columns. 1, pDB1 markers; 2, pDG1 control. Blue A samples: 3, sample flow-through and wash; 4, second wash; 5, ATP elution; 6, urea regeneration. RedA samples: 7, sample flow-through and wash; 8, second wash; 9, ATP elution; 10, urea regeneration.



b) Samples from Green A column. 1, pDB1 markers; 2, pDG1 control; 3, sample flow-through and wash; 4, second wash; 5, ATP elution; 6, urea regeneration.

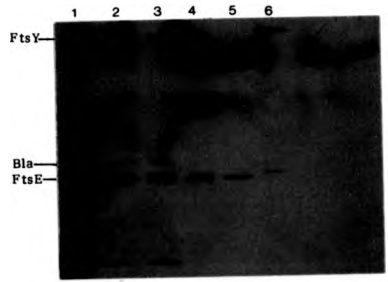
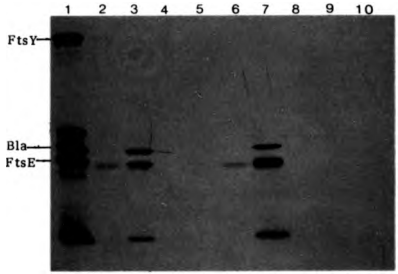
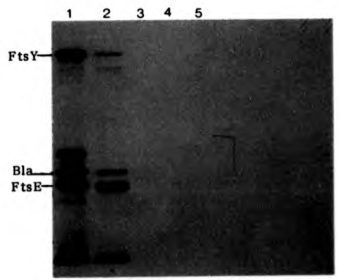


Figure 5.5 Autoradiogram of Samples from Blue A, Red A, and Green A columns Using NADH as Eluant

a) Samples from Blue A and Red A columns. 1, pDB1 markers; 2, pDG1 control. Blue A samples: 3, sample flow-through and wash; 4, second wash; 5, NADH elution; 6, urea regeneration. RedA samples: 7, sample flow-through and wash; 8, second wash; 9, NADH elution; 10, urea regeneration.



b) Samples from Green A column. 1, pDB1 markers; 2, sample flow-through and wash; 3, second wash; 4, NADH elution; 5, urea regeneration.



was only detected in the flow-through and wash. FtsY was not detected at all in any sample. This suggests that FtsY was binding very tightly (maybe irreversibly) to the column matrix. Most of the FtsE and FtsY were seen in the flow-through and wash and second wash on the Green A columns. On the column where ATP was used as eluant some FtsY and a little FtsE were seen in the urea regeneration fraction. On the Green A column where NADH was used as eluant these proteins were only seen in the sample flow-through and wash. It is hard to reconcile these results and those for the Blue A regeneration fraction as the 8M urea should remove all proteins from the columns, therefore FtsY should be present in the regeneration fraction of both columns irrespective of the nucleotide used as eluant. It could be argued that there is a specific interaction between FtsY and ATP that does not exist between FtsY and NADH, such that the presence of ATP in the column destabilises the FtsY binding but does not elute it. When the urea is added it elutes FtsY that is associated with ATP, but not FtsY that has been exposed to NADH, however it is hard to see how treatment as harsh as 8M urea could fail to strip these proteins off the columns.

There are obviously differences between the column matrices with respect to the binding of FtsE and FtsY. This suggested that it might be wise to try the other dye-ligand matrices with the maxi-cell lysates and perform replicate experiments. It was felt that if the cell division proteins could be shown to bind onto any one dye-ligand matrix and then be clearly eluted by any eluant that it would give a base-level against which different eluants could be tested. The whole range of Amicon dye-ligand columns (Blue A, Red A, Blue B, Orange A, Green A, and a control

column packed with agarose) was assessed following the manufacturers instructions; that is regenerating the columns at room temperature and then running them at 4°C, using 1.5M KCl as eluant. The sample flow-through and wash were pooled, resulting in three samples per column, viz. flow through and wash, elution, and regeneration. The protocol is given in Table 5.4. The columns were run identically three times. Because a large KCl precipitate formed when the proteins in the samples were acetone precipitated, a variety of strategies were tried to overcome this. These included dialysis and the use of gel-filtration desalting columns on the 'KCl' samples, and freeze-drying instead of acetone precipitation for all samples. Suitable controls were performed to test the dialysis and then the desalting steps but virtually no radiolabelled protein was found in any of the experimental samples or controls. This was surprising but concurred with the early experiment performed with just the Blue A column at 4°C where there appeared to be very poor recovery of proteins. The conclusion made from this series of experiments was that these proteins could not be successfully manipulated at 4°C.

One final experiment was performed using the complete set of dye-ligand media. The columns were run at room temperature using ATP as eluant. Sample flow-through and ATP elution fractions were freeze-dried and urea regeneration fractions were desalted then freeze-dried. The whole of each fraction was then run on a PAGE gel. The dye-front on these gels ran extremely unevenly and was very diffuse. These gels were stained with Coomassie blue and very little protein was visible in any of the tracks. The gels were then treated with the scintillant diphenyloxazole (PPO) or Amplify (Amersham) and long autoradiograph exposures were made. FtsY

Table 5.4 Chromatography Protocol for Amicon Columns

All solutions except KCl and 8M urea were made up in equilibration buffer.

Temperature	Regeneration: Room Temperature All other steps: 4°C
Column Bed Volume	2ml
Equilibration buffer	10mM Tris.HCl pH7.4
Load volume	0.5ml plus 0.1ml equilibration buffer 'wash in'
Wash	10ml
Eluant	10ml of 1.5M KCl
Regeneration	10ml of 8M urea

and FtsE were apparent in the sample flow-through from all the columns with a larger amount present in the samples from the Orange A and Control columns. The bands seen were exceedingly diffuse, making it impossible to distinguish clearly between the FtsE and Cat proteins. FtsY was not detected in any of the ATP elution samples and FtsE was not clearly detected in any of these samples either, although there was a diffuse band in the right region of the gel for both the Red A and the Green A columns. No radiolabelled material was present in the urea regeneration fractions from any of the columns.

5.5 Use of Alcohol Dehydrogenase as a Control Protein

In order to test that the columns were functioning correctly a purified protein of known performance was processed on the whole Amicon kit. Alcohol dehydrogenase (ADH, EC 1.1.1.1) has been successfully purified on the Blue A matrix from a number of sources including bakers' yeast (Easterday and Easterday, 1974). The eluant used for the purification of ADH is NAD in concentrations of up to 10mM, in buffers such as 20mM Tris.HCl, pH 8.5. This was the enzyme chosen for use to test the columns. The actual enzyme used was from Saccharomyces cerevisiae and was purchased in a purified form from Sigma. The advantage of using purified protein was that it gave no detection problems, with as little as 1ug of protein being easily visualised on a Coomassie stained PAGE gel.

Although some problems were initially experienced with recovery of protein when the columns were run in the cold room, when they were run at room temperature the ADH behaved as expected on the Blue A column. 0.5ml of 5mg/ml ADH in equilibration buffer (20mM Tris pH 8.4,

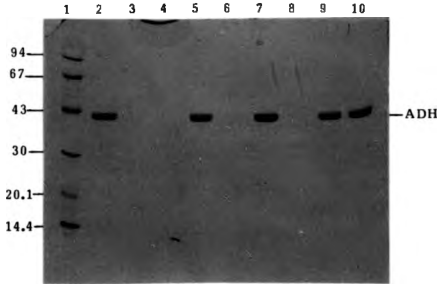
5mM $MgCl_2$, 0.4mM EDTA and 2uM βmercaptoethanol) was loaded onto each column and washed in with 100ul of equilibration buffer. Following a 30 minute equilibration period each column was washed with a further 10ml of equilibration buffer. 10ml of 5mM NAD in equilibration buffer was then passed down each column as eluant. The columns were finally stripped with 12ml of 8M urea. 50ul from each flow through, elution or regeneration was mixed with 50ul of 2X SDS PAGE loading buffer and run on a PAGE gel.

Figure 5.6 shows the Coomassie stained gels from this experiment. ADH bound to Blue A, Red A, and Green A, flowing straight through the Blue B, Orange A, and Control columns. The majority of the bound protein was then recovered in the NAD eluate. A small proportion of the ADH was bound, presumably non-specifically, to all the columns and was recovered in the urea regeneration step. The control loading of 12.5ug ADH on the second PAGE gel in Figure 5.6 represents the total amount of ADH that would be seen in these samples from any one column assuming 100% recovery. It is apparent from the band intensities that recovery was virtually 100% on all the columns. In summary, the use of ADH demonstrated that the columns did appear to give reasonable results with a pure control protein, suggesting that poor column performance was not the cause of the difficulties encountered when working with FtsY and FtsE. However it is notable that the performance of the columns at room temperature differed substantially from that at 4°C.

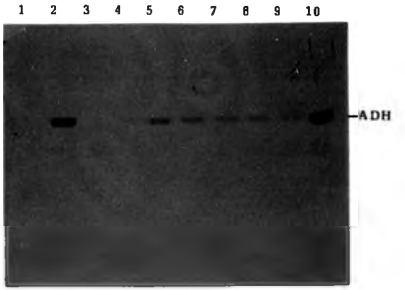
Figure 5.6 Alcohol Dehydrogenase Control Experiment

Coomassie stained PAGE gels of samples from alcohol dehydrogenase control experiment. a) Lane 1, molecular weight markers (sizes in kD). Lanes 2-7 sample flow-through and wash: 2, control column; 3, BlueA; 4, RedA; 5, Blue B; 6, Green A; 7, Orange A. Lanes 8-10 NAD elution: 8, control column; 9, Blue A; 10, Red A. b) Lanes 1-3 NAD elution: 1, Blue B; 2, Orange A; 3, Green A. Lanes 4-9 urea regeneration: 4, control column; 5, Blue A; 6, Red A; 7, Blue B; 8, Orange A; 9, Green A. Lane 10 control loading of ADH.

a)



b)



5.6 Discussion

Very poor recovery of proteins typified all the dye-ligand chromatography experiments with radiolabelled proteins and was probably largely responsible for the ambiguity of the results. The major problem with this sort of analysis of FtsE and FtsY is, as stated in the introduction to this chapter, the difficulty of detecting the proteins. Lengthy protein preparation, sample processing, and autoradiograph exposures meant that it was a minimum of three weeks from the start of any experiment to the analysis of the results, and in many cases this time period was increased further by the need for even longer autoradiograph exposures. This limited the number and nature of experiments that could be performed.

The column system clearly requires optimisation if it is to yield unambiguous results even with a pure protein that is easily detected on PAGE gels. In the case of the cell division proteins investigated here, not only is there a great excess of contaminating protein but there is only a very small amount of the protein of interest and this is time-consuming to detect. If a more sensitive assay were to be developed, such as the raising of FtsE and FtsY specific antibodies, and enrichments of the proteins of interest (or purified proteins) were available these experiments would be greatly facilitated.

Laying this aside I feel that there is some evidence for ATP having an interaction with at least FtsY and probably FtsE. Results suggestive of this were seen with the Blue A column in the first and third experiments which used total maxi-cell lysates, and the fourth experiment, in the column loaded with soluble proteins. In these experiments some FtsE and FtsY protein was seen in the ATP elutions, although the majority of the

protein clearly remained on the columns. It is certainly to be expected that at least FtsE will bind, and quite probably hydrolyse, ATP, from the results of the investigation of other members of the ABC superfamily of proteins. It remains a matter of some importance to demonstrate that this actually occurs. Interestingly, the recently reported homology between FtsY and eukaryotic docking protein includes the putative nucleotide binding domain, and docking protein has been demonstrated to bind GTP (Bernstein et al., 1989). This appears to confirm the validity of looking for nucleotide binding in FtsY.

If nucleotide binding and hydrolysis could be demonstrated in either FtsY or FtsE then this protein would be only the second cell division protein to have a biochemical function assigned to it. The other protein is FtsI (PBP 3) which is one of the penicillin-binding (murein synthetic) proteins. If the system was optimised and demonstrated elution of FtsE by a specific nucleotide it was envisaged that this approach might then be used to assess the FtsE proteins produced by the range of *ftsE* missense mutants described in Chapters 3 and 4.

Other approaches for assessing nucleotide binding should perhaps be investigated. A simple experiment that could be performed is to attempt to bind ^{32}P labelled ATP to FtsE and FtsY *in vitro* and show that the ^{32}P label co-migrates with the Fts proteins on non-denaturing PAGE gels. Alternatively photoaffinity labelling ATP analogues could be reacted with FtsE and FtsY and co-migration of the radio-labelled analogue and the cell division protein could be investigated. In the case of these cell division proteins all these techniques would be complicated by the fact that the proteins can only be visualised by radiolabelling them, and the

results compromised by the fact that the cell division proteins represent only a very small component of a whole cell lysate. It is clearly possible for there to be a nucleotide binding protein of similar molecular weight to the Fts proteins on PAGE gels that is not labelled in the maxi-cell system. This would obviously give rise to false positive results in terms of the labelled nucleotides co-migrating with the cell division proteins. It was for this reason, as well as the difficulty of demonstrating the double labelling of the proteins, that affinity chromatography was the technique investigated in this chapter. It appears that until there is an improvement in detection methods or purified proteins become available the question of whether FtsE and FtsY protein bind and hydrolyse nucleotides will remain unanswered.

Chapter 6: MAPPING OF A NEW ESSENTIAL GENE AND ISOLATION OF COMPLEMENTING DNA

6.1 Introduction

The isolation of a range of temperature-sensitive mutants using a localised mutagenesis protocol was described in Chapter 3. The vast majority of these were mapped by complementation with defined plasmids to known essential genes in the 76 minute region of the Escherichia coli chromosome as was expected. However there were two mutants that were not complemented by any of the available plasmids carrying DNA cloned from the 76 minute region. These were the missense mutants SG28 and SG33. When these strains were lysogenised with λS3D which carries ORF4 to rpoH plus a flanking region counter-clockwise of rpoH, ie. all the known essential genes from this region, the strains remained temperature-sensitive. This result suggested that SG28 and SG33 do not carry multiple mutations in the known essential genes at 76 minutes, hence they were tentatively assigned to a new essential gene or genes. If this is correct then it raises the exciting possibility that there may be more, as yet undiscovered, essential genes in this region of the E. coli chromosome, as predicted by Salmond and Plakidou (1984). This could imply a large 76 minute essential gene cluster analogous to the large morphogene cluster at 2 minutes on the E. coli map.

This chapter describes the analysis of the two strains SG28 and SG33 that appeared to carry mutations in a new gene, or genes. Initially, some physiological assessment was made of these two temperature-sensitive mutants. Concurrently attempts were made to transductionally map the

two mutations, especially that in SG33, relative to several genetic markers in the region. The chapter concludes with a description of the isolation of DNA that complements the temperature-sensitive mutation in SG33 and which was also found to complement SG28.

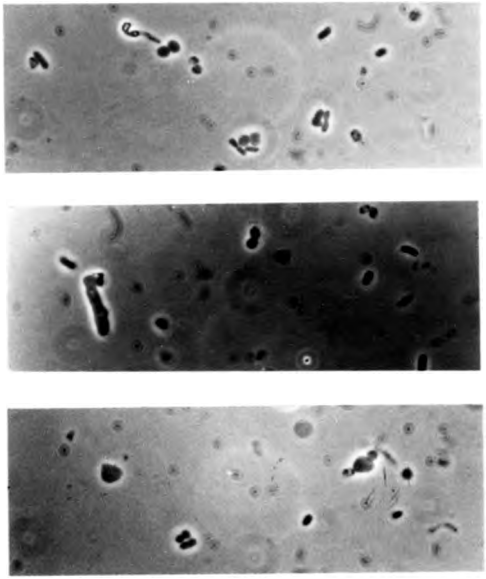
6.2 Physiology

SG28 was found to grow weakly at the restrictive temperature when streaked out on NA plates. SG33 exhibited virtually no growth at the restrictive temperature on NA. The presence of some revertants however suggested that it was probably due to a single mutation. Because the temperature-sensitivity of SG33 was so clear cut it was mapped and SG33 complementing DNA was cloned in preference to using SG28. Viable count results at permissive and restrictive temperature were identical for both strains. Both exhibited less than 0.001% of the 30°C viability at 42°C on NA plates. The cells of both strains had a pleomorphic appearance including lemon shaped cells and short filaments when examined under the microscope following incubation at the restrictive temperature on NA plates, although the paucity of growth of SG33 under these conditions meant that very few cells could be found. Figure 6.1 shows the appearance of SG33 from NA plates incubated at 30°C and 42°C.

In order to properly assess the appearance of SG33 at the restrictive temperature, temperature-shift experiments were performed using liquid cultures. Overnight NB cultures of SG33 and OV2 grown at 30°C with shaking were diluted 1 in 20 and incubated for a further 1 hour at 30°C. These cultures were split in two, one half remaining at 30°C, the other

Figure 8.1 Appearance of SG33 at 42°C

Photomicrographs were taken using a Leitz Dialux 22 camera system.



half being incubated at 42°C. Samples were taken at regular intervals and examined using phase contrast microscopy. After 90 minutes at 42°C the cells of the SG33 culture started to increase in diameter, giving short cells the appearance of lemons and longer cells the appearance of thick rods. At 150 minutes at 42°C many of the cells appeared to have lysed resulting in large amounts of debris and few intact cells. After 210 minutes at 42°C almost all of the cells were lysed and all cells that remained were either spherical or had the shape of lemons. The culture that was incubated at 30°C, and both the OV2, cultures contained typical wild type short rods. Interestingly SG33 (pH3C) when subjected to the same experimental procedure showed slight filamentation in addition to the SG33 phenotype described above. This resulted in the production of dumbell shaped cells and filaments with one end enlarged into a sphere at 42°C and slight filamentation at 30°C. This slight lengthening of cells carrying pH3C is also seen in OV2 and is presumably an effect of the multicopy cell division genes carried on this plasmid. It is interesting that this effect should still be seen in SG33 at the restrictive temperature where it appears to have lost control of the cellular morphology, perhaps suggesting that the morphological control that is disturbed in SG33 is independent of the action of the cell division genes carried on pH3C.

Viable counts of SG33 at the permissive and restrictive temperature were performed on LB plates with a range of NaCl concentrations in order to establish whether the SG33 phenotype was salt reversible. SG33 displayed a reduced viability on LB0 at 30°C (0.01% of the viability at 30°C on LB5 or LB10). In addition the viability of SG33 at 42°C on LB0, LB5, and LB10 was less than 0.001% of that on LB10 at 30°C. Thus SG33 is not salt

reversible and the strain is also susceptible to reduced salt levels at the permissive temperature. Cells from LB0 and LB10 plates were examined under phase contrast. On LB0 at 30°C the cells were slightly longer than OV2 under the same conditions. At 42°C on the same medium virtually no cells were found, those that were found appeared to be ghosted and of a larger diameter than normal. On LB10 at 30°C cells appeared to be generally normal although some were of a larger than normal diameter. At 42°C the cells from the LB10 plate showed the typical SG33 morphology of large diameter spheres, lemon and dumbell shapes, many of the cells showing signs of lysis.

A growth curve was determined for SG33 growing in NB. Duplicate cultures were inoculated and split into two. These four cultures were grown at 30°C for 4 hours to mid-logarithmic growth phase (OD_{600}=0.3) then one of each duplicate was transferred to 42°C. The cultures were incubated for a further 3½ hours, optical density readings being taken every 30 minutes. The growth curves generated were indistinguishable from those for OV2, indicating that SG33 is not a growth rate mutant.

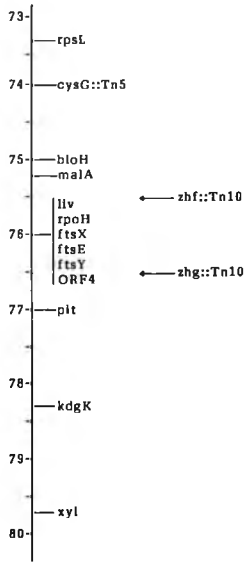
6.3 Transductional Mapping

6.31 Approaches taken to mapping the mutations in SG28 and SG33

Figure 6.2 shows a map of the genetic markers near 76 minutes that are referred to in this section. In order to establish whether SG28 and SG33 carry mutations in a hitherto un-mapped locus it was necessary to show that the temperature-sensitive lethal mutations in SG28 and SG33 (Ts28 and Ts33) lay in the "silent" region clockwise of the 76 minute *ftsYEX*

Figure 6.2 Transductional Markers Located Near 78 Minutes

Adapted from Bachmann (1983). Approximate locations of Tn10 insertions marked.



operon. This is because the region anti-clockwise of *ilv* (the gene immediately anti-clockwise of *rpoH*) up to least *bioH* has been shown by deletion not to contain any essential genes (Anderson and Oxender, 1977), therefore is unlikely to be the site of a mutation that results in a temperature-sensitive lethal phenotype. Unfortunately, because the region clockwise of 76 minutes is "silent" there are virtually no markers with which to map any mutation thought to lie there. One strategy would be to map Ts28 and Ts33 relative to a known auxotrophic marker in the 75 to 76 minute region and *zhf::Tn10* in three factor crosses. If this demonstrated that the temperature-sensitive mutations in SG28 and SG33 were clockwise of these markers then it could be assumed that they lie in a hitherto un-mapped gene or genes in the 'silent' region as the mutations do not appear to lie in the essential genes between *rpoH* and ORF4. In order to confirm this the Ts28 and Ts33 mutations should be shown to be linked to a marker in the 'silent' region. The most useful marker would be an antibiotic resistance determinant such as that carried by strain JGC127 (*zhg::Tn10*). An alternative strategy would be to map the temperature-sensitivities relative to the few natural markers that lie in the "silent" region. These markers include *pit* at 77 minutes (an inorganic phosphate transport gene, mutations in which give rise to arsenate resistance), *kdgK* at 78 minutes (ketodeoxygluconokinase, loss of which results in an inability grow on gluconate) and the *xyl* operon at 79 minutes, essential for growth on xylose. All three of these mapping procedures were attempted.

6.32 Mapping Ts28 and Ts33 relative to malA and zhf::Tn10

In order to confirm the location of the Tn10 which was used as the marker in the original localised mutagenesis, a preliminary mapping experiment was performed to map the Tn10 relative to ftsE and rpoH. T4GT7 lysates were prepared on the original strains carrying the SG20 (ftsE) and SG25 (rpoH) mutations. These were used to transduce POP2239 (malA), selecting for tetracycline resistance on MacConkey plates containing maltose. This was expected to give an immediate result for the cotransduction frequency of malA and the Tn10. However although there was some colour variation apparent in the colonies that grew on the MacConkey plates this was insufficient for the accurate classification of the transductants into Mal^+ and Mal^-. Therefore the transductants were patched onto NA plates in duplicate, one plate being incubated at the restrictive temperature and one at the permissive temperature to test for temperature-sensitivity, and POP2239 minimal medium with maltose as the sole carbon source to test for maltose utilisation. The cotransduction figures are given in Table 6.1. In both of the transductions only a low percentage of the Tet^R, Mal^+ transductants had co-inherited the temperature-sensitivity. This demonstrates that the temperature-sensitivity (ftsE or rpoH) lies outside the region between the Tn10 and malA. Similarly only a low percentage of the Tet^R, Ts^- transductants co-inherit maltose utilisation, showing that malA is external to the region from the Tn10 to ftsE/rpoH. This confirms the expected location of the zhf::Tn10 as being between malA and the 76 minute morphogenes.

A similar experiment was performed on SG28 and SG33 using T4GT7 lysates made on these strains to transduce POP2239, selecting for

Table 8.1 Cotransduction Frequencies for Tn10, malA, and ftsE or rpoH

T4GT7.SG20 (*zhf::Tn10*, *ftsE*, *malA*$^+$) and T4GT7.SG25 (*zhf::Tn10*, *rpoH*, *malA*$^+$) were used to transduce POP2239 (*malA*) selecting for tetracycline resistant (Tet^R) transductants. Transductants were tested by patching out on the apprpriate media for temperature-sensitivity (Ts$^-$) and utilisation of maltose (Mal$^+$).

Lysate (Number of transductants)	Phenotype	Cotransduction Frequency (marker and %)
T4GT7.SG20	Tet^R	Ts$^-$ 41
(300)	Tet^R	Mal$^+$ 65
	Tet^R, Mal$^+$	Ts$^-$ 38
	Tet^R, Ts$^-$	Mal$^+$ 60
T4GT7.SG25	Tet^R	Ts$^-$ 58
(400)	Tet^R	Mal$^+$ 79
	Tet^R, Mal+	Ts$^-$ 51
	Tet^R, Ts$^-$	Mal$^+$ 70

transductants on NA plus tetracycline and screening the transductants for temperature-sensitivity and maltose utilisation by patching out 300 transductants on the appropriate media. The cotransduction figures for this experiment are given in Table 6.2. These figures do not clearly define the order of the genes although they might be taken to suggest that the temperature-sensitivity is the included marker in both cases because of the high co-inheritance of temperature-sensitivity in the Tet^R, Mal^+ transductants. However in the transduction with the T4GT7.SG28 lysate maltose utilisation was also highly co-transduced with the other two markers making it difficult to realistically assign a gene order. In addition to this it was apparent in both transductions that the temperature-sensitivity when transduced into POP2239 was very difficult to accurately assess on patches, therefore the percentage cotransduction of the temperature-sensitivities could be wrong. In fact POP2239 (Ts28) was found to be so poorly temperature-sensitive that little further mapping was done on Ts28. For these reasons the transduction experiment was repeated with the T4GT7.SG33 lysate, 500 transductants being screened for temperature-sensitivity by streaking out on duplicate NA plates, one plate being incubated at the permissive temperature and one at the restrictive temperature. These figures are given in Table 6.2 and are broadly similar to the results when mapping the zbf::Tn10 relative to ftsE and rooH. The temperature-sensitivity again shows a low co-inheritance in TetR, Mal^+ transductants, and the maltose utilisation shows a low co-inheritance in Tet^R, Ts^- transductants, demonstrating that the zbf::Tn10 is the included marker. In other words these results show that Ts33 appears to map on the other side of the Tn10 to malA. As the

Table 6.2 Cotransduction Frequencies for Tn10, malA, and Ts28 & Ts33

T4GT7.SG28 (zhf::Tn10, malA$^+$, Ts28) and T4GT7.SG30 (zhf::Tn10, malA$^+$, Ts33) were used to transduce POP2238 (malA) selecting for tetracycline resistant (Tet^R) transductants. 300 colonies from each transduction were tested by patching out on the appropriate media for temperature-sensitivity (Ts$^-$) and utilisation of maltose (Mal$^+$). The transduction using the T4GT7.SG33 lysate was repeated and 500 of these transductants were tested by streaking out on the appropriate media for the same phenotypic markers.

Lysate (Number of transductants)	Phenotype	Cotransduction Frequency (marker and %)
T4GT7.SG28	Tet^R	Ts$^-$ 88
(300,patched)	Tet^R	Mal$^+$ 84
	Tet^R, Mal$^+$	Ts$^-$ 92
	Tet^R, Ts$^-$	Mal$^+$ 87
T4GT7.SG33	Tet^R	Ts$^-$ 78
(300,patched)	Tet^R	Mal$^+$ 57
	Tet^R, Mal+	Ts$^-$ 87
	Tet^R, Ts$^-$	Mal$^+$ 64
T4GT7.SG33	Tet^R	Ts$^-$ 25
(500,streaks)	Tet^R	Mal$^+$ 37
	Tet^R, Mal$^+$	Ts$^-$ 46
	Tet^R, Ts$^-$	Mal$^+$ 68

initial transductions showed that zhf::Tn10 lies clockwise of malA (between malA and the 76 minute morphogenes), this must mean that Ts33 lies clockwise of zhf::Tn10. There are no essential genes between the Tn10 and the 76 minute morphogenes, and Ts33 is not complemented by constructs carrying any of the known 76 minute morphogenes, therefore Ts33 must lie in the silent region clockwise of 76 minutes. To confirm this result it would be desirable to demonstrate linkage of the Ts33 mutation to a marker in the 'silent' region.

6.33 Construction of JGC127 ts^+ and fusaric acid curing of SG28 and SG33

JGC127 would seem to be a very good strain to use for the transductional mapping of Ts28 and Ts33 to the 'silent' region. This is because it has the following mutations either side of 76 minutes: rpsL (streptomycin resistance, at 73.5 minutes), malA (maltose utilisation, at 75.2 minutes), zhg::Tn10 (tetracycline resistance, at 76 to 77 minutes), and xyl (xylose utilisation, at 79.5 minutes). By using the phage T4GT7 (which transduces approximately 4 minutes of DNA) to transduce these markers from JGC127 into SG28 and SG33 the Ts28 and Ts33 mutations should be easily mapped. However there are two disadvantages with this strategy. Firstly Ts28 and Ts33 are already linked to zhf::Tn10 and thus SG28 and SG33 would need to be cured of Tn10 to make them tetracycline sensitive. Secondly JGC127 is a dnaM (an allele of rpoH) mutant and this temperature-sensitive lethal phenotype would have to be reverted so that Ts28 and Ts33 could be mapped relative to the Tn10. In order to revert the rpoH mutation JGC127 was initially simply plated out on NA plates which were incubated at the restrictive temperature. No revertants were isolated in this manner. The rpoH mutation was therefore reverted by

transducing in a wild-type rpoH allele. For this purpose a T4GT7 lysate was made on the strain JCB421 (relevant markers: cysG::Tn5 malA rpoH$^+$). This was used to transduce JGC127 selecting for transductants on NA plates with 50ug/ml kanamycin. 100 transductants were screened for growth at 42°C and 6 were found to be temperature-insensitive. These transductants carry the full range of useful markers between 74 and 79 minutes listed above plus cysG::Tn5 (Figure 6.2).

In order to take advantage of these strains attempts were made to cure SG28 and SG33 of their resident Tn10 by means of positive selection in the presence of fusaric acid and autoclaved chlortetracycline (Bochner et al., 1980). In the first attempt 100ul of 10^{-1}, 10^{-2}, and 10^{-3} dilutions of overnight cultures were spread on fusaric acid medium and the plates incubated at 30°C. Approximately 40 colonies of each strain grew up with a strong background on the plates spread with the 10^{-1} dilutions. These colonies were tested for temperature-sensitivity and tetracycline sensitivity. All the colonies isolated in this manner were found to be temperature-sensitive and tetracycline resistant. The isolation procedure was repeated using the standard fusaric acid medium, medium with twice the amount of autoclaved chlortetracycline, and medium with twice the standard amount of fusaric acid. The medium with twice the normal chlortetracycline concentration gave decreased background growth and 8 discrete colonies from each strain. These colonies were streaked out on the medium with double chlortetracycline then the resultant colonies tested for resistance to tetracycline and temperature-sensitivity. All the isolates were found to be tetracycline resistant and temperature-sensitive.

Maloy and Nunn (1981) suggested that the medium of Bochner et al was only really effective with slow growing strains of E. coli. They provided a recipe for fusaric acid medium with a decreased nutrient concentration that is more universally applicable. This medium was tried in parallel with a batch of the original fusaric acid medium. Colonies were isolated on both media. These colonies were tested for tetracycline sensitivity. All the colonies were found to still be tetracycline resistant.

A final attempt was made to generate a tetracycline sensitive SG33. The principle behind this was to transduce from SG33 into a malA mutant, selecting for maltose utilisation, then screen for transductants that were temperature-sensitive and tetracycline sensitive. If the temperature-sensitive mutation was clockwise of the Tn10 (ie the other side of the Tn10 to malA) this could be expected to be an extremely rare class of transductants. The original T4GT7 lysate used to transduce SG33 into a 'clean' OV2 background (Chapter 2) was used to transduce the strain POP2239 (relevant marker malA) selecting transductants on the appropriate minimal medium with maltose as the sole carbon source. 200 transductants were screened for temperature-sensitivity and resistance to tetracycline. 65 of the 200 transductants tested were found to be tetracycline resistant but all 200 appeared to be temperature-insensitive. This implied that finding a temperature-sensitive transductant that was also tetracycline sensitive would involve a very large screening program. Following these results alternative strategies were considered for mapping SG33 that did not necessitate curing the strain of zhf::Tn10.

6.34 Mapping Ts33 relative to pit, kdgK, and xyl

Strains with markers clockwise of the 78 minute region include K10 (pit-10; Willsky et al., 1973) and AB1157 (kdgK51, xyl-5; DeWitt and Adelberg 1962). Neither strain carries a temperature-sensitive lethal mutation or tetracycline resistance determinant, therefore transducing from these strains into SG33 and scoring cotransduction of markers should be unambiguous. pit-10 has been reported to give resistance to 20mM arsenate (Willsky et al., 1973). It would be convenient to have this marker in an AB1157 background so that there would be three markers that could be used in transductional mapping viz. kdgK51, xyl-5, and pit-10. To test whether the pit-10 marker can be used to select for transductants by means of arsenate resistance when mapping in OV2 based strains the following experiment was performed. NA plates were made with a range of arsenate concentrations (0, 20, 40, 60, 80, and 100mM). K10, AB1157 and OV2 were all streaked out on these media and the plates incubated at 37°C. Following overnight incubation the plates were examined for growth. AB1157 only grew well in the absence of arsenate. OV2 grew weakly in the presence of 20mM arsenate and progressively more weakly with increasing concentrations of arsenate. K10 grew well up to 40mM arsenate and then grew progressively more weakly (although more strongly than OV2) with increasing concentrations of arsenate. These results suggested that it would be possible to use arsenate resistance as a selection for transductants.

A T4GT7 lysate was prepared on K10. This was used to transduce AB1157, selecting for transductants on NA plus 20mM arsenate. Two of these transductants were tested for growth in the presence of arsenate

and growth on glucuronate and xylose. Both were found to be arsenate resistant, and to be unable to utilise glucuronate and xylose. Thus all three markers, pit, kdgK, and xyl, were in one strain. This strain was named AB1157 Pit$^-$.

A P1 transducing lysate was made on AB1157 Pit$^-$. This was used to transduce SG33 and the transduction mixture was plated out on NA plates containing 40mM arsenate. After 3 days incubation colonies were found on the arsenate plates. However when these colonies were patched out onto the appropriate media to test for temperature-sensitivity and glucuronic acid utilisation they were all found to be temperature-sensitive and capable of growing on glucuronic acid. There are two possible explanations for this. Firstly the colonies could be genuine transductants, and this would imply that pit and kdgK are further than 2 minutes away from the temperature-sensitive mutation in SG33. Secondly the colonies could simply be spontaneous arsenate resistant mutants of SG33. As a similar number of colonies was observed when the same inoculum of control (non-transduced) SG33 was plated out on arsenate plates, and given the previous transduction results which imply that the temperature-sensitivity in SG33 ought to map near pit, the colonies from the transduction were assumed to be spontaneous arsenate resistant SG33 mutants rather than transductants. This result demonstrated that it would not be possible to select for transductants in this genetic background (OV2) using arsenate resistance as a selectable marker.

The obvious solution to this problem appeared to be to transduce the Ts33 mutation into a background in which the pit-10 mutation is known to confer selectable or at least easily screened resistance to arsenate. The

natural choice for this was AB1157. This would mean that the SG33 temperature-sensitive mutation would be in an isogenic strain to one carrying the pit-10 mutation. Ts33 was transduced into AB1157 using a T4GT7 lysate and selecting for transductants on NA plates containing tetracycline. Transductants were screened for temperature-sensitivity and temperature-sensitive tetracycline resistant transductants (AB1157 Tn10 Ts) were retained. The genotype of these transductants was therefore as AB1157 but zbf::Tn10 and Ts33. This strain was tested for temperature-sensitivity and resistance to 20mM arsenate when streaked out in parallel with AB1157 and AB1157 Pit$^-$. The results are shown in Table 6.3 (growth is indicated by +, no growth by -). These results indicate that the growth of the strains is as expected. A T4GT7 transducing lysate was made on AB1157 Tn10 Ts. This was used to transduce AB1157 Pit$^-$, selecting for transductants on NA plates with tetracycline. After 2 days incubation many colonies were found growing on the plates. 200 of these were patched onto duplicate NA plates which were incubated at 30°C and 42°C and NA plates containing 20mM arsenate. Following overnight incubation there was some difficulty identifying with any certainty which patches were temperature-sensitive and which were arsenate resistant. To overcome this 200 transductants were streaked out on the same media. Following overnight incubation the transductants were scored for temperature-sensitivity and arsenate resistance. These results are presented in Table 6.4. Once again the scoring was difficult (although easier than with the patches) and the results are not all that clear-cut. There was an unexpectedly high cotransduction frequency of Tet^R and either Pit$^+$ or Ts$^-$. This may be due to the difficulty in scoring both

Table 6.3 Growth of AB1157 Derived Strains

Growth is indicated by '+', no growth by '-'

Strain	Media/Temperature		
	NA/30°C	NA/42°C	NA plus 20mM Arsenate/30°C
AB1157	+	+	-
AB1157 Plt$^-$	+	+	+
AB1157 Tn10 Ts	+	-	-

Table 6.4 Cotransduction Frequencies for Ts33, zbf::Tn10, and pit

T4GT7.AB1157 *zbf::Tn10* Ts33 was used to transduce AB1157 *pit*$^-$, selecting for tetracycline resistant (Tet^R) tranductants. 200 transductants were screened for temperature-sensitivity (Ts$^-$) and arsenate sensitivity (Pit$^+$) by streaking out on the appropriate media.

Phenotype	Cotransduction Frequency (marker and %)
Tet^R	Ts$^-$ 88
Tet^R	Pit$^+$ 89
Tet^R, Pit$^+$	Ts$^-$ 87
Tet^R, Ts$^-$	Pit$^+$ 88

temperature-sensitivity and arsenate resistance as neither phenotype was entirely 'clean'. Interpreted in the light of the cotransduction frequency with malA and zhf::Tn10 which showed Ts33 to lie on the pit side of this transposon one can only conclude that Ts33 and pit-10 must be close together as virtually all of the Pit^+ transductants were also Ts^- and vice versa.

6.35 Conclusions from the mapping of Ts33

SG33 carries a temperature-sensitive lethal mutation that is linked to zhf::Tn10. By implication this mutation lies in an essential gene, and mapping experiments have shown that it lies clockwise of zhf::Tn10. The only known essential genes that lie clockwise of this transposon are carried on λS3D which does not complement the mutation in SG33. Therefore the Ts33 mutation must lie in a new essential gene or genes that map in the virtually 'silent' region clockwise of ORF4. One marker that is in this region is pit. Attempts were made to map Ts33 relative to this marker and although the relative positions can not be assigned from this data it appears that Ts33 lies very near pit and is thus defines a new essential gene.

6.4 Cloning the New Essential Gene

6.41 Shotgun Cloning

Initial attempts to clone SG33 complementing DNA involved digesting OV2 chromosomal DNA (partially, or to completion) with HindIII, EcoRI, PstI or KpnI. The resultant fragments were ligated into pBR325 that was digested with the corresponding enzyme and treated with calf intestinal

phosphatase, with the exception of the KpnI digested DNA which was ligated into KpnI cut and phosphatase treated pJRD184 due to the absence of KpnI sites in pBR325. The ligation mixes were used to transform SG33, aliquots of which were then plated out at 30°C and 42°C. The enzymes chosen were used because, according to the map of Kohara et al. (1987), they should produce suitable sized fragments clockwise of where the 4.5 kb HindIII fragment maps.

No colonies were isolated at 42°C with the HindIII cloning. Large numbers of colonies were however isolated at 30°C. 300 were patched out on duplicate NA plates that were incubated at 30°C and 42°C and one patch was found that grew reasonably well at 42°C. Viable counts were performed on this isolate at 30°C and 42°C. The viability of this isolate at 42°C was approximately 0.1% of that at 30°C as compared to the plasmid-less mutant which has a viability at 42°C <0.001% of that at 30°C. This suggested that part of the gene had been cloned and, as this was from a library that was made with partially digested chromosomal DNA other clones might contain the whole gene. Therefore all the remaining colonies from the transformation were patched out at 30°C and 42°C and another 4 temperature-insensitive isolates were found. These were streaked out in duplicate on NA and the plates incubated at 30°C and 42°C. The isolates were found to be temperature-insensitive. DNA was "miniprepped" from these and they were also re-streaked out at 30°C and 42°C. These re-streaks were found to only grow poorly at 42°C. The miniprep DNA was used to re-transform SG33 and 20 colonies from each transformation were patched out in duplicate at 30°C and 42°C. These transformants also grew very poorly at 42°C. The weakness of growth at

42°C and the apparent instability of the clones suggested that the whole complementing gene had not been isolated or that trans-complementation was poor with these clones in this mutant. The possibility that the weak complementation was due to "marker rescue" was thought to be unlikely as the initial transformants did not maintain their temperature-insensitivity upon re-streaking, although this possibility could have been more fully addressed by the use of a recA derivative of SG33 as recipient in the transformations.

This led to the construction of three more shotgun libraries using the enzymes EcoRI, KpnI, and PstI. These were used to transform competent SG33 and cloning of trans-complementing DNA was selected for by incubating the selection plates at 42°C. 7 colonies were isolated at 42°C in the KpnI cloning, but no colonies were isolated in the other cloning experiments. The colonies from the KpnI cloning were streaked out to test for temperature-sensitivity and only 3 of the 7 isolates exhibited temperature-insensitive growth. On re-streaking, all 7 of the isolates appeared temperature-sensitive. DNA minipreps were performed on the three isolates that had originally appeared temperature-insensitive. This DNA was used to transform SG33 and 50 colonies from each transformation were tested for temperature-sensitivity by patching. Only one of the 3 clones gave growth at 42°C. Four of these transformants were tested for temperature-sensitivity by streaking out. They were all found to grow weakly at 42°C. Under the microscope the cells grown at 42°C were found to be pleomorphic, consisting of normal shaped rods, some plump rods and some short filaments. Because only poorly

complementing clones were isolated using plasmid based shotgun libraries a cosmid library was constructed using OV2 DNA.

6.42 Screening the Cosmid Library

The DNA preparation from the pooled OV2 cosmid library (Chapter 2) was used to transform competent SG33. Selection for transformants was on NA plates containing ampicillin that were incubated at 30°C and 42°C. However no colonies grew on these plates. This was thought to be due to inefficient transformation of the large cosmids.

Fortunately some of the *in vitro* packaged DNA had been retained and as this had given high levels of transductants with DH1 it seemed worth trying to directly isolate SG33 complementing cosmids from this 'lysate'. Consequently SG33 was transduced with the *in vitro* packaged DNA and plated out on 5 NA plates with ampicillin. One of these plates was incubated at 30°C and four at 42°C. More than one thousand colonies grew on the 30°C plate and approximately 40 colonies grew on each of the 42°C plates. 12 of the colonies isolated at 42°C were tested for temperature-sensitivity when streaked out. All 12 were temperature-insensitive suggesting that the cosmids in these isolates carried DNA that complemented the Ts33 mutation. The cosmids carried by these transductants were named cTOM1 to cTOM12.

6.43 Checking the Cosmid Complementation

Cosmid DNA was "miniprepped" from colonies carrying cTOM1 to cTOM12. No cosmid DNA was isolated from strains carrying cTOM7 and cTOM8. The successful cosmid minipreps were used to transform SG33. Transformants were isolated on NA plates containing ampicillin that were

incubated at 30°C. 10 colonies from each of the 10 transformations were patched out on NA plates in duplicate that were incubated at 30°C and 42°C to test for temperature-sensitivity. All of these patches grew at 42°C. This confirmed that the cosmids isolated really do complement Ts33.

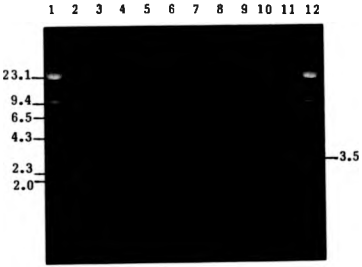
The cosmid minipreps were also used to transform SG28 to see if there was any complementation. This was tested in exactly the same way as for SG33 (above) and gave exactly the same results. That is, all 10 cosmids that complemented Ts33 also complemented Ts28, which would suggest that the mutation in SG28 is near to, or possibly in the same gene as, that in SG33. The same process was repeated for SG1 (ftsE) in order to see whether any of the cosmids bridged the gap between ftsE and Ts33. However none of the cosmids that complemented SG33 complemented SG1.

6.44 Subcloning fragments from the complementing cosmids

The miniprep DNA of cosmids cTOM1 to cTOM8 and cTOM9 to cTOM12 was digested with EcoRI. The pattern of restriction fragments of the 10 minipreps when digested with EcoRI showed that all 10 cosmids had a common 3.5kb band (Figure 6.3). There was also a 1.7kb fragment in all but one of the cosmids (cTOM5) and a 5.2kb fragment in all but cTOM3. It would seem logical that the 3.5kb fragment carries the Ts33 complementing DNA as it is common to all the cosmids. Therefore the 3.5kb EcoRI fragment was subcloned from each of the 10 cosmid minipreps and tested for complementation of the SG33 temperature-sensitive phenotype.

Figure 6.3 EcoRI Digestion of SG33 Complementing Cosmids

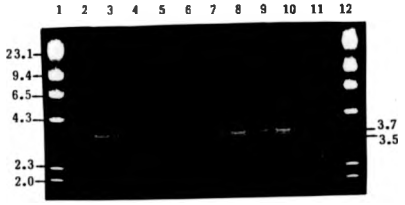
Lanes 1 & 12, HindIII molecular weight markers (sizes in kb). Lane 2, cTOM1; 3, cTOM2; 4, cTOM3; 5, cTOM4; 6, cTOM5; 7, cTOM6; 8, cTOM9; 9, cTOM10; 10, cTOM11.



The 3.5kb EcoRI fragment was prepared from all 10 miniprepped cosmids and ligated with EcoRI cut and phosphatase treated pDMW140. This vector has a polylinker, insertions in which interrupt lacZ expression giving blue/white screening for inserts on media containing X-Gal and IPTG. It also has an F1 origin allowing for the recovery of single stranded DNA template upon superinfection of the strain carrying the plasmid with a helper phage. The ligation mixes were used to transform TG1 and the transformations plated out on X-Gal ampicillin plates. Approximately one hundred white colonies were isolated in each case, including the control where the vector was simply religated. Presumably this was due to the religation of damaged vector. In order to check that the white colonies from the subcloning transformations carried the desired insert, plasmid DNA from 6 white colonies each from two of the subclonings was "miniprepped" and restricted with EcoRI. One of these minipreps did not cut with this enzyme but the other 11 digestions all exhibited a 3.7kb vector and 3.5kb insert band when run on an agarose gel (Figure 8.4). Following this result 2 white colonies from each of the other 8 subclonings were used to set up minipreps. These were restricted with EcoRI and they were all found to contain the 3.5kb insert. These 10 subclone plasmids were named pTWG1 to pTWG6 and pTWG9 to pTWG12 corresponding to the relevant cTOM cosmids from which they were subcloned. One of each of these pTWG series plasmids was used to transform SG33, selecting for transformants on ampicillin containing plates at 30°C. Only a few colonies were isolated in each case. Four colonies from each transformation where available were tested for temperature-sensitivity by streaking out. All the transformants were found to be still

Figure 6.4 EcoRI Digestion of pTWG6 and pTWG11 'Minipreps'

Lanes 1 and 14 HindIII molecular weight markers (sizes in kb). Lanes 2 to 7 EcoRI digestion of pTWG6. Lanes 8 to 13 EcoRI digestion of pTWG11. 3.5kb insert is resolved from 3.7kb vector band.



temperature-sensitive. Thus the subcloned 3.5kb EcoRI fragment does not complement the temperature-sensitive mutation in SG33.

6.5 Discussion

One of the original purposes of performing a localised mutagenesis on the 76 minute region (Chapter 3) was to see if any mutations could be isolated that mapped in previously undiscovered essential genes in this region of the E. coli chromosome. The present chapter demonstrates that this aim has been fulfilled. The transductional mapping data show that the temperature-sensitive lethal mutation in strain SG33 lies clockwise of the 76 minute cell division operon in a largely silent region of the chromosome. The mutation has been shown to be close to the pit locus although the actual order of the genes could not be determined. This is largely due to the nature of the small number of markers available. The latest edition of the E. coli linkage map (Bachmann, 1990), despite showing several new loci in the 77 minute region does not reveal any obvious candidate loci for Ts28 and Ts33.

The function of the locus that is mutated in SG33 is still unclear. The strain is clearly not a growth rate mutant. It is not a cell division mutant either (in the rigorous sense of failing to divide at the restrictive temperature). The morphology under restrictive conditions (pleomorphism, large numbers of swollen rods and spheres) suggests that the locus may code for a protein involved in cell wall synthesis or control of cell shape. However the susceptibility of SG33 to low NaCl levels (and low osmolarity in general?) implies more of a membrane or permeability/transport function. There is obviously scope for more

experimentation on the physiology of SG33. SG28 resembles SG33 under the microscope and is complemented by all of the SG33 complementing cosmids. This suggests that the two strains may carry mutations in the same gene. Further physiological assessment of SG28 could give more evidence for this possibility.

Less equivocal would be to subclone a small SG33 complementing fragment from the cTOM cosmid series and see if this also complemented SG28. It remains a matter of conjecture as to why the common 3.5kb EcoRI fragment did not complement SG33 when subcloned. One possibility is that the vector's lacZ promoter was counter-driving or over-expressing the putative subcloned gene. However as the fragment had identical ends and there were ten subclones it seems unlikely that all would contain the same orientation of insert, therefore if read-through from a promoter in the vector was causing non-complementation one would not expect all the subclones to exhibit this. Alternatively the lack of complementation could simply be an effect of the increased copy number of pJRD184 compared with that of the cosmid. Another alternative is that the complementing gene could contain an EcoRI site so that the gene straddles two EcoRI fragments. The reason for not isolating two EcoRI fragments common to all ten cosmids being that in one or more of the cosmids one of the two common EcoRI fragments was at the extreme end of the cosmid insert, hence had been reduced in size by digestion with Sau3A, therefore this fragment would be common to 9 or less of the cosmids. This is perhaps the most likely explanation and it is easy to imagine variations on this theme such as there being a repressor or activator carried on the hypothetical second common EcoRI fragment. The only evidence that

suggests that the cosmids do not carry complementing DNA is the fact that the sizes of the EcoRI fragments from the cosmids do not correlate well with the physical map of the E. coli chromosome for the region around 77 minutes (Kohara et al., 1987). It is therefore possible that the cosmids actually give "marker rescue" (which could be tested using a recA derivative of SG33), or carry a gene that gives a suppression effect when in multicopy in trans, rather than giving true complementation. However the SG33 transformants grew very strongly at what had been their restrictive temperature which makes these possibilities less likely.

Having a range of complementing cosmids is very useful and several experiments immediately suggest themselves. Firstly it would be sensible to try to subclone the Ts33 complementing DNA. This could be done for example by subcloning using a restriction enzyme other than EcoRI to digest the cosmids and cloning any common fragments or by subcloning from an EcoRI partial digest of the cosmids. Alternatively one could randomly subclone from the cosmids selecting for cloning of the complementing DNA by plating out SG33 transductants at 42°C. If complementing subclones could be isolated restriction mapping of these might show more clearly where Ts33 lies on the Kohara physical map. Another useful experiment would be to test if any of the cosmids complement the pit-10 mutation. If complementation was found it would support the hypothesis that Ts33 lies near pit.

To summarise, a temperature-sensitive mutant has been isolated carrying a mutation that has been mapped to a hitherto silent region of the E. coli chromosome. This mutation is lethal at the non-permissive

temperature, hence is thought to lie in an essential gene. The mutation also confers sensitivity to low salt concentrations at the permissive temperature and high salt concentrations have no effect at the restrictive temperature. A range of cosmids has been isolated that complement this mutation and an attempt was made to subclone complementing DNA from these.

Chapter 7: DISCUSSION

7.1 Summary of Results

It has been thought for some time that the 76 minute region may represent a large morphogene cluster. The finding that the ftsYEX operon and rpoH are contiguous has given encouragement to this speculation. The extended homology of the FtsE protein with the superfamily of ABC proteins has created extra interest in this gene product in particular and the possibility of ascribing a function to it. This study of the 76 minute region has given the results below:

1) A localised mutagenesis procedure was performed on the 76 minute region and a screen for temperature-sensitive growth yielded 28 temperature-sensitive mutants. There were three classes of mutants based on their microscopic appearance at the restrictive temperature, that is filamentous, rpoH-like (having short filaments with inclusions), and pleomorphic.

2) The mutations carried by these mutants were mapped by complementation with pH3C and the pH3C deletion series: pR2; pDBS9; and λS3D. Of the mutations giving rise to a filamentous phenotype, nine mutations were found to map in ftsE, five in ftsX, two appeared to carry mutations in both ftsE and ftsX, and two appeared to be complemented but were not fully mapped. Of the mutations giving rise to an rpoH-like phenotype all nine mapped in rpoH. The remaining two mutations that gave a pleomorphic phenotype were not complemented by any of these

constructs. Use of a temperature-stable suppressor by construction of λsupF lysogens enabled the mutants to be further characterised as having missense or amber mutations. No temperature-sensitive lethal mutations were isolated in ftsY or ORF4.

3) The ftsE missense mutants were analysed phenotypically and were not easily classified into groups on the basis of temperature-sensitivity, microscopic appearance, salt reversibility of phenotype, or the requirement for de novo protein synthesis for recovery of septation following a period of incubation at the restrictive temperature.

4) The ftsE mutant alleles were cloned and the DNA sequenced. This showed the mutations to be clustered in a region of extensive homology with the ABC superfamily of proteins.

5) Nucleotide binding of FtsE and FtsY proteins was investigated using radio-labelled proteins from maxicells and dye-ligand chromatography columns. The results were very ambiguous due to the low amounts of the labelled proteins in the maxicell lysates but they are suggestive of a possible interaction between ATP and these proteins.

6) One of the mutations (Ts33) that was not complemented by any of the available constructs carrying DNA from the 76 minute region was mapped by transduction to the 'silent' region clockwise of 76 minutes, and appeared to lie very close to pit. It is therefore thought to define a new essential gene.

7) A cosmid library was constructed and ten cosmids were isolated that contained Ts33 complementary DNA. The one common EcoRI fragment from these cosmids was subcloned into pDMW40 but these subclones were

found not to complement the Ts33 mutation. The Ts33 complementary cosmids were found to also complement Ts28, the other mutation that gave rise to a pleomorphic appearance at the restrictive temperature.

7.2 Possible Significance of the Results

Because of the range of mutations mapping to the 76 minute region there was originally thought to be a large number of morphogenes located there. A large 76 minute morphogene cluster however has not been identified. From the findings of workers Gill and Crickmore described in Chapter 1, all of the mutations that have morphogene-like effects that were available at that time map in only three genes, ftsE, ftsX, and rpoH. The extensive mutagenesis and screening program described in Chapter 3 resulted in the isolation of 26 mutants with mutations in one or more of these genes. However only two mutants were isolated that carried mutations that appeared to map in a new essential gene or genes, and these did not appear to map very close to the ftsYEX operon. It therefore appears unlikely that there are a lot more essential genes to be found at 76 minutes that are susceptible to temperature-sensitive lethal mutations. However, the fact that this was the screening criterion employed, probably means that phenotypically more subtle mutations were missed. This may explain the lack of isolation of ftsY mutations, even though the work of Gill and Salmond (1990) described in Chapter 3, Section 3.4 strongly suggests that ftsY is essential. The proximity of ftsY to ftsE and ftsX, and the presence of a promoter for ftsE and ftsX within the coding sequence of ftsY imply that the expression of these

three genes is inter-related, which also suggests a morphogene-like role for *ftsY*.

The clustering of mutations seen in the *ftsE* mutant alleles appears to be of some importance. The fact that they map in regions of homology (at the protein level) with the other members of the ABC family of nucleotide-binding proteins, and that the CFTR mutations isolated by Cutting *et al.* (1990) also map in the same region, is an exciting discovery and suggests that these regions have a functional significance. This work reinforces the idea that FtsE is likely to be nucleotide binding and hydrolysing, and it appears that this is an important avenue of research. Preliminary work described in Chapter 5 is encouraging, although a different approach is probably necessary in order to adequately address this problem.

Even if FtsE is found to hydrolyse ATP, the cellular function that this would be coupled to is not immediately apparent, although it seems safe to assume that loss of this activity or coupling would result in a cell division phenotype. The FtsE homology encourages speculation that the *ftsY ftsE ftsX* operon may be involved in a transport process. The homology between FtsY protein and docking protein ([illegible] *et al.*, 1989) may suggest that there is a second (*secA* independent) secretory pathway in *E. coli* in which these Fts proteins are involved. In the light of this it is interesting to note that *secA* lies in the 2 minute morphogene cluster, perhaps implying that protein secretory pathways are intimately involved with morphogenesis.

The continuing isolation of mutants carrying mutations defining hitherto unmapped morphogenes, such as Ts33, suggests that there are still more

genes remaining to be discovered whose protein products, directly or indirectly, affect the cell division process and control of morphology.

Because cell division is the ultimate product of all of a cell's metabolic activity, we should not be surprised to find that the control and execution of this process is very complex. However it is only by the systematic isolation of the genes or proteins involved and the co-ordination of genetic, biochemical, and physiological research on them that we can hope to finally put together a picture of this most fundamental of processes.

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