SFIB AND THE CONTROL OF CELL DIVISION IN E.COLI

Thesis submitted for the degree of Doctor of Philosophy at the University of Leicester

by

Christopher Andrew Jones B.Sc. (Bristol)

Department of Genetics, University of Leicester

December 1984
DEDICATION

To all my family and friends.........especially Sclub.
Firstly I'd like to thank Barry Holland for his "interesting" style of supervision. Secondly I'd like to thank all the members of labs 145 and 136, past and present, in particular: Julie Pratt, Maria Jackson and Neil Stoker (for their patient supervision), Nigel (for being a mate) and Deborah Gill (for help and friendship). I am very grateful to everyone inside and outside of Leicester for providing strains and many helpful discussions. Finally, I'd also like to thank everyone in the Genetics department who has helped in the preparation of this thesis.

I'd also like to acknowledge the support of all my friends and drinking companions who have made these three years (and a bit!) so enjoyable; "the lads", Alister, Randa, all my listening friends (esp. Dinah, John(s), Carrie, Gaynor, Adrian), Ian, John..............

I acknowledge the financial assistance of the Science and Engineering Research Council and the Dept. of Health and Social Security.
### ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>APS</td>
<td>ammonium persulphate</td>
</tr>
<tr>
<td>bp</td>
<td>base pairs</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumen</td>
</tr>
<tr>
<td>Ci</td>
<td>Curies</td>
</tr>
<tr>
<td>DMSO</td>
<td>diemethyl sulphoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>DTT</td>
<td>dithiothreitol</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetra-acetic acid</td>
</tr>
<tr>
<td>h</td>
<td>hours</td>
</tr>
<tr>
<td>kb</td>
<td>kilobases</td>
</tr>
<tr>
<td>kD</td>
<td>kilodaltons</td>
</tr>
<tr>
<td>min</td>
<td>minutes</td>
</tr>
<tr>
<td>PEG</td>
<td>polyethylene glycol</td>
</tr>
<tr>
<td>PPO</td>
<td>2,5-diphenyloxazone</td>
</tr>
<tr>
<td>rpm</td>
<td>revolutions per minute</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulphate</td>
</tr>
<tr>
<td>TCA</td>
<td>trichloroacetic acid</td>
</tr>
<tr>
<td>Tris</td>
<td>tris(hydroxymethyl) aminomethane</td>
</tr>
<tr>
<td>TEMED</td>
<td>N,N,N',N' tetramethyl ethylenediamine</td>
</tr>
</tbody>
</table>

### Genetic nomenclature

- **recA** gene
- **RecA** protein
- **RecA** phenotype
CONTENTS

CHAPTER ONE:  Introduction  1

CHAPTER TWO:  Methods and Materials  30

CHAPTER THREE:  Investigations into the location of sfiB  47

CHAPTER FOUR:  Cloning of sfiB114 and sfiB\(^+\) genes from chromosomal DNA  54

CHAPTER FIVE:  Characterisation and sub-cloning of plasmids pLG550 and pLG551  62

CHAPTER SIX:  Mapping of sfiB114 by transpositional mutagenesis  69

CHAPTER SEVENTH:  Analysis of polypeptides produced by pLG552 and pLG552::Tn1000  76

CHAPTER EIGHT:  The design of a model system for studying sfiA–sfiB interactions  82

CHAPTER NINE:  Interactions between plasmids pLG558 and pLG552/pLG554 in maxi-cells  92

CHAPTER TEN:  Studies on the cellular location of SfiA and SfiB  100

CHAPTER ELEVEN:  Discussion  104

REFERENCES  112
CHAPTER 1

Introduction

In the study of cell division in *Escherichia coli* four principle approaches have been used.

1) An analysis of the relationships between different physiological parameters over the cell cycle has led to the formulation of a mathematical description of cell surface growth, DNA replication and cell division in the cell cycle.

2) Studies on the biosynthesis of the peptidoglycan layer of the cell envelope have been used in an attempt to establish the enzymology of cell length extension and the specific events involved in cell division.

3) The genetic analysis of cell division has involved the isolation of mutants defective in the division process.

4) The transient division inhibition seen on treating *E. coli* with DNA damaging agents has uncovered at least one specific mechanism for the control of cell division in *E. coli*.

It is this fourth approach that is the major concern of this investigation.

Although these approaches will be discussed separately, their development was largely concurrent and so the compartmentalisation presented here simply reflects the historical interests of various research groups. A critical discussion of all these studies is clearly beyond the scope of this chapter and several reviews have analysed the data in depth (Pritchard, 1974.; Daneo-Moore and Shockman, 1977.; Helmstetter et al., 1979.; Donachie, 1981.; Nanninga et al.,
I Coupling of division and DNA replication

1.1 The E.coli cell cycle

The cell cycle of *E.coli* growing with a generation time $T$, can normally be resolved into two distinct periods, "C" the time taken to replicate the chromosome, and "D" the period between the termination of DNA replication and cell division (Cooper and Helmstetter, 1968).

Maaloe and Kjeldgaard (1966) first suggested that the time taken to replicate the chromosome (C) was constant, regardless of growth rate. Maaloe and Kjeldgaard (1966) also integrated the then recent discovery of dichotomous replication by Oishi *et al.* (1964) to propose that at high growth rates successive rounds of replication overlap. Helmstetter and Cooper (1968) and Cooper and Helmstetter (1968) developed the model of Maaloe and Kjeldgaard (1966) and introduced the terms C and D. They also attempted to quantify them, assigning values of approximately 40 and 20 min respectively, and confirmed their constancy over different growth rates. Churchward and Bremer (1977) combined a review of previous work and original data to conclude that C is not in fact invariable and that it decreases continuously with growth rate such that it approaches a constant value (approximately 37 min) at high growth rates. Where $C + D$ there is a period ("B") between cell division and initiation of DNA replication (Helmstetter and Pieurucci, 1976) and thus the B, C and D periods have been suggested to correspond to the $G_1$, $S$ and $G_2$ periods observed in the cycles of eukaryotic
1.2 Theoretical models for cell division
The observed relationships between growth rate, DNA replication and division have been the basis of a mathematical description of the cell cycle (Donachie, 1968; Pritchard et al., 1969) later reviewed by Pritchard (1974). The mathematical studies were based on the initiation of DNA replication as the crucial event in the bacterial cell cycle. Donachie (1968) suggested that initiation occurred at a constant cell mass and depended upon the synthesis of a new protein(s) required for initiation each cycle. It was also suggested that initiation could be controlled by the inhibitory action of a repressor, (acting either directly on initiation or by inhibiting the action of an initiator protein) which is diluted out by growth (Pritchard et al., 1969). Pritchard et al. proposed this inhibitor could either be stable and synthesised only at initiation or unstable and synthesised throughout the cell cycle. The requirement of the model of Pritchard et al. (1969) that an initiator protein must comprise a constant proportion of cellular protein led Sompeyrac and Maaloe (1973) to postulate an autorepressor model of DNA replication control whereby an initiator protein is part of an operon controlled by an autorepressor.

1.3 The coupling of cell division and DNA replication
Two general models were put forward to explain how cell division is timed with respect to DNA replication. Firstly, it has been proposed that the termination of DNA replication
provides a signal for cell division, which occurs $D$ minutes later (Clark, 1968; Helmstetter and Pierucci, 1968). Jones and Donachie (1973) suggested a second method of coordinating DNA replication with cell division. They suggested that termination of DNA replication triggered the synthesis of a protein that promoted division only when the independent requirement for a period of protein synthesis concurrent with DNA replication was completed. In order to distinguish between these two models, Meacock and Pritchard (1975) used thymine limitation of a thymine requiring strain of *E. coli* B/r to artificially increase the length of C. Meacock and Pritchard (1975) showed that as C increases, $D$ decreases, which is in agreement with the second model. However in experiments studying the kinetics of cell division on transferring cultures from media of one thymine concentration to another they also showed that cell division is controlled by a late event in the cell cycle, which is in agreement with the former model.

Work by Koch (1977) on the coefficient of variation of the size of cells at initiation and cell division failed to show any significant difference that might indicate a specific point in the cell cycle from which the cell division signal originates. For example, if the distribution of the sizes of cells is greater at initiation than at division it is difficult to explain how initiation can regulate cell division. Donachie *et al.* (1976) proposed a minimum 'unit cell' size from measurements of cell length under different growth conditions, suggesting that division was triggered by the achievement of a certain cell length. This further approach to the study of DNA replication-coupling i.e. that
of using light and electron microscopy to examine quantitatively the dynamics of cell length and cell diameter through the cell cycle using both synchronised and unsynchronised cells has been reviewed by Nanninga et al., (1982). Unfortunately, results obtained from these studies are frequently contradictory and their significance is difficult to assess at this stage.

Experiments relating to the ideas described above have helped greatly to stimulate interest in bacterial cell division nevertheless, the results have been disappointing and no universally accepted model has been put forward for division control in E.coli.

There are two major criticisms of much of the early work on the relationship between DNA synthesis and division. Firstly, these experiments involved a perturbation of the normal DNA replication period and it was assumed that this would not affect division in any 'active' way. On the contrary we now know that the SOS response to DNA damage includes at least one specific link between replication and cell division i.e. the induced synthesis of an inhibitor of division. The contribution of this effect to early experiments is not completely clear. The second criticism is that many of the early experiments on E.coli cell division were performed using E.coli B/r strains, now beleived to carry 2 mutations affecting division control (particularly that affected by DNA damage) and again the precise effects of these mutations on the results of early experiments is unclear.
II Cell shape and the septation process

1.4 The structure and function of peptidoglycan

The component of the *E. coli* cell wall chiefly responsible for maintaining the rod shape of *E. coli* is the peptidoglycan layer, between the inner and outer membranes (Fig. 1.1). Bacteria lacking an intact peptidoglycan layer lose their cell shape and lyse in media of normal osmotic strength. However, the role played by other components of the cell wall is unclear. For example, cells lacking both lipoprotein and protein OmpA (protein II*) from the outer membrane grow as osmotically stable spheres (Sonntag et al., 1978).

The peptidoglycan layer or cell sacculus consists of alternating residues of N-acetyl glucosamine (GlcNAc) and N-acetyl muramic acid (MurNAc). Pentapeptide side chains are attached to each muramic acid residue and covalent bonds between side chains from different glycan strands form a net like structure (Fig. 1.2). 10% of peptide side chains are covalently attached to lipoprotein in the outer membrane (Wensink et al., 1982) further suggesting the importance of lipoprotein in determining the shape and strength of the peptidoglycan layer.

1.5 Peptidoglycan and cell wall assembly

Studies on the biosynthesis and assembly of peptidoglycan in *Streptococcus* have identified a system of growth zones which result in the introduction of more polymers into the peptidoglycan layer (Daneo-Moore and Shockman, 1977). The activity of such zones appears to provide the basis for successive cycles of cell wall synthesis and septum formation. Attempts to identify such
Fig. 1.1
Structure of the *E. coli* cell envelope (redrawn with modifications by M. Jackson from Lugtenberg and van Alphen, 1983)

In *E. coli* K12 strains the O antigens chains of LPS are absent

- **A** - OmpA protein
- **BP** - periplasmic binding protein
- **IM** - inner membrane
- **IMP** - inner membrane protein
- **L** - lipoprotein
- **Lip. A** - lipid A
- **O Ag** - O antigen
- **OM** - outer membrane
- **PG** - peptidoglycan
- **PMP** - periplasmic protein
- **PMS** - periplasmic space
- **PP** - pore forming protein trimer
Figure 1.2
The structure of part of the peptidoglycan of *E. coli*.

MurNac - N-acetyl muramic acid
GlcNac - N-acetyl glucosamine
L-ala - L-alanine
D-glu - D-glutamate
m-dap - meso-diaminopimelate
D-ala - D-alanine

Taken from Stoker (1983).
zones in *E. coli* (Ryter et al., 1973, 1975; Begg and Donachie, 1973, 1977) by studying the incorporation of either radioactive peptidoglycan precursors or new bacteriophage receptors have shown that peptidoglycan growth is more complex than in the spherical *Streptococcus* and Nanninga et al. (1982) for example, concluded that it is not possible to distinguish between zonal and diffuse growth in *E. coli*.

The biosynthesis of the sacculus is complex and has been reviewed by Daneo-Moore and Shockman (1977), Tipper and Wright (1979), Wright and Tipper (1979), Inouye (1979), Rogers et al. (1980) and Mirelman (1981).

1.6 The action of penicillins on peptidoglycan assembly and cell division

The major source of information on the role of peptidoglycan synthesis in cell division has come from the identification of the targets of action of penicillins. Although transpeptidation was originally thought to be the target step for penicillin, Izaki et al. (1966) showed that two other activities (D-alanine carboxypeptidase and endopeptidase) were also penicillin sensitive (Fig 1.3). Seven penicillin binding proteins (PBP's) have been identified (Spratt, 1977a) (Table 1.1). The use of penicillins with specific binding properties has been most informative.

The penicillin mecillinam binds specifically to PBP2 (the product of the pbpA gene) and causes the production of spherical cells (Spratt, 1977a). Temperature sensitive mutations in the pbpA gene have been isolated (Spratt,
<table>
<thead>
<tr>
<th>PBP</th>
<th>MW</th>
<th>Molecules per cell</th>
<th>Enzymic activity</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>IA</td>
<td>92,000</td>
<td>170</td>
<td>Transpeptidase/Transglycosylase</td>
<td>Elongation</td>
</tr>
<tr>
<td>IB</td>
<td>90,000</td>
<td>60</td>
<td>Transpeptidase/Transglycosylase</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>66,000</td>
<td>20</td>
<td>Transpeptidase</td>
<td>Cell shape</td>
</tr>
<tr>
<td>3</td>
<td>60,000</td>
<td>50</td>
<td>Transpeptidase/Transglycosylase</td>
<td>Cell division</td>
</tr>
<tr>
<td>4</td>
<td>49,000</td>
<td>110</td>
<td>DD-carboxypeptidase/DD-endopeptidase</td>
<td>?</td>
</tr>
<tr>
<td>5</td>
<td>42,000</td>
<td>1800</td>
<td>DD-carboxypeptidase/model transpeptidase</td>
<td>?</td>
</tr>
<tr>
<td>6</td>
<td>40,000</td>
<td>570</td>
<td>DD-carboxypeptidase/model transpeptidase</td>
<td>?</td>
</tr>
</tbody>
</table>
Figure 1.3

A diagram illustrating the action of transpeptidase, DD-carboxypeptidase and endopeptidase.

NAM - N-acetyl muramic acid

NAG - N-acetyl glucosamine

L-ala - L-alanine

D-glu - D-glutamate

m-dap - meso-diaminopimelate

D-ala - D-alanine

Taken from Stoker (1983).
1977a) and strains containing this mutation form spheres at the restrictive temperature and therefore the product of the pbpA gene has been assumed to be involved in elongation of the cell wall. In agreement with this, minicells were reported to be deficient in PBP2 compared to normal cells (Buchanan, 1981).

A different set of antibiotics (e.g. cephalaxin and furazlocillin) bind to PBP3 and block cell division. A temperature sensitive mutation which causes filamentation and failure to bind $^{14}$C-benzyl penicillin to PBP3 at the restrictive temperature was described by Spratt (1975). PBP3 was therefore assigned to be directly involved in peptidoglycan synthesis associated with septum formation (Botha and Park, 1981). Enzymic activities of PBP2 and PBP3 have been investigated and Ishino et al. (1982) showed that PBP2 probably has transpeptidase activity and Ishino and Matsukeshi (1981) claimed that purified PBP3 catalyses both transpeptidase and transglycosylase reactions. Both of these conclusions however, were based on very low activities measured in-vitro. Markiewicz et al. (1982) showed that an increase in the level of PBP5 caused the formation of spherical cells and interpreted this as showing that PBP5 is involved in the switching of peptidoglycan synthesis between cell elongation and cell division.

Canepari et al. (1984) measured the effects of adding mecillinam to three E. coli strains having conditional cell division defects (BUG6, ftsA or pbp3) (see section III) growing at the restrictive temperature (42°C). Mecillinam stimulated cell division in two of the mutants studied (BUG6 and pbp3) but not ftsA. Canepari et al. interpreted this as
showing that during the growth of BUG6 and pbp3 strains at 42°C although septum formation is blocked, the sites for septation are not destroyed but are unable to compete with the sites for lateral wall extention. Addition of mecillinam then inactivates PBP2 and thus lateral wall elongation allowing cell division sites to compete more effectively. These results agree well with the idea that division involves a change in the balance between the incorporation of peptidoglycan into the septum and lateral wall elongation (Vicente, 1984).

1.7 Peptidoglycan synthesis and the cell cycle
Mirelman et al. (1976) measured peptidoglycan synthesis in cultures of E.coli strain PAT84 (ftsZ) at 30°C and 42°C and showed that at 42°C (when cell division was blocked) both the transpeptidase/carboxypeptidase ratio and the degree of cross linking were increased (relative to growth at 30°C). Mirelman et al. (1977) later showed that these effects were also produced by blocking cell division either by treatment with nalidixic acid or cephalxin, or growing a pbp3 mutant at the restrictive temperature. In later work, Mirelman et al. (1978) synchronised E.coli B cells and claimed that an increase in the transpeptidase/carboxypeptidase ratio and the level of crosslinking occurred immediately after division. All this work implies an enzymic basis to the balance between septation and cell wall extension suggested above.
III Genetic analysis of division control

1.8 The isolation and study of cell division mutations

In attempts to identify genes controlling division many mutants defective in septum formation have been isolated (Helmstetter et al., 1979; Mendelson, 1982; Donachie et al., 1983; Donachie, 1984). Some of these are now known to be involved in the SOS response (see Section IV), but there remains 15-20 genes whose primary defect appears to be in cell division (Table 1.2; Fig 1.4).

1.9 Filamenting mutants

Many of the cell division loci in Table 1.4 have been identified by the isolation of mutants temperature sensitive for cell division. The criteria used to define such mutations are normally that growth at the restrictive temperature (usually 42°C) blocks cell division without hindering accumulation of cell mass leading to filament formation and in addition that cells returned to 30°C after growth at 42°C for short periods (1-2 h) are still viable. It is of course possible that division genes other than those in Fig. 1.4 exist and have yet to be identified.

A major problem in studying the literature on the early isolation of division mutants is that different groups of workers ascribed separate names to mutations whose location was later found to be in the same gene. The most important example of the confusion in nomenclature concerns PAT84 (Hirota et al., 1968) one of the earliest and intensively studied division mutants, claimed to have a mutation at ftsA. Luktenhaus et al. (1980) showed that the division mutation in PAT84 (previously ftsA84) was adjacent but distinct from
### Table 1.2

**Classification of genes affecting cell morphology in E.coli (Donachie, 1984)**

<table>
<thead>
<tr>
<th>Class</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Class I</td>
<td>Genes required for net peptidoglycan (PG) biosynthesis.</td>
</tr>
<tr>
<td>Class II</td>
<td>Genes for outer membrane proteins involved in morphogenesis.</td>
</tr>
<tr>
<td>Class III</td>
<td>Genes required for cell elongation.</td>
</tr>
<tr>
<td>Class IV</td>
<td>Genes linking DNA replication with cell division.</td>
</tr>
<tr>
<td>Class V</td>
<td>Genes required for nucleoid segregation and septum localisation.</td>
</tr>
<tr>
<td>Class VI</td>
<td>Genes required for septum initiation.</td>
</tr>
<tr>
<td>Class VII</td>
<td>Genes required for septum formation.</td>
</tr>
<tr>
<td>Class VIII</td>
<td>Genes required for septum separation (cell division).</td>
</tr>
<tr>
<td>Class IX</td>
<td>Genes required for the inactivation of sites of septation.</td>
</tr>
<tr>
<td>Location (min)</td>
<td>Gene</td>
</tr>
<tr>
<td>---------------</td>
<td>-------</td>
</tr>
<tr>
<td>0</td>
<td>dnaK</td>
</tr>
<tr>
<td>2</td>
<td>mraA</td>
</tr>
<tr>
<td>2</td>
<td>mraB</td>
</tr>
<tr>
<td>2</td>
<td>ftsI</td>
</tr>
<tr>
<td>2</td>
<td>murE</td>
</tr>
<tr>
<td>2</td>
<td>murF</td>
</tr>
<tr>
<td>2</td>
<td>murG</td>
</tr>
<tr>
<td>2</td>
<td>murC</td>
</tr>
<tr>
<td>2</td>
<td>ddl</td>
</tr>
<tr>
<td>2</td>
<td>ftsQ</td>
</tr>
<tr>
<td>2</td>
<td>ftsA</td>
</tr>
<tr>
<td>2</td>
<td>ftsZ</td>
</tr>
<tr>
<td>2</td>
<td>envA</td>
</tr>
<tr>
<td>2</td>
<td>secA</td>
</tr>
<tr>
<td>2</td>
<td>azi</td>
</tr>
<tr>
<td>4</td>
<td>mrcB</td>
</tr>
<tr>
<td>4</td>
<td>sefA</td>
</tr>
<tr>
<td>10</td>
<td>lon</td>
</tr>
<tr>
<td>10</td>
<td>minA</td>
</tr>
<tr>
<td>15</td>
<td>dacA</td>
</tr>
<tr>
<td>15</td>
<td>rodA</td>
</tr>
<tr>
<td>15</td>
<td>pbpA</td>
</tr>
<tr>
<td>22</td>
<td>ompA</td>
</tr>
<tr>
<td>22</td>
<td>sfIA</td>
</tr>
<tr>
<td>26</td>
<td>minB</td>
</tr>
<tr>
<td>29-31</td>
<td>ftsG</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>36</td>
<td><strong>lpp</strong></td>
</tr>
<tr>
<td>49</td>
<td><strong>ftsB</strong></td>
</tr>
<tr>
<td>69</td>
<td><strong>dacB</strong></td>
</tr>
<tr>
<td>69</td>
<td><strong>ftsH</strong></td>
</tr>
<tr>
<td>71</td>
<td><strong>envB</strong></td>
</tr>
<tr>
<td>74</td>
<td><strong>crp</strong></td>
</tr>
<tr>
<td>74</td>
<td><strong>fic</strong></td>
</tr>
<tr>
<td>74</td>
<td><strong>cha</strong></td>
</tr>
<tr>
<td>75</td>
<td><strong>mrcA</strong></td>
</tr>
<tr>
<td>75</td>
<td><strong>envZ</strong></td>
</tr>
<tr>
<td>76</td>
<td><strong>ftsE</strong></td>
</tr>
<tr>
<td>76</td>
<td><strong>ftsS</strong></td>
</tr>
<tr>
<td>76</td>
<td><strong>fam</strong></td>
</tr>
<tr>
<td>81</td>
<td><strong>envC</strong></td>
</tr>
<tr>
<td>82</td>
<td><strong>pcsA</strong></td>
</tr>
<tr>
<td>82</td>
<td><strong>gyrB</strong></td>
</tr>
<tr>
<td>82-83</td>
<td><strong>ftsF</strong></td>
</tr>
<tr>
<td>85</td>
<td><strong>cya</strong></td>
</tr>
<tr>
<td>86</td>
<td><strong>ftsD</strong></td>
</tr>
<tr>
<td>86</td>
<td><strong>fcsA</strong></td>
</tr>
<tr>
<td>90</td>
<td><strong>mrbA</strong></td>
</tr>
<tr>
<td>90</td>
<td><strong>mrbB</strong></td>
</tr>
<tr>
<td>90</td>
<td><strong>mrbC</strong></td>
</tr>
<tr>
<td>90</td>
<td><strong>ftsH</strong></td>
</tr>
</tbody>
</table>

References for the above are contained in Bachmann (1983) or the text. It is possible that some of the closely located genes are in fact allelic (for example, **pcsA** and **gyrB**).
Figure 1.4
A diagram showing the position on the *E.coli* chromosomal map of many of the loci involved in cell morphology (from Donachie, 1984).
mraA mraB...ftsI murE murF murG murC ddl ftsQ ftsA ftsZ envA "U" secA azi

mrbA
mrbb
mrBC
ftsA
ftsO
cya
fts'124''
(lexA)

minA
lon

dacA ("54K") rodA pbpA

dnaK
mrcB
sfpA

minB

(sfiA
ompA

ftsG

lpp

recA

ftsB

(envZ
mrcA

chas
crp

dacB

ftsH

fam
ftsS
ftsL}
other \textit{ftsA} alleles and renamed it \textit{ftsZ}. In addition there are instances of two different genes being given the same name, for example, \textit{ftsH} (Santos and D'Almeida, 1975; Holland and Darby, 1976) and \textit{ftsS} (Dwek \textit{et al.}, 1984; Salmond and Plakidou, 1984).

Although many of the division genes in Fig 1.4 have been known for many years, few have been studied intensively with regard to their function. The physiology of these mutants is summarised by Helmstetter \textit{et al.} (1979) and studies have mainly concentrated on elucidating the following:

\begin{itemize}
  \item [a)] The possible osmoreversibility of the conditional cell division phenotype, in anticipation that envelope proteins might respond to this treatment.
  \item [b)] The residual increase in cell number upon shifting to the non-permissive temperature in attempts to distinguish between genes involved in initiating or late stages in the division process.
  \item [c)] The effect of filamentation on peptidoglycan metabolism (Pages \textit{et al.}, 1975).
  \item [d)] The necessity of protein synthesis for recovery of cell division after the release of the temperature induced division block (Ricard and Hirota, 1975). This, it was presumed, would indicate the stability or temporal sequence of division gene activity.
\end{itemize}

\textbf{1.10 Spherical mutants of E.coli}

In Section 1.6 temperature sensitive alleles of the \textit{pbpA} gene were described which produced spherical cells at high temperature. In addition, mutations in two other genes, \textit{envB}
(Adler et al., 1968; Normark, 1969) and rodA (Henning et al., 1972; Spratt, 1977a; Stoker et al., 1983) are capable of forming apparently spherical cells. Cells defective in adenyl cyclase (cya<sup>-</sup>) or in cAMP receptor protein (crp<sup>-</sup>) have also been described as round. However, the apparently spherical cells produced by envB, cya and crp mutations maintain the ability to form filaments upon treatment with nalidixic acid (Donachie, 1984) and Scott and Harwood (1981) examined cya cells and found them to be short rods. This suggests that the alleles of envB, cya and crp studied retain some capacity for cell wall elongation under restrictive conditions (where appropriate).

The cAMP-CRP complex is in some way involved in cell morphology. Donachie (1984) suggests that the cAMP-CRP complex exerts negative regulation on septal peptidoglycan synthesis. This is supported by the isolation of mutants, fic, that filament when treated with exogenous cAMP (Utsumi, 1982).

Both Mendelson (1982) and Donachie (1984) have classified mutations in genes involved in cell division and cell morphology according to their phenotype. Table 1.2 shows the classification of Donachie (1984) and includes genes concerned with murein and cell wall biogenesis. Donachie (1984) has also used observations of the phenotype of various cell division mutations, and the effects of constructing double division mutants, to compile a temporal map of mutations affecting cell morphology and the division process (Fig. 1.5).

Of the division genes studied so far only pbp3 (ftsI,sep) has been correlated with any specific cellular
A schematic diagram (from Donachie, 1984) showing a temporal map, within the cell cycle, of the phenotypes produced by mutations affecting cell morphology.
1.11 The 2 minute region of the E.coli chromosome

One of the most striking aspects of the distribution of the cell division genes on the E.coli chromosome (Fig 1.4) is the cluster of genes at approximately 2 min (Bachmann, 1983). Fig 1.4 contains an expansion of the 2 min region and shows the presence of at least 12 apparently contiguous genes all involved with cell division or cell wall synthesis (Wijsman, 1972; Fletcher et al., 1978; Luktenhaus et al., 1980). These also include the three most intensively studied cell division genes, pbp3, ftsA and ftsZ. Luktenhaus and Donachie (1979) isolated a λ-transducing phage (λ16-2) capable of complementing an amber ftsA mutation and identified its gene product. This transducing phage (λ16-2) was shown to carry a chromosomal segment extending from murC to envA and the use of deletion derivatives of λ16-2 allowed the identification and location of ftsZ (Luktenhaus et al., 1980) as well as the newly discovered murG (Salmond et al., 1980) and ftsQ (Begg et al., 1980) genes. Luktenhaus and Wu (1980), again using deletion derivatives of λ16-2, identified the gene product and direction of transcription of the murC, ddl, ftsA, ftsZ and envA genes although the direction of transcription of envA reported by Luktenhaus et al. (1980) was later shown to be incorrect (V.Darby, 1981; Sullivan and Donachie, 1984).

It is intriguing that although many different alleles of ftsA, ftsQ and envA have been isolated, only one allele of ftsZ (ftsZ84(Ts)) has been reported.
1.12 *ftsA* and *ftsZ* and cell division control

The precise role of *ftsZ* in cell division is unclear, although both microscopic studies (Burdett and Murray, 1974) and temperature shift experiments (Walker et al., 1975) have implied that FtsZ acts early in the cell division cycle. Mirelman et al., (1977) measured the effects on peptidoglycan metabolism in PAT84 at 30°C and 42°C however, their observations related to the block in division rather than any specific mechanism of *ftsZ* action. Similarly, although PAT84 has been used intensively in studies on the coupling between DNA replication and division (Nanninga et al., 1982) this was purely because of its very long doubling time when grown under certain conditions.

Work by Donachie et al. (1979) and Tormo et al. (1980), using almost identical approaches, showed that the product of the *ftsA* gene was required during the last 10-15 min before division and that the FtsA protein is necessary in an active form throughout the whole septation process. Tormo et al. (1980) concluded that FtsA could therefore be a termination protein as described by Jones and Donachie (1973).

1.13 Minicell formation

One class of division mutants that are not characterised by a temperature sensitive cell division phenotype are minicell producing mutants (*minA, minB*) (Adler et al., 1967; Frazer and Curtiss, 1975). Minicells are small chromosome less cells formed by a septation unusually close to one end of the bacterial rod. Their formation is blocked under conditions where normal cell division is inhibited. For example either by shifting a *minA, minB*, BUG6 strain to the
permissive temperature (Khachatocrians et al., 1973) or UV-irradiation of a minA, minB, lon strain (Helmstetter et al., 1979). Recent work by Davie et al. (1984) has cast doubt upon the existence of minA, or at least its requirement for minicell formation in a minB strain. Although intensively used as the basis for an E.coli gene expression system, the molecular basis behind minicell formation remains unknown.

To conclude, although the isolation of many mutants defective in septum formation has as yet yielded little to advance our understanding of the mechanism of cell division, the application of molecular biology to their study should be highly informative.
IV The SOS response and its effect on cell division

1.14 The model of the SOS regulatory system

When *E. coli* cells are exposed to DNA damaging agents, a complex response is induced termed the SOS response. A number of recent reviews have discussed the current state of knowledge on the SOS system in depth (Little and Mount, 1982; Witkin, 1982; Kenyon, 1983; Gottesman and Neidhardt, 1984; Walker, 1984).

Some treatments known to induce the SOS response are listed in Table 1.3, the common factor between them being damage to DNA and/or a halt in DNA replication. The responses associated with SOS induction are shown in Table 1.4. Although the physiological changes accompanying SOS induction are complex, the overall result is to increase DNA repair capacity in response to a DNA damage signal.

With the exception of some of the early stages in SOS induction, in particular the precise triggering event, the control of the SOS network is well understood both genetically and biochemically.

Two genes are primarily involved in SOS regulation, *recA* and *lexA*. In the current model of the SOS regulatory system, the product of the *lexA* gene is postulated to be a repressor of a number of unlinked genes involved in the SOS response (including *recA*) and the *lexA* gene itself. During normal growth, when the SOS system is switched off, the LexA protein represses its target genes (Fig. 1.6a). When DNA damage occurs, or DNA replication is blocked in certain ways, an inducing signal apparently activates a proteolytic activity in RecA (RecA*). RecA* then cleaves the LexA protein, resulting in increased expression of *recA* and other LexA
Table 1.3

**SOS inducing treatments**

UV, and X-irradiation.

Nalidixic acid, novobiocin, bleomycin, mitomycin-C, methyl methane sulphonate, NMG and other mutagens.

Thymine starvation.

Expression of temperature sensitive alleles of *lig* and several other *dna* genes.

Introduction of irradiated (double stranded) replicons.
Table 1.4

SOS induced responses

Prophage induction.
Weigle reactivation.
Weigle mutagenesis.
Inhibition of cell division.
Increased synthesis of RecA protein.
Error prone repair of chromosomal lesions.
Induction of colicins in col⁺ strains.
Induction of excision repair.
Alleviation of restriction.
Stable replication.
Cessation of respiration.

References to the above can be found in Walker (1984).
Figure 1.6
A diagram showing the accepted model of the control of the SOS response.

A) At rest the SOS system is switched off and the LexA protein represses the synthesis of itself, RecA and the products of SOS inducible genes.

B) Following DNA damage RecA becomes "activated" and LexA becomes cleaved, releasing repression on all LexA controlled genes causing induction of the SOS response.

C) Following the disappearance of the SOS inducible signal, RecA becomes "de-activated" and LexA levels rapidly rise to repress SOS inducible genes.
B) inducing signal 'activates' RecA
regulated genes (Fig. 1.6b). When DNA repair is completed and/or DNA replication resumes, RecA loses its proteolytic activity and due to the autoregulatory nature of lexA control, LexA levels rapidly rise, repressing the expression of the SOS inducible genes (Fig. 1.6c).

1.15 Genetic evidence for the SOS regulatory system
Much of the detail concerning the regulation of the SOS system has been elucidated by the isolation of mutations in the recA and lexA genes (Table 1.5).

The lexA gene was originally identified by a class of dominant mutations (lexA\textsuperscript{+}, (lexA(Ind\textsuperscript{+}))) blocked SOS induction and conferred extreme sensitivity to DNA damaging agents (Mount et al., 1972). In addition, two classes of recessive lexA mutant were isolated as suppressors of lexA(Ind\textsuperscript{+}); tsl (lexA(Ts)) which show induction of the SOS system at high temperature and spr (lexA(Def)) which causes SOS functions to be expressed constitutively.

Many recA\textsuperscript{-} mutants have been isolated which show no induction of SOS functions (Table 1.5) and consequently are highly sensitive to DNA damaging agents such as UV-irradiation. However when a recA mutation is combined with a tsl mutation, both recA and other SOS genes are induced at high temperature (Gudas and Pardee, 1975), indicating that lexA is epistatic to recA. In addition, work on the production of recA mRNA in strains carrying various mutations in recA or lexA (McPartland et al., 1980) led to the conclusion that lexA is a negative regulator and recA is a positive regulator of recA expression.
<table>
<thead>
<tr>
<th>Allele</th>
<th>Phenotype</th>
<th>Biochemical change</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>lexA alleles</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>lexA(Ind⁻) (lexA⁻)</td>
<td>Dominant, no SOS induction</td>
<td>Protease resistant repressor</td>
</tr>
<tr>
<td>lexA(Ts) (tsl)</td>
<td>Recessive, filamentation and recA induction at 42°C</td>
<td>Thermosensitive repressor</td>
</tr>
<tr>
<td>lexA(Def) (spr)</td>
<td>Recessive, constitutive expression of target genes</td>
<td>Defective repressor</td>
</tr>
<tr>
<td><strong>recA alleles</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>recA</td>
<td>Cannot induce SOS functions</td>
<td>Defective protein</td>
</tr>
<tr>
<td>recA441 (tif)</td>
<td>Spontaneous SOS expression at 42°C</td>
<td>Protease thermoactivated</td>
</tr>
<tr>
<td>recA430</td>
<td>Cannot induce SOS system but recombination proficient</td>
<td>Specific defect in protease</td>
</tr>
<tr>
<td>recA0</td>
<td>Constitutive recA expression</td>
<td>Operator defect</td>
</tr>
</tbody>
</table>
1.16 RecA* and the inducing signal

The mechanism of λ prophage induction during the SOS response was elucidated when it was discovered that the RecA protein cleaves the λcI repressor (Roberts and Roberts, 1975; Roberts et al., 1977, 1978). The RecA protein was subsequently shown to cleave LexA in-vitro (Little et al., 1980) and the specific site of cleavage identified (Horii et al., 1981a). The repressor activity of both λcI and LexA proteins is greatly reduced by cleavage with RecA (Little and Mount, 1982). Moreover, both in-vivo and in-vitro data shows that LexA is a much better substrate for RecA than the λcI repressor (Little et al., 1981; Little and Mount, 1982).

The nature of the inducing signal for the SOS response has been intensively studied (reviewed by Little and Mount, 1982 and Walker, 1984). Data has been presented for the role of both specific oligonucleotides (Irbe et al., 1981) and single stranded DNA (Craig and Roberts, 1980; 1981) in the process of SOS induction. However, definitive evidence for the precise nature of the effector is still lacking.

It is possible that genes other than recA and lexA are involved in the induction of the SOS response. For example the RecBC enzyme is required for RecA induction following treatment with nalidixic acid but not with UV-irradiation (Little and Hanawalt, 1977; Bockrath and Hanawalt, 1980) and the recF gene is required for RecA induction following UV-irradiation but not nalidixic acid treatment (McPartland et al., 1980). The ssb gene is also thought to play a role in SOS induction. For example, ssb mutations suppress a number of SOS responses (e.g. λ-prophage induction (Vales et al.,
1980) and derepression of RecA synthesis (Baluch et al., 1980). However, ssb mutations do not prevent expression of the SOS system in tif strains grown at high temperature and so it has been proposed that the ssb gene is involved in mediating the action of the inducing signal.

Whatever the mechanism of induction, it appears that some activation of RecA is required for the efficient cleavage of LexA since the derepression of RecA synthesis alone is not sufficient for the induction of λ and several other SOS phenotypes. This is best demonstrated in \(\text{lexA}(\text{Def})\) cells or cells carrying multiple copies of \(\text{recA}\) (Uhlin and Clark, 1981). However, as a further complication Little (1984) has recently shown that both purified LexA and λCI proteins are capable of autodigestion in the absence of RecA, with the breakdown of CI occurring at a considerably slower rate than LexA. Little (1984) interpreted this as showing that RecA plays an indirect stimulatory role in the cleavage of LexA (and λCI) perhaps acting as an allosteric effector.

### 1.17 Identification and analysis of SOS inducible functions under \(\text{lexA}\) control

Of the SOS phenotypes in Table 1.4 some (for example prophage λ induction) may be explained almost purely by the increase in synthesis and activation of the RecA protein. Others are due to the derepression of genes whose transcription is under the control of \(\text{lexA}\).

In the identification of SOS inducible genes the most fruitful approach has involved the use of Mud\(1(\text{Ap, lac})\) bacteriophage. The Mud\(1(\text{Ap, lac})\) bacteriophage allows the
isolation of *in-vivo* gene fusions in a single step (Casadaban and Cohen, 1980). Kenyon and Walker (1980) took an *E. coli* strain deleted for *lac* and used Mudl(Ap, *lac*) to produce ampicillin resistant derivatives with the bacteriophage integrated into the chromosomal DNA. These colonies were replica plated onto agar plates containing the chromophore X-gal + or - the mutagenic chemical mitomycin-C. Five different insertion derivatives were isolated that only produced easily detectable β-galactosidase activity when grown in the presence of mitomycin-C. These were presumed to have the Mudl(Ap, *lac*) β-galactosidase gene fused to the promoters of SOS inducible genes. These SOS regulated genes were named din (damage inducible).

The Mudl(Ap, *lac*) insertion in dinE was subsequently shown to be in uvrA (Kenyon and Walker, 1981). In addition to the din genes identified by Kenyon and Walker (1980), the Mudl(Ap, *lac*) technique has been used to show the lexA dependent control, following DNA damaging treatments of the uvrB, sfiA, umuDC, recA, himA, uvrD, ruv and recN genes (Table 1.6; Fig. 1.7). Gene fusions engineered *in-vitro* have been used to demonstrate the SOS dependent inducibility of lexA, uvrC, and ssb (Fig. 1.8). McPartland *et al.* (1980) showed that the transcription of the recA gene was repressed by LexA protein. These studies on the inducibility of SOS genes confirmed the identity of genes under lexA control. It must be remembered that the screening for damage inducible genes used by Kenyon and Walker (1980) can only identify genes whose gene product is not essential for cell viability. One method for identifying more din genes whilst avoiding this limitation would be to repeat the experiments of Kenyon
<table>
<thead>
<tr>
<th>Gene</th>
<th>Gene location</th>
<th>Function</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>recA</td>
<td>58</td>
<td>Recombination</td>
<td>McPartland et al. (1980), Little et al. (1981)</td>
</tr>
<tr>
<td>uvrB</td>
<td>17</td>
<td>Excision repair</td>
<td>Fogliano and Schendel (1981)</td>
</tr>
<tr>
<td>umuDC</td>
<td>25</td>
<td>Mutagenesis</td>
<td>Bagg et al. (1981)</td>
</tr>
<tr>
<td>himA</td>
<td>38</td>
<td>Site-specific recombination</td>
<td>Miller et al. (1981)</td>
</tr>
<tr>
<td>dinA</td>
<td>2</td>
<td>Unknown</td>
<td>Kenyon and Walker (1980)</td>
</tr>
<tr>
<td>dinB</td>
<td>8</td>
<td>Unknown</td>
<td>Kenyon and Walker (1980)</td>
</tr>
<tr>
<td>dinD</td>
<td>80-85</td>
<td>Unknown</td>
<td>Kenyon and Walker (1980)</td>
</tr>
<tr>
<td>dinF</td>
<td>9^1</td>
<td>Unknown</td>
<td>Kenyon and Walker (1980)</td>
</tr>
<tr>
<td>sfiA</td>
<td>22</td>
<td>Division inhibition</td>
<td>Huisman and D'Ari (1981)</td>
</tr>
<tr>
<td>recN</td>
<td>57</td>
<td>RecF dependent recombination</td>
<td>Lloyd et al. (1983), Lovett and Clark (1983)</td>
</tr>
<tr>
<td>ssb</td>
<td>92</td>
<td>Single strand binding protein</td>
<td>Brandsma et al. (1983)</td>
</tr>
<tr>
<td>ruv</td>
<td>41</td>
<td>UV resistance</td>
<td>Shurvington and Lloyd (1982)</td>
</tr>
<tr>
<td>uvrD</td>
<td>85</td>
<td>Excision repair (DNA helicase II)</td>
<td>Seigel (1983)</td>
</tr>
<tr>
<td>rpsU- dnaG-rpoD</td>
<td>67</td>
<td>See below</td>
<td>Lupski et al. (1983)</td>
</tr>
</tbody>
</table>

The rpsU-dnaG-rpoD operon has been sequenced and a LexA binding site found in the operator region. The rpsU gene encodes 30S ribosomal protein subunit S21, dnaG encodes DNA primase and rpoD encodes the a subunit of RNA polymerase.
Figure 1.7

A diagram showing the position on the *E.coli* chromosomal map of the SOS inducible genes described in Table 1.6.
Figure 1.8

A diagram of the processes occurring during the induction of the SOS system as seen by Kenyon (1983).
and Walker (1980) in strains carrying F' plasmids containing large portions of the E.coli chromosome. Finally it is important to note that of the 5 din genes identified by Kenyon and Walker (1980) only dinE has been correlated with a known SOS phenotype (uvrA). Several other functions therefore remain to be identified.

1.18 The LexA binding site

The LexA binding site for several of the SOS inducible genes has been investigated and a consensus DNA sequence for LexA binding derived ("SOS box") (Table 1.7). Three genes, lexA, umuDC and the colicin E1 gene have been shown to possess 2 closely located SOS boxes but in the case of umuDC LexA binding has yet to be demonstrated.

Kenyon and Walker (1980) showed that the kinetics of induction of β-galactosidase production from the dinA, dinB, dinD and dinF promoters on treatment with mitomycin-C varied considerably between the different din genes. In addition, it has been shown that different SOS boxes bind LexA with characteristic affinities. For example, the $K_d$ value for LexA binding to the recA operator is 2 mM whereas that for LexA binding to the uvrB, lexA and colEl operators is 20 mM, 20 mM and 0.04 mM respectively (Brent and Ptashne, 1981; Ebina et al., 1983). It is clear therefore that the induction of different SOS functions may be co-ordinated by providing genes whose product is required early in the SOS response with LexA binding sites with lower affinities for LexA than those required at a later time.
Table 1.7

LexA binding sites of SOS inducible genes

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequence</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>recA</td>
<td>TACTGTATGAGCATAAGTA</td>
<td>Brent and Ptashne (1981)</td>
</tr>
<tr>
<td>uvrA</td>
<td>TACTGTATATTCCATTCCAGGT</td>
<td>Sancar et al. (1982a)</td>
</tr>
<tr>
<td>uvrB</td>
<td>AACTGTTTTTTATCCAGTA</td>
<td>Sancar et al. (1982b)</td>
</tr>
<tr>
<td>sfiA</td>
<td>TACTGTACATCCATAAGTA</td>
<td>Cole (1983)</td>
</tr>
<tr>
<td>uvrD</td>
<td>ATCTGTATATATACAGCT</td>
<td>Walker (1984)</td>
</tr>
<tr>
<td>lexA-1</td>
<td>TGCTGTATATACTCACAGA</td>
<td>Horii et al. (1981b)</td>
</tr>
<tr>
<td>lexA-2</td>
<td>AACTGTATACCCAGGG</td>
<td>Horii et al. (1981b)</td>
</tr>
<tr>
<td>clel-1</td>
<td>TGCTGTATATCTCACAGA</td>
<td>Ebina et al. (1981)</td>
</tr>
<tr>
<td>clel-2</td>
<td>CAGTGGTTATATGTACAGA</td>
<td>Ebina et al. (1981)</td>
</tr>
</tbody>
</table>

Consensus: TACTGTatata-a-aCAGta

Capital letters indicate strongly conserved bases and small letters indicate weakly conserved bases. Other LexA binding sites have been found by comparison with the consensus sequence shown above but LexA binding has not yet been demonstrated.
1.19 SOS induced filamentation

As seen in Table 1.4, one of the responses to DNA damaging treatments is a block in cell division resulting in filament formation. In wild type E.coli K12 strains the length of this division inhibition is exponentially related to DNA damage (measured using varying doses of UV-irradiation) after which cell division capacity is restored (Burton, 1980). Witkin (1967) suggested that this filamentation could be due the derepression of a gene coding for an inhibitor of septum formation. Both recA\textsuperscript{-} and lexA(Ind\textsuperscript{-}) mutations block UV-induced filamentation and lead to a high rate of production of DNA less cells (Howe and Mount, 1978), suggesting that one function of SOS induced division inhibition is to co-ordinate DNA replication to cell division.

1.20 Mutants used to study the SOS division inhibition

Three mutations have been used to study SOS induced filamentation due to their ability to uncouple the effects of DNA damage from division inhibition.

a) tif (recA\textsuperscript{441}) : The tif (recA\textsuperscript{441}) allele of recA shows constitutive expression of the SOS response (including division inhibition) when grown at high temperature (Kirby et al., 1967). Castelazzi et al. (1972a) showed that tif expression did not increase recombinational capacity and went on to show that a lexA\textsuperscript{-} mutation blocked tif induced filamentation but not prophage \textlambda\textsuperscript{ind} induction (Castelazzi et al., 1972b). This showed that division inhibition was an induced response under recA-lexA control and led George et al. (1975) to propose the existence of a division inhibitor designated RADI (Radiation Associated Division Inhibitor).
The expression of the tif allele is highly medium dependent. Plating a culture of tif cells followed by growth at 42°C results in a $2.4 \times 10^{-4}$ reduction in plating efficiency (compared to growth at 30°C) on minimal medium but no reduction on Luria broth medium (Huisman et al., 1980a). Tif expression is in fact potentiated by addition of exogenous adenine yet is inhibited by guanosine and cytidine.

b) tsl (lexA(Def)) : The tsl mutation causes thermal induction of SOS functions (including division inhibition and RecA synthesis) without inducing λ prophage (Mount et al., 1972; Gudas, 1976). The effect of the tsl mutation is thought to be due to the production of a mutant LexA (Tsl) protein unable to repress SOS inducible genes at high temperature. Tsl expression is again medium dependent, the phenotype being amplified in rich rather than minimal media (Huisman et al., 1980a).

c) lon : lon mutants continue to filament for long periods after UV-irradiation (Adler and Hardigree, 1965) and other SOS inducing treatments although their DNA repair capacity is normal (Howard-Flanders et al., 1964). Darby and Holland (1979) showed that following UV-irradiation of a lon mutant, division inhibition continued when both DNA replication and RecA synthesis had returned to the pre-irradiation state.

The lon mutation is highly pleiotropic. For example, lon mutants overproduce capsular polysaccharide (colanic acid) and show extreme mucoidy when grown on solid minimal media (Markovitz, 1977). Mackie and Wilson (1972) showed that the mucoidy of lon strains (also known as capR) was due to derepression of the gal operon leading to overproduction of
colanic acid.

The *lon* gene has been shown to be involved in the degradation of abnormal proteins and is the location for mutations originally designated *degT* (Shineberg and Zipser, 1973; Gottesman and Zipser, 1978). The product of the *lon* gene has been identified as a 94 kD polypeptide (Zehnbauer and Markovitz, 1980) and demonstrated to possess ATP'ase dependent protease activity (Chung and Goldberg, 1981; Chung *et al.*, 1983).

Mutations in *lon* reduce the lysogenisation frequency of some bacteriophages (for example λ and P1). Walker *et al.* (1973) showed that λ multiplies normally in *lon* strains but forms lysogens very poorly. Truitt *et al.* (1976) demonstrated that the level of λcII repressor in a *lon*−, λ+ strain was half of that in a *lon*+, λ+ strain. Subsequently Gottesman and Gottesman (1981) demonstrated that the *lon* mutation reduced the half-life of the λcII protein by 50% and suggested that this was the basis of the *lon* lysogenisation deficiency. This observation is compatible with the previously discovered role of *lon* in protein degradation.

Other properties of *lon* mutants include the spontaneous formation of a low frequency of filaments in the absence of an SOS inducing treatment and the loss of viability of *lon* cells grown in minimal medium and plated onto rich solid media ("complex medium killing").

The effect of growing *tif* or *tsl* mutants at 42°C or the treatment of *lon* mutants with DNA damaging agents is essentially to inhibit cell division leading to filamentation and eventual lysis.
1.21 The isolation of suppressors of SOS induced division inhibition

A number of groups independently isolated mutations capable of suppressing the persistent filamentation seen on growing ts1 and tif cells at 42°C or following UV-irradiation of lon mutants.

Gayda et al. (1976) and Johnson (1977) isolated extragenic suppressors of lon mutants by examining survivors of a lon strain plated onto solid media containing either nitrofurantoin or methyl methane sulphonate (MMS). 2 classes of suppressor mutation were identified, sulA and sulB and their location on the E.coli chromosome found to be 22 min and 2 min respectively (Bachmann, 1983). Gayda et al. (1976) and Johnson (1977) showed that both sulA and sulB suppressed all of the tested phenotypes of lon strains (for example filamentation after DNA damage, lysogenisation deficiency and complex medium killing) with the exception of mucoidy. Gayda et al. (1976) showed that the one allele of sulA tested (sulA17) was recessive to sulA⁺.

George et al. (1975) isolated temperature resistant revertants of a lon, tif strain plated onto solid media and grown at 41°C. Approximately 1% of the revertants studied carried extragenic suppressor mutations of the lon and tif induced filamentation (sfi). These sfi mutations (sfiA and sfiB) were mapped to the same chromosomal locations as the sulA and sulB loci respectively. Although George et al. (1975) concluded that sfi and sul were distinct, no differences have been found between sfiA and sulA, and sfiB and sulB mutations isolated in E.coli K12, and sfi and sul
are now thought to be identical. Except when referring to alleles originally isolated as sulA or sulB, in this work the names sfiA and sfiB will be used throughout. Huisman et al. (1980b) showed that of six sfiA mutations studied, three were recessive (sfiA23, sfiA85 and sfiA121) and three were dominant (sfiA11, sfiA67 and sfiA91), in a tif, lon background strain. Huisman et al. (1980b) also examined two sfiB alleles (sfiB103 and sfiB114) and found both to be recessive, again in a tif, lon chromosomal background. Of the 15 sfi mutations isolated by George et al. (1975), 13 were sfiA and 2 sfiB. Likewise, of 16 sul mutations studied by Gayda et al. (1976) only one was sulB.

Both sfiA and sfiB alleles have no detectable phenotype in the absence of tif, tsl or lon mutations (with the exception of a minor growth and cell morphology defect of some sfiB alleles particularly when grown at high temperature (Huisman et al., 1980b)). The effect of sfi mutations therefore appears to be specific to the SOS division inhibition response. The ability of sfi mutations to suppress the reduced lysogenisation frequency for phage P1 and λ shown by lon strains is intriguing (Huisman et al., 1980c). In Section 1.20 evidence was presented which suggested that the effect of the lon mutation on lysogenisation is due to an altered stability of λ proteins involved in the establishment of lysogeny. D'Ari and Huisman (1982) have shown that the introduction of λ or P1 into E.coli causes a mild induction of RecA synthesis. It is thus possible that the introduction of λ or P1 into lon cells derepresses the synthesis of the RADI proposed by George et al. (1975). This could be sufficient to cause persistant filamentation and cell death
which is seen in Ion strains after mild exposure to DNA damaging agents. The effect of sfi mutations could therefore be to block this persistent filamentation and allow lysogen formation.

A further class of mutations has been isolated which suppress tif mediated division inhibition, infA (Bailone et al., 1975) and infB (Huisman et al., 1980a; Huisman and D'Ari, 1983). However, both infA and infB strains are defective in tif induced λ prophage induction and infB also reduces mutagenesis seen in tif strains grown at 42°C. Therefore infA and infB are probably involved in a more general aspect of the regulation of the SOS system than division inhibition (Huisman and D'Ari, 1983).

1.22 A model for SOS associated division inhibition

Recent work, mainly on the sfiA gene has allowed the formulation of a plausible model of cell division inhibition following induction of the SOS system.

Huisman and D'Ari (1981) isolated a Mudl(Ap, lac) insertion into the sfiA gene and showed that sfiA is an SOS inducible gene. Cole (1983) showed that the sfiA promoter was indeed repressed by LexA protein and sequencing of the sfiA promoter showed the presence of an SOS box (Beck and Bremer, 1980) (Table 1.7). The isolation of a Mudl(Ap, lac) into the chromosomal sfiA gene demonstrates that sfiA is not an essential gene in E.coli (Huisman et al., 1983). The product of the sfiA gene is thought to be the division inhibitor predicted by George et al. (1975). The role of the Ion gene in the regulation of SOS mediated division inhibition was
elucidated by Mizusawa and Gottesman (1983), who measured the stability of SfiA protein in lon\(^-\) and lon\(^+\) cells. The lon mutation increased the half-life of SfiA from 1.2 min to 19 min.

The current model of SOS regulated division inhibition is shown in Fig 1.9. sfiA is normally repressed by the LexA protein and upon induction of the SOS system, LexA is cleaved, sfiA is derepressed and SfiA protein blocks division. SfiA is rapidly broken down by the product of the lon gene (or a protease under lon control) such that when LexA repression on the sfiA gene is restored, cell division can quickly resume. In lon cells, once sfiA is derepressed SfiA is not rapidly broken down and continues to block cell division.

Having assigned a function to the sfiA gene, what then is the role of sfiB? The sfiB locus has been mapped to the 2 min region of the E. coli chromosome (Johnson, 1977), amongst a group of genes involved in cell wall synthesis and septum formation (Fig. 1.4). A number of workers have suggested that the sfiB locus corresponds to a gene required for cell division and is the "target" for the inhibitory action of the SfiA protein. Mutations at sfiB would presumably suppress SOS induced division inhibition by preventing the inhibitory action of SfiA.

1.23 sfiA, sfiB independent division inhibition

Although sfiA and sfiB mutations suppress the persistant filamentation seen in tif, tsl and lon strains under "restrictive" conditions, sfiA and sfiB mutants still show a transient division inhibition when UV-irradiated (Burton and
A schematic representation of the control of sfiA during the SOS response.

Rest - At rest the sfiA gene is repressed by the product of the lexA gene.

SOS induction - Following induction of the SOS system repression on the sfiA promoter by LexA is released and SfiA proceeds to block cell division.

Recovery - LexA repression on the sfiA gene resumes and either the product of the lon gene or a protease under lon control degrades SfiA and cell division can continue.
Rest

SOS induction

Recovery

SfiA blocks cell division
Holland, 1983). To explain this, Burton and Holland (1983) proposed a second, \textit{sfi} independent pathway of division inhibition. In addition to this D'Ari and Huisman (1983) have identified a third locus, \textit{sfiC}, involved in division inhibition in certain strains of \textit{E.coli}.

1.24 The aims of this project

The aim of this work was to investigate the location of the \textit{sfiB} gene and its role in the regulation of cell division during the SOS response.
CHAPTER 2

Methods and materials

2.1. Bacterial strains

All the bacterial strains used were E. coli K-12 derivatives and are listed in table 2.1. Strains were kept at 4°C on agar plates, or at -80°C in nutrient broth or M9-minimal medium containing 20% glycerol.

2.2. Bacteriophage strains

Pl and λ strains were stored, at 4°C, in buffer containing a few drops of chloroform.

2.3 Media

Nutrient broth: 2.5% Oxoid agar No. 2
Luria broth: 1% w/v Oxoid tryptone, 0.5% w/v Oxoid yeast extract, 0.5% w/v NaCl (pH7.4).
M9-minimal medium: 40 mM Na₂HPO₄, 20 mM KH₂PO₄, 8mM NaCl, 20 mM NH₄Cl, 1 mM CaCl₂, 10 mM MgSO₄.
Added as required: glucose (0.4%), maltose (0.2%), L-amino acids (50 μg/ml), thiamine (2 μg/ml), thymine (50 μg/ml), Difco casamino acids (0.4%).

Solid media: nutrient broth, Luria broth or M9-minimal media were solidified with 1.45% Oxoid No. 3 agar.
Trypticase agar: 1% w/v Trypticase peptone (Baltimore Biological Laboratories), 1.5% w/v NaCl, 1.5% agar. Soft Trypticase agar contained only 0.7% agar.
<table>
<thead>
<tr>
<th>Name</th>
<th>Genotype</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>MC4100</td>
<td>araD, ΔlacIPOZYA, rpsL, thi</td>
<td>P.Bassford</td>
</tr>
<tr>
<td>PAM162m+</td>
<td>thi, thr, leuB6, proA, his, lon,</td>
<td></td>
</tr>
<tr>
<td></td>
<td>lacY, galK, ara, xyl, mtl, rpsL,</td>
<td></td>
</tr>
<tr>
<td></td>
<td>tsx, supE, sfiB26</td>
<td></td>
</tr>
<tr>
<td>PAM162/1</td>
<td>thi, thr, leuB6, proA, his, lon,</td>
<td></td>
</tr>
<tr>
<td></td>
<td>lacY, galK, ara, xyl, mtl, rpsL,</td>
<td></td>
</tr>
<tr>
<td></td>
<td>tsx, supE, sfiB26 azi</td>
<td></td>
</tr>
<tr>
<td>PAMsfi+</td>
<td>thi, thr, leuB6, proA, his, lon,</td>
<td></td>
</tr>
<tr>
<td></td>
<td>lacY, galK, ara, xyl, mtl, rpsL,</td>
<td></td>
</tr>
<tr>
<td></td>
<td>tsx, supE, sfiB26+</td>
<td></td>
</tr>
<tr>
<td>RB308 recA</td>
<td>F, deoC, thyA, lacY, recA srl::Tn10</td>
<td>R.Buxton</td>
</tr>
<tr>
<td>D22</td>
<td>trp, pro, his, rpsL, ampA, envA</td>
<td>A.Salem</td>
</tr>
<tr>
<td>TKF12</td>
<td>thr, leu, thi, pyrF, thyA, ilv,</td>
<td>W.Donachie</td>
</tr>
<tr>
<td></td>
<td>ftsA12, his, arg, lac tonA</td>
<td></td>
</tr>
<tr>
<td>SP63</td>
<td>trp(am), tyr(am), ilv, pbpB63</td>
<td>B.Spratt</td>
</tr>
<tr>
<td>GC2490</td>
<td>thr, pro, his, lac, gal leuA, tsl,</td>
<td>R.D'Ari</td>
</tr>
<tr>
<td></td>
<td>rpsL, sfiB114</td>
<td></td>
</tr>
<tr>
<td>GC24901</td>
<td>thr, pro, his, lac, gal leuA, tsl,</td>
<td></td>
</tr>
<tr>
<td></td>
<td>rpsL, sfiB</td>
<td></td>
</tr>
<tr>
<td>GC4293</td>
<td>tsl, recA-99, rpsL</td>
<td>R.D'Ari</td>
</tr>
<tr>
<td>CSH26ΔF6</td>
<td>ara, Δ(lac, pro), Δ(recA,srl)F6,</td>
<td>D.Oliver</td>
</tr>
<tr>
<td></td>
<td>rpsL, thi</td>
<td></td>
</tr>
<tr>
<td>JFL100</td>
<td>ilv, his, thyA, deo, ara(am),</td>
<td>J.Lutkenhaus</td>
</tr>
<tr>
<td></td>
<td>lac(am), galU(am), tyrT, supF,</td>
<td></td>
</tr>
<tr>
<td></td>
<td>ftsZ84</td>
<td></td>
</tr>
<tr>
<td>MM52</td>
<td>araD, ΔlacIPOZYA, rpsL, thi</td>
<td>J.Beckwith</td>
</tr>
<tr>
<td></td>
<td>secA</td>
<td></td>
</tr>
<tr>
<td>5K</td>
<td>hsdR, thr, leu, thi tonA</td>
<td>W.J.Brammar</td>
</tr>
</tbody>
</table>
Pi bottom layer agar : nutrient broth + 1.45% Oxoid No. 3 agar made 2.5 mM CaCl\(_2\) and 0.1% w/v glucose immediately before pouring.

Pi top layer agar : nutrient broth + 0.5% Oxoid No. 3 agar made 2.5 mM CaCl\(_2\) before use.

Antibiotics were used in the following final concentrations:

- Sodium ampicillin 25 μg/ml
- Kanamycin sulphate 25 μg/ml
- Tetracycline 10 μg/ml
- Chloramphenicol 25 μg/ml
- Streptomycin sulphate 200 μg/ml

Ampicillin, kanamycin and streptomycin were dissolved in distilled water and tetracycline and chloramphenicol were dissolved in 50% ethanol.

Bacterial buffer : 20 mM KH\(_2\)PO\(_4\), 50 mM Na\(_2\)HPO\(_4\), 70 mM NaCl, 0.4 mM MgSO\(_4\) (pH 7.0).

Buffer : 6 mM Tris HCl pH 7.2, 10 mM MgSO\(_4\), 0.05% w/v gelatin.

Sodium phosphate buffer : 10 mM Na\(_2\)HPO\(_4\) and 10 mM NaH\(_2\)PO\(_4\) titrated together to pH 7.2.

2.4. Growth of bacterial cultures

Bacterial liquid cultures were shaken in a New Brunswick Gyrotary Shaker at 37°C unless otherwise indicated in the text. Bacterial cell mass was measured using a Gilford Microsample spectrometer 300N. Normally cultures were monitored by determining absorbance \(A_{450}\).
2.5. UV-irradiation of bacteria

Cultures of bacteria (normally in M9-minimal medium) were irradiated in sterile plastic petri-dishes, with gentle swirling, under a Hanovia Bacteriocidal lamp (254nm). The dose rate was calibrated with a Latarjet dosimeter.

2.6. Generalised transduction using Plvir

(a) Preparation of Pl lysates

Donor strains were grown in nutrient broth to $A_{450}$ 0.5 and $\text{CaCl}_2$ added to 2.5 mM. 0.3 ml of cells were mixed with 0.1 ml of a Pl lysate at a range of dilutions and left for 15 min at $37^\circ C$ to allow the phage to adsorb. 3.5 ml of molten Pl top layer agar was then added to each tube and the contents poured onto Pl bottom layer agar plates and incubated overnight. The plates on which Pl plaques were just confluent were selected and the top layer scraped off into a sterile bottle. 0.4 ml of chloroform and 1 ml of buffer per plate was added. The mixture was mixed and incubated at $37^\circ C$ for 15 min after which the tubes were centrifuged and the supernatant taken off and stored at $4^\circ C$ over chloroform. Yields were usually 2-10 x $10^{10}$ phage/ml.

(b) Pl transduction

10 ml of recipient cells were grown in nutrient broth to $A_{450}$ 0.5, centrifuged and the cells resuspended in 1 ml of Luria broth. 0.5 ml of these cells were added to 0.5 ml donor Pl lysate (titre approximately $5 \times 10^9$ phage/ml) and 0.5 ml of prewarmed Luria broth made 30 mM with respect to $\text{MgCl}_2$ and 15 mM with respect to $\text{CaCl}_2$ and the tubes incubated for 15 min at $37^\circ C$. The cells were then harvested, washed twice in bacterial buffer + 0.25% sodium citrate and the cells plated
onto selective plates at a range of serial dilutions. Where necessary cells were grown in nutrient broth + 0.25% sodium citrate prior to plating out to allow the expression of transduced markers.

2.7. Tests for transduced markers

The cotransduction of unselected markers was tested by patch plating transductants onto agar plates under conditions where the relevant phenotypes were expressed.

lon: lon mutants show extensive filamentation (as confirmed by phase contrast microscopy) and poor growth on M9-minimal media agar plates + 250 μl/l methyl methane sulphonate (MMS).

ftsA, pbp3, secA and tsl: all of these mutants show poor growth and filamentation when grown on nutrient agar plates at 42°C.

sfiB: suppression of filamentation shown by lon mutants when grown on MMS containing media or tsl mutants when grown at 42°C on nutrient agar.

envA: envA mutants fail to grow on nutrient agar plates + 2-10 μg/ml rifamycin depending on the genetic background of the host.

azi: azi mutants are able to grow on nutrient agar plates + 150 μg/ml sodium azide.

2.8. Preparation of bacteriophage λ

(a) Plate lysates

This was carried out as for phage P1 except that cells were grown in nutrient broth + 10 mM MgCl₂ and 0.4% w/v maltose. Trypticase agar was used as the bottom layer agar and soft
Trypticase agar as the top layer agar.

(b) Thermoinduction of $\lambda$cl$^{857}$ and derivatives

Lysogens of $\lambda$cl$^{857}$ were grown in Luria broth + 10 mM MgCl$_2$ at 30°C to A$_{450}$ 0.5, shifted to 42°C for 20 min and then grown at 37°C until lysis occurred. Chloroform was added and the culture centrifuged to separate any bacterial debris from the phage lysate.

(c) UV induction of lysogens

$\lambda$ lysogens were grown in M9-minimal medium + casamino acids and 10 mM MgCl$_2$ to A$_{450}$ and UV-irradiated with 40 Jm$^{-2}$. After 90-120 min further incubation the cells lysed, chloroform was added and the culture centrifuged to remove bacterial debris.

2.9. Isolation of $\lambda$lysogens

Lysogens were isolated by placing drops of phage onto a bacterial lawn on a Trypticase agar plate. After incubation overnight cells were taken from the turbid zone of lysis and streaked onto a nutrient agar plate to single colonies. These were tested for lysogeny by stabbing into a bacterial lawn on a Trypticase agar plate and incubating overnight. Lysogens gave halos of lysis on the test plate.

2.10. $\gamma$ (Tn1000) mutagenesis

The plasmid to be mutagenised was first transformed into the F$^+$ strain RB308recA, str$^S$. Both the donor and the recipient strain (str$^R$) were grown in nutrient broth, with only very gentle aeration, to A$_{450}$ 0.2 and the two cultures mixed in the ratio of 1:10, donor to recipient. The mating culture was left incubating for 2-3 h and then serial dilutions plated out onto selective plates.
2.11. Preparation of chromosomal DNA

This was essentially the method of Chou et al. (1977). 200 ml of stationary phase cells were grown in nutrient broth and washed twice in an equal volume of cold TE buffer made 0.1 M with respect to NaCl. After resuspending in 25 ml of the same buffer, 5 ml of lysozyme (10 mg/ml) was added and incubated at 37°C for 10 min. To this was added 30 ml of TE buffer, containing 2% sarkosyl NL97 and 20 μg/ml ribonuclease A and incubation continued for another 60 min at 42°C. Pronase (pre-digested by incubation at 37°C for 1 h) was added to a final concentration of 1 mg/ml. After incubation at 42°C for 4 h, the preparation was extensively phenol extracted to remove all protein. The DNA was then dialysed against several changes of TE buffer and stored at -20°C.

2.12. Ethanol precipitation of DNA

DNA samples to be precipitated were adjusted to 0.3 M in NaAc, 2.5 volumes of cold 100% ethanol were added and the sample cooled in a dry ice/IMS bath for 10 min. The tube was then centrifuged in a precooled centrifuge for 10 min. After washing the DNA pellet with 70% ethanol to remove salt, the tube was centrifuged again and the supernatant carefully removed. The DNA pellet was dried under vacuum and resuspended in TE buffer or sterile H₂O.

2.13. Preparation of plasmid DNA

(a) Rapid plasmid DNA preparation

An adaption of the method described by Birnboim and Doly (1979) was used to prepare small amounts of plasmid DNA.
### Table 2.2

**Reagents used in the preparation of DNA**

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Composition</th>
</tr>
</thead>
</table>
| Tris-sucrose           | 50 mM Tris-HCl pH 8.0  
                          | 25% sucrose (w/v)                                                        |
| TE buffer              | 10 mM Tris-HCl pH 7.5  
                          | 1 mM EDTA                                                                |
| TES buffer             | 50 mM Tris-HCl pH 8.0  
                          | 5 mM EDTA  
                          | 50 mM NaCl                                                               |
| TEG buffer             | 25 mM Tris-HCl pH 8     
                          | 10 mM EDTA  
                          | 50 mM Glucose                                                           |
| Alkaline SDS           | 200 mM NaOH  
                          | 1% SDS                                                                   |
| Potassium acetate      | 60 ml 5 M KAc  
                          | 11.5 ml glacial acetic acid  
                          | 28.5 ml H₂O                                                              |
| Triton lysis mix       | 2% Triton X-100 (v/v)  
                          | 50 mM Tris-HCl pH 8.0                                                   |
| PEG-NaCl               | 25% polyethylene glycol 6000  
                          | 1.25 M NaCl                                                              |
| CsCl-EtBr mix          | 80 g caesium chloride  
                          | 52 ml TES buffer  
                          | 8 ml EtBr (5 mg/ml)  
                          | refractive index = 1.3990–1.4000                                       |
| Phenol mix             | 100 g phenol dissolved in 100 ml CHCl₃  
                          | 4 ml isoamyl alcohol  
                          | 0.1 ml 8-hydroxyquinoline                                              |
(Maniatis et al., 1982). 3ml of an overnight culture of plasmid bearing cells were taken and pelleted in an eppendorf tube. The cells were resuspended in 0.1 ml of a 1 mg/ml lysozyme solution in TEG buffer and left on ice for 5 min, 0.2 ml of alkaline SDS was added and after 5 min on ice 0.15 ml of cold potassium acetate solution was added. After a further 5 min on ice, tubes were centrifuged (4 min ) and extracted once with phenol. 1 ml of 100% ethanol at room temperature was added and the tubes centrifuged for 7 min. The pellets were washed with 70% ethanol, vacuum dried and resuspended in 50μl of sterile water. RNA was removed, if required, by the addition of 5μl of DNA'ase free RNA'ase (1 mg/ml) and samples incubated at 37°C for 30 min.

(b) Large scale plasmid preparation

400 ml of nutrient broth + antibiotics were inoculated with cells containing the required plasmid and incubated overnight with vigorous agitation. After harvesting (Sorvall GS3, 5,000 rpm, 4°C) the cells were chilled, washed with 10 ml bacterial buffer, and resuspended in 3 ml Tris-sucrose, and 0.5 ml lysozyme (10 mg/ml) / RNase (300 μg/ml) solution was added. This mixture was incubated at room temperature for 5 min, 1 ml 0.25 M EDTA was added, and the mixture again incubated for 5 min. 4 ml triton lysis mix were added and the tube inverted until lysis was complete. The lysate was cleared by centrifugation (Sorvall SS34, 18,000 rpm, 20 min, 4°C) and 2/3 volume of PEG/NaCl added to precipitate the DNA. After 2-4 h on ice the precipitate was collected by centrifugation and resuspended in 1.1 ml of TES buffer. The 1.1 ml lysate was transferred to a Beckman VTi65 self-sealing tube, and
underlayed with 4 ml CsCl-EtBr solution. The tube was centrifuged to equilibrium in a Beckman VTi65 rotor (either 55,000 rpm, 3 h, 15°C, or 50,000 rpm, 16-20 h, 15°C). When plasmid DNA was clearly visible as a quite separate band from the chromosomal DNA it was removed, from the side, with a syringe needle. Ethidium bromide was removed from the DNA by extraction with NaCl-saturated propan-2-ol and the DNA then dialysed to remove CsCl. If required the plasmid DNA was phenol extracted and/or concentrated by ethanol precipitation before use.

2.14. Agarose gel electrophoresis

Horizontal slab gels were prepared by boiling agarose in Tris-acetate electrophoresis buffer (40 mM Tris, 1 mM EDTA, adjusted to pH7.5 with glacial acetic acid), adding ethidium bromide (0.5 μg/ml) and pouring into a mould. Samples were prepared by adding 1/3 volume loading buffer (0.01% w/v bromophenol blue, 10% glycerol, 250 mM Tris-HCl pH8.0). Samples were electrophoresed at 100 V with the gel submerged in Tris-acetate buffer + 0.5μg/ml ethidium bromide. DNA was visualised by transillumination with short wave UV light (260 nm) and photographed through an orange filter using a Polaroid MP-3 land camera with Polaroid 4 x 5 land film (types 52, 55 or 57). As DNA molecular weight standards in agarose gel electrophoresis, λ+ bacteriophage DNA with digested with HindIII was used. This generated fragments having sizes of 23.131, 9.418, 6.557, 4.361, 2.322, 2.028, 0.564 and 0.125 kb.
2.15. Restriction enzyme digestions

Wherever possible restriction endonuclease enzymes were used in BRL core buffer (particularly for double enzyme digestions). Where this was not possible individual buffers were used as shown in Table 2.3. Digestions were performed at 37°C for 1 h unless indicated in the text. Where difficulty was encountered in digesting DNA, spermidine-HCl was added to a final concentration of 4 mM. Reactions were stopped by heating at 65°C for 10 min or adding 1/5 th volume of 0.1 M EDTA pH 8.0. All DNA manipulations were performed using sterile tubes and reagents to minimise nuclease contamination.

2.16. Alkaline phosphatase treatment

(a) Preparation of the alkaline phosphatase

The ammonium sulphate suspension of calf intestinal alkaline phosphatase was mixed well and 50 units removed and pelleted by brief centrifugation. The supernatant was discarded and the pellet resuspended in 0.5 ml TE buffer containing 2 mM ZnCl₂. This was dialysed against 200 ml TE containing 0.1 mM ZnCl₂ for 30 min at 4°C, aliquoted and stored at 4°C.

(b) Use of alkaline phosphatase (Maniatis et al., 1982)

DNA was digested with restriction endonucleases, phenol extracted, and ethanol precipitated. After resuspending in a minimal volume of 10 mM Tris-HCl (pH 8.0), 5 μl of 10 x CIP buffer (Table 2.3) and 48 μl of H₂O were added followed by 0.005-0.02 units of calf intestinal phosphatase depending on the DNA size and concentration. Incubation was performed for 15 min at 37°C followed by 15 min at 56°C, a further aliquot of enzyme was added and incubations repeated at both
Table 2.3

Restriction buffers (x5)

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Tris/Cl</th>
<th>pH</th>
<th>MgCl$_2$</th>
<th>NaCl</th>
<th>KCl</th>
<th>DTT</th>
<th>SH</th>
<th>EDTA</th>
</tr>
</thead>
<tbody>
<tr>
<td>EcoRI</td>
<td>500</td>
<td>7.4</td>
<td>50</td>
<td>250</td>
<td>-</td>
<td>2.5</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>BamHI</td>
<td>30</td>
<td>7.4</td>
<td>30</td>
<td>250</td>
<td>-</td>
<td>-</td>
<td>30</td>
<td>-</td>
</tr>
<tr>
<td>HindIII</td>
<td>30</td>
<td>7.4</td>
<td>50</td>
<td>250</td>
<td>-</td>
<td>2.5</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>core buffer</td>
<td>50</td>
<td>8.0</td>
<td>10</td>
<td>50</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

figures represent mM concentrations

Ligation buffer (x10)

- 1M Tris/Cl pH 7.4 660μl
- 1M MgCl$_2$ 100μl
- 10mM ATP 100μl
- DTT 15mg
- BRL nuclease free BSA 10μl
- H$_2$O 90μl

DTT - dithiothreitol; SH - β-mercaptoethanol.

CIP buffer (x10)

- Tris/HCl pH 9.0 0.5 M
- MgCl$_2$ 10 mM
- ZnCl$_2$ 1 mM
- spermidine 10 mM

STE (x10)

- Tris/HCl pH 8.0 10 mM
- EDTA 1 mM
- NaCl 100 mM
temperatures. To this mixture was added 40 μl of H₂O, 10 μl of 10x STE and 5 μl of 10% SDS and heated to 65°C for 15 min. The DNA was extracted twice with phenol and passed through a Sephadex G-50 column equilibrated with TE buffer. After precipitation with ethanol DNA was ready for ligation.

2.17. Recovery of DNA from agarose gels

a) DE81 method (Dretzen et al., 1981)

Whatman DE81 chromatography paper was cut to a convenient size, and pieces soaked for several h in 2.5 M NaCl. After being rinsed in distilled water, the paper strips were stored in 1 mM EDTA at 4°C until required. DNA was digested as required, loaded onto an agarose gel, and electrophoresed until acceptable band separation had been achieved. The DNA bands were visualised under UV light, the gel cut in front of the band to be purified, and DE81 paper inserted. If any fragments ran closely behind, paper was inserted behind the band also. After electrophoresing the DNA onto the paper, the paper was removed, washed well in distilled water and blotted dry. It was transferred to a 1.5 ml Eppendorf tube and 0.5 ml of high salt buffer (1 M NaCl, 50 mM Tris-HCl pH 8.0, 1 mM EDTA) added. The paper was shredded by vortexing and incubated at 37°C for 2 h followed by 65°C for 10 min. The eluted DNA was separated from the paper by centrifugation through a hole in the bottom of the tube, and passed through a polyallomer plug to remove any remaining paper. The DNA was then extracted once with butan-2-ol to remove EtBr, ethanol precipitated twice (the addition of NaAc being unnecessary for the first precipitation), and resuspended in a small
b) Freeze-squeeze method (Tautz and Renz, 1983)

DNA restriction fragments were loaded onto an agarose gel and electrophoresed until well separated and the gel slice containing the fragment of interest removed using a razor. The gel slice was equilibrated for 15-45 min in 0.3 M NaAc, 1 mM EDTA in the dark and then transferred to a small eppendorf centrifuge tube (previously pierced at the bottom and plugged with polyallomer wool). This tube was frozen in dry ice/IMS and centrifuged (still frozen) in an eppendorf centrifuge for 10 min allowing eluant to pass into a large eppendorf centrifuge tube below. To the eluant was added 1/100 vol of 1 M MgCl₂ and 1/10 vol of acetic acid before precipitation of the DNA by ethanol. The isolated DNA fragment was washed in 70% ethanol before resuspension in TE buffer. Yields were comparable with those obtained using the DE81 method.

2.18. Ligation

DNA samples were digested with restriction endonucleases, phenol extracted, ether extracted and ethanol precipitated. The DNA pellet was resuspended in a small volume of sterile H₂O. Ligation buffer (10 x ,Table 2.3) and T4 DNA ligase were added and the mixture incubated at 14°C overnight. The efficiency of ligation of the DNA was monitored by agarose gel electrophoresis.

2.19. Transformation

(a) Preparation of competent cells

Recipient cells were grown in nutrient broth to A₄₅₀ 0.4-0.5 and chilled in ice. 10 ml of cells were pelleted and
resuspended in 5 ml of cold 0.1 M MgCl₂ (all centrifugation was done at 4°C). Cells were again pelleted and resuspended in 5 ml of cold 0.1 M CaCl₂ and left on ice. After 20 min the cells were harvested and resuspended in 0.6 ml cold 0.1 M CaCl₂. Cells stored at 4°C remained competent for up to 7 days but highest frequencies of transformation were achieved with cells prepared 24 h before use.

(b) Transformation

200 μl of competent cells were taken and up to 10 μl of DNA added. After 1 h on ice, cells were heat shocked for 5 min at 42°C (2 min if the strain was temperature sensitive) and added to 2 ml of prewarmed nutrient broth and shaken for 1-2 h at 37°C (30°C for temperature sensitive strains). Serial dilutions were plated out onto selective agar plates and grown under appropriate conditions.

2.20. The maxicell system

The strain used for this in vivo expression system was CSH26ΔF6, which carries a recA deletion and is therefore highly sensitive to ultraviolet irradiation. The method used here is based on that of Sancar et al. (1979), who found that low UV doses cause extensive degradation of chromosomal DNA in recA strains, while most plasmid DNA copies remain intact due to a lower probability of receiving a UV hit. Plasmid encoded proteins can therefore be identified by labelling the proteins synthesised after chromosomal degradation has occurred.

CSH26ΔF6 carrying the plasmid of interest was grown in minimal medium + casamino acid to an A₄₅₀ 0.5 and 5 ml of
cells UV-irradiated with $3.75 \text{ Jm}^{-2}$ over 10 s. The irradiated culture was transferred to a foil wrapped flask (to prevent DNA repair by photoreactivation). In order to kill any cells undamaged by the UV-irradiation either ampicillin ($25 \mu g/ml$) or cycloserine ($250 \mu g/ml$) was added to the irradiated culture before incubation overnight. 3 ml of Maxi-cells were harvested in eppendorf tubes and washed three times with minimal medium + methionine assay casaminoacids (which contains only a very low concentration of methionine) and finally resuspended in 0.5 ml of this medium. After 1 h incubation at $37^\circ C$ 2 $\mu l$ of $35S$-methionine was added to label the plasmid encoded proteins and incubation continued, normally for 30-60 min after which 40 $\mu l$ of cold methionine (44mg/ml) was added. The bacteria were then pelleted in an eppendorf centrifuge tube and resuspended in 40 $\mu l$ SDS-PAGE buffer A and 40 $\mu l$ SDS-PAGE sample buffer and heated to $100^\circ C$ for 5 min. The sample was then ready for analysis by SDS-PAGE.

2.21 Zubay in vitro transcription-translation system

The *E. coli* extracts were prepared as described by Pratt et al (1984) using strain N138 ($\text{recB}^{\text{ts}}$). Transcription-translation incubations were carried out at $37^\circ C$ and contained:

- 7.5 $\mu l$ low molecular weight mix
- 2.0 $\mu l$ $^{35}S$-methionine (25 Ci)
- 3.5 $\mu l$ 0.1M magnesium acetate
- 5.0 $\mu l$ S30 extract (strain indicated in text)
- 2 - 5$\mu g$ DNA in 10mM Tris/Cl 1mM EDTA pH 7
- 10mM Tris/acetate pH 7 to 30$\mu l$
After 30 min incubation, 5μl of prewarmed 44mg/ml unlabelled methionine was added and the incubation continued for a further 5 min. The protein products were analysed by SDS PAGE and autoradiography.

2.22 SDS polyacrylamide gel electrophoresis

a) Preparation and running of gels

The procedure was based on that of Laemmli (1970), using a Biorad 220 slab gel apparatus without cooling. The buffers, solutions and gel recipes used are given in Table 2.4. Gels were usually 1mm thick, and composed of a 7% acrylamide stacking gel with an 11% or 15% acrylamide separating gel, with 1cm of effective stacking distance between the sample wells and the surface of the separating gel. All samples were boiled for 5 min before loading and electrophoresis carried out at 25 mAmps/gel, until the dye front was within 5mm of the bottom of the gel. Gels were then either fixed by shaking in 200 - 300ml of destain (Table 2.4) for at least 30 min, or shaken in 200 - 300ml of stain overnight. Stained gels were destained by diffusion in several changes of destain, shaking throughout.

b) Molecular weight markers

For radioactive molecular weight markers, a ¹⁴C-methylated protein mixture was used, which contained myosin (200kD), phosphorylase B (100kD and 92.5kD), bovine serum albumin (69kD), ovalbumin (46kD), carbonic anhydrase (30kD) and lysozyme (14.3kD). 5nCi of this mixture, with sample buffer added, was loaded per gel slot, or, if the gel was to be fluorographed, 1nCi per slot.
### Table 2.4: SDS PAGE solutions, buffers and gel recipes

<table>
<thead>
<tr>
<th>Gel composition</th>
<th>15%</th>
<th>11%</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Separating gel:</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Buffer A</td>
<td>13.5</td>
<td>13.5ml</td>
</tr>
<tr>
<td>Acrylamide</td>
<td>9.2</td>
<td>6.8ml</td>
</tr>
<tr>
<td>H₂O</td>
<td>3.6</td>
<td>6.0ml</td>
</tr>
<tr>
<td>Ammonium persulphate (APS)</td>
<td>1.0</td>
<td>1.0ml</td>
</tr>
<tr>
<td>N,N,N',N'-tetramethyl ethylenediamine (TEMED)</td>
<td>75</td>
<td>75µl</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>Stacking gel:</strong></th>
<th>Buffer B</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Acrylamide</td>
<td>3.3ml</td>
<td></td>
</tr>
<tr>
<td>H₂O</td>
<td>6.7ml</td>
<td></td>
</tr>
<tr>
<td>APS</td>
<td>0.5ml</td>
<td></td>
</tr>
<tr>
<td>TEMED</td>
<td>40µl</td>
<td></td>
</tr>
</tbody>
</table>

TEMED was always added immediately before the gel was poured.

- **Buffer A**
  - Tris/Cl pH 8.8
  - SDS: 0.75M
  - 0.2% w/v
- **Buffer B**
  - Tris/Cl pH 6.8
  - SDS: 0.25M
  - 0.2% w/v
- **Acrylamide**
  - 44% w/v
- **Electrophoresis buffer**
  - Trisma base: 0.125 M
  - Glycine: 0.192 M
  - SDS: 0.1% w/v
- **Sample buffer**
  - Tris/Cl pH 6.8
  - Glycerol: 20% v/v
  - 3-mercaptoethanol: 10% v/v
  - SDS: 4% w/v
  - Bromophenol blue: 0.05% w/v

1/3 volume of this sample buffer was added to each sample before boiling and electrophoresis unless indicated otherwise.

- **Destain**
  - Isopropanol: 25% v/v
  - Acetic acid: 10% v/v

- **Stain**
  - Coomassie brilliant blue: 0.05% w/v in destain
c) Autoradiography and fluorography

For autoradiography, fixed gels were dried onto a sheet of Whatman No. 17 chromatography paper using a Biorad slab gel drier model 1125. The dried gels were placed in a cassette with a sheet of Kodak XR P5 X-ray film for exposure, and the films developed using Kodak DX-80 developer, a 1% acetic acid wash, and Kodak FX-40 fixer.

Fluorography was carried out using the method of Bonner and Lasky (1974): fixed gels were dehydrated by two 30 min washes in 300ml dimethylsulphoxide (DMSO), after which the gels were soaked for 90 min in 200ml 22% PPO in DMSO and then rehydrated by soaking in water for 60 min. The gels were then dried and autoradiographed as above, except that cassettes were stored at -80°C during exposure.

d) Densitometric scanning of autoradiographs

Autoradiographs were densitometrically scanned using an LKB 2202 Ultrascan laser densitometer and data was analysed using an LKB 2220 recording integrator.

2.23 Cell fractionation

The basic procedure was that described by Churchward and Holland (1976). Exponentially growing cells at $A_{450} = 0.5$ or $^{35}$S-methionine labelled maxi-cells were harvested and resuspended in 10ml ice cold 10mM sodium phosphate buffer, pH7.2 (+ or - 10 mM MgCl$_2$). Samples were transferred to a 25ml beaker, and sonicated for 3 x 30 sec with 30 sec cooling on ice, at amplitude 6 m, using the 3/4 inch end diameter probe in a 150 Watt MSE ultrasonic disintegrator. The sonicated samples were centrifuged in a Sorvall SM24 rotor for 5 min at 7krpm to remove unlysed cells. If required,
0.5ml of the supernatant was removed at this stage as a total cell sample, before pelleting the membranes in a Beckman 50Ti rotor for 30 min at 30krpm and 5°C. The supernatant was retained and proteins precipitated by the addition of 50% TCA to a final concentration of 10% (w/v) as the cytoplasmic fraction. The membrane pellet was resuspended in 1ml of 10mM sodium phosphate buffer (+ or - MgCl$_2$), and repelleted in the 50Ti rotor for 30 min at 30krpm and 5°C.

The washed membrane pellet was resuspended in 200µl of 0.5% w/v sarkosyl NL97, and 50µl removed for a total envelope fraction, if required. The remainder was incubated at room temperature for 30 min before centrifuging in the 50Ti rotor for 1 h at 35krpm and 15°C. The supernatant from this centrifugation represented the solubilised inner membrane proteins; the pellet, containing the sarkosyl insoluble outer membrane proteins, was then resuspended in 150 µl of 10mM sodium phosphate buffer, pH 7.2. 1/3 volume of acrylamide gel sample buffer was added to each fraction in an eppendorf tube, and the samples boiled for 5 min and frozen at -20°C. Resuspension of membrane pellets at each stage was found to be easier if the pellets were frozen in the resuspension solution and resuspended as they thawed.

When loading the samples thus prepared onto acrylamide gels, sample volumes used represented equivalent cellular proportions.
Table 2.5: Sources of chemicals

Radiochemicals were obtained from Amersham International. S-methionine used had a specific activity of 50-55 TBq/mmol and a radioactive concentration of 500-560 MBq/ml; all other chemicals were obtained from Fisons (AR grade) except:

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetic acid</td>
<td>Fisons SLR</td>
</tr>
<tr>
<td>Acrylamide</td>
<td>Eastman Kodak</td>
</tr>
<tr>
<td>Amino acids</td>
<td>Sigma</td>
</tr>
<tr>
<td>Ammonium persulphate</td>
<td>Biorad</td>
</tr>
<tr>
<td>Ampicillin</td>
<td>Sigma</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>Sigma</td>
</tr>
<tr>
<td>Coomassie brilliant blue</td>
<td>Gurr's</td>
</tr>
<tr>
<td>cycloserine</td>
<td>Sigma</td>
</tr>
<tr>
<td>Gelatin</td>
<td>Difco</td>
</tr>
<tr>
<td>8-hydroxyquinoline</td>
<td>AnalaR</td>
</tr>
<tr>
<td>Isoamylalcohol</td>
<td>Sigma</td>
</tr>
<tr>
<td>Isopropanol</td>
<td>Fisons SLR</td>
</tr>
<tr>
<td>Kanamycin</td>
<td>Sigma</td>
</tr>
<tr>
<td>Lysozyme</td>
<td>Sigma</td>
</tr>
<tr>
<td>β-mercaptoethanol</td>
<td>Sigma</td>
</tr>
<tr>
<td>Methyl methane sulphonate</td>
<td>BDH</td>
</tr>
<tr>
<td>N,N'-methylene-bis-acrylamide</td>
<td>Eastman Kodak</td>
</tr>
<tr>
<td>Restriction endonucleases</td>
<td>BRL</td>
</tr>
<tr>
<td>Sodium dodecyl sulphate</td>
<td>Biorad</td>
</tr>
<tr>
<td>Streptomycin</td>
<td>Glaxo</td>
</tr>
<tr>
<td>T4 ligase</td>
<td>Biolabs</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>Sigma</td>
</tr>
<tr>
<td>N,N,N',N'-tetramethylethylenediamine</td>
<td>Eastman Kodak</td>
</tr>
<tr>
<td>Trisma base</td>
<td>Sigma</td>
</tr>
<tr>
<td>Tryptone</td>
<td>Oxoid</td>
</tr>
<tr>
<td>Thiamine HCl</td>
<td>Sigma</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>Oxoid</td>
</tr>
</tbody>
</table>
3.1 Introduction

Two considerations were paramount in devising a cloning strategy for sfiB. Firstly, the only well documented phenotype of sfiB mutants was the suppression of the persistent filamentation shown by lon, or by recA441(tif) or tsl (lexA(Ts)) strains under restrictive conditions (i.e. after UV-irradiation or shifting to 42°C respectively). Secondly sfiB mutants had been shown by Huisman et al. (1980c) to be recessive. This was achieved by the introduction of an F' (F' 104) covering the region to which sfiB had been mapped, into a recA441, lon, sfiB114 strain. This F' restored the recipient to temperature and UV sensitivity thereby demonstrating the complementation of the recessive chromosomal sfiB mutation by the incoming wild type sfiB\textsuperscript{+} gene. The continued presence of the sfiB114 allele was confirmed by using a P1 generalised transducing lysate grown on the presumed diploid strain in order to transduce a recipient strain from SfiB\textsuperscript{+} to SfiB114. On the basis of these observations a cloned sfiB\textsuperscript{+} gene could only be identified by its ability to reverse the suppression of filamentation shown by a sfiB mutant. This might be detected by screening for complementation of a sfiB allele, but a simple direct selection for an sfiB\textsuperscript{+} clone was clearly precluded.

sfiB mutants show very slow growth at high temperature (42-44°C) (George et al., 1975; Johnson, 1977), and indeed Huisman et al. (1980c) observed a small, approximately 6\%,
reduction in growth rate of sfiB\textsuperscript{-} cells compared to sfiB\textsuperscript{+} cells even at 37\textdegree C. In addition, PAM162m\textsuperscript{+} (lon, sulB26) showed highly abnormal cell morphology when grown in nutrient broth or on nutrient agar plates at 44\textdegree C. Nevertheless, these properties were not considered to be a suitable basis for use as a selection for complementation of sulB26 in cloning experiments. However, when PAM162m\textsuperscript{+} was grown for 30 generations in nutrient broth at 44\textdegree C and plated on nutrient agar plates, after overnight incubation at 44\textdegree C large and small colonies were obtained. Large colonies were phenotypically SfiB\textsuperscript{+} (i.e. normal cell morphology when grown at 44\textdegree C and MMS\textsuperscript{S}) and were therefore assumed to be sfiB\textsuperscript{+} revertants. One such revertant, PAM162sfi\textsuperscript{+} (leu, lon) was used in later work.

In the absence of a suitable selection procedure for the cloning of sfiB by "shotgun" cloning methods, the decision was made to map the sfiB locus precisely using P1 transduction and then to clone the gene along with the nearest selectable marker.

3.2 P1 transduction mapping

sfiB mutations were provisionally mapped by Johnson (1977) and George et al. (1975) and found to lie between leu and azi at approximately 2 min on the E.coli chromosomal map and this formed the starting position for the genetic analysis described below.

The 2 min region of the E.coli chromosome is composed almost entirely of genes involved in either cell division or cell wall synthesis (Fig. 3.1) (Table 3.1) and this created a
Figure 3.1
Organisation of genes in the 2 minute region of the E.coli chromosome.
Table 3.1

<table>
<thead>
<tr>
<th>gene</th>
<th>phenotype of mutant</th>
</tr>
</thead>
<tbody>
<tr>
<td>leuA</td>
<td>leucine prototrophy</td>
</tr>
<tr>
<td>pbp3</td>
<td>filaments when grown at high temperature</td>
</tr>
<tr>
<td>murE, murF, murC, ddl</td>
<td>lyse when grown at high temperature</td>
</tr>
<tr>
<td>ftsQ, ftsA, ftsZ</td>
<td>filament when grown at high temperature</td>
</tr>
<tr>
<td>envA</td>
<td>resistance to antibiotics and forms chains during growth</td>
</tr>
<tr>
<td>secA</td>
<td>lethal defect in secretory mechanism when grown at high temperature</td>
</tr>
<tr>
<td>azi</td>
<td>resistance to azide and cells filament when grown at high temperature</td>
</tr>
</tbody>
</table>
number of problems in the mapping of sfiB. Firstly, only leu could be used as a primary selection in P1 transduction experiments. Other available mutant markers in this region show only reduced growth and/or filamentation under restrictive conditions and so residual growth was considerable, preventing the routine identification of transductants. Secondly, three factor crosses involving the formation of multiple cell division/cell wall mutants (including sfiB) often gave classes of recombinants showing poor viability and growth. This indicated that such recombinants could be underrepresented and the results of crosses could be seriously distorted. Consistant data for 2 factor crosses was obtained however, and some examples are summarised in Table 3.2. These results show that sfiB lies between ftsA and azi, and very close to envA. This was consistent with previously reported genetic analysis and the use of additional markers resulted in a more specific localisation. However, despite a great deal of effort to improve the identification of specific transductional classes and attempts to obtain additional selectable markers in this region, further attempts to improve the analysis were abandoned for the technical reasons indicated above.

3.3 Specialised $\lambda$-transducing phages

Shortly after P1 transduction experiments had implicated the ftsA-secA region as being the location for the sfiB locus, a number of specialised $\lambda$-transducing phages covering this region became available (e.g. Fletcher et al., 1978) as an alternative approach to mapping sfiB more precisely. Tests for complementation of sfiB by these transducing phages were
Table 3.2

<table>
<thead>
<tr>
<th>Donor</th>
<th>Recipient</th>
<th>Co-transduction</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\text{leuB}^+, \text{pbp3}^-$</td>
<td>$\text{leuB}^-, \text{pbp3}^+$</td>
<td>88% (198/225)</td>
</tr>
<tr>
<td>$\text{leuB}^+, \text{ftsA}^+$</td>
<td>$\text{leuB}^-, \text{ftsA}^+$</td>
<td>59% (159/270)</td>
</tr>
<tr>
<td>$\text{leuA}^+, \text{sfiB}^+$</td>
<td>$\text{leuA}^-, \text{sfiB}^{114}$</td>
<td>41% (111/270)</td>
</tr>
<tr>
<td>$\text{leuA}^+, \text{envA}^-$</td>
<td>$\text{leuA}^-, \text{envA}^+$</td>
<td>41% (92/225)</td>
</tr>
<tr>
<td>$\text{leuB}^+, \text{azi}^+$</td>
<td>$\text{leuB}^-, \text{azi}^-$</td>
<td>25% (56/225)</td>
</tr>
</tbody>
</table>
always carried out in a \textit{tsl} chromosomal background. The \textit{tsl} mutation is expressed at $42^\circ$C and leads to induction of all LexA repressed SOS functions (including division inhibition) but without inducing $\lambda$-prophage.

\textbf{a) $\lambda$DO2 and $\lambda$16-2}

Specialised transducing phages $\lambda$DO2 (Oliver and Beckwith, 1982) and $\lambda$16-2 (Luktenhaus and Donachie, 1979) carrying \textit{ftsA-secA} and \textit{murC-envA} regions respectively (Fig. 3.2) were used to lysogenise strain GC2490 (\textit{tsl, sfiB114}) and lysogens tested by growth at $42^\circ$C on nutrient agar plates for complementation of \textit{sfiB114} (i.e. restoration of filamentation at $42^\circ$C). Neither lysogen class showed such filamentation when examined microscopically, and did not therefore show complementation of \textit{sfiB114}, although the same lysates of $\lambda$DO2 and $\lambda$16-2 used to lysogenise GC2490 were able to complement temperature sensitive mutations in \textit{secA} (MM52) and \textit{ftsA} (TKF12) respectively.

\textbf{b) $\lambda$sep$^+$82, $\lambda$ sep$^+$3 and $\lambda$sep$^+$46}

Fletcher \textit{et al.} (1978) described a set of defective $\lambda$-transducing phages carrying carrying \textit{E.coli} chromosomal segments extending from \textit{leuA} in a clockwise direction towards \textit{secA}. These phages were obtained from the induction of a lysogen having $\lambda$cl$^{857}$ integrated into the \textit{leu} operon. Such a lysate provided a range of defective specialised transducing phages, the largest covering \textit{leuA-envA} (Table 3.3) (Fig. 3.2). Although all phages carried only a portion of \textit{leuA}, an intact gene could be reformed upon integration into the chromosome of a \textit{leuA} mutant by homologous recombination. A helper phage, either $\lambda^+$ or $\lambda$cl$^{857}$, is required for the
Table 3.3

<table>
<thead>
<tr>
<th>Phage</th>
<th>leuA</th>
<th>pbp3</th>
<th>murE</th>
<th>murF</th>
<th>murC</th>
<th>ddl</th>
<th>ftsA</th>
<th>envA</th>
</tr>
</thead>
<tbody>
<tr>
<td>XleuA13</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>λsep⁺82</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>λsep⁺69</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>λsep⁺27</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>λsep⁺3</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>λsep⁺24</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>λmurF⁺121</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>λsep⁺46</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>
Figure 3.2
A schematic diagram of the chromosomal DNA carried by transducing phages $\lambda$sep$^+$82, $\lambda$sep$^+$3, $\lambda$sep$^+$46, $\lambda$16-2 and $\lambda$DO2.
leuB  leuA  pbpB  mur E F C  ddl  ftsQ  ftsA  ftsZ  envA  secA

\[\lambda\text{sep}_82\]

\[\lambda\text{sep}_3\]

\[\lambda\text{sep}_46\]

\[\lambda\text{l}6-2\]

\[\lambda\text{D}02\]
production of a high frequency transducing lysate (HFT) from a lysogen of these defective transducing phages, although a helper phage is not required for lysogeny.

Lysogens carrying phages $\lambda cI^{857} \lambda sep^{+82}$ (leuA-pbp3), $\lambda^+ \lambda sep^{+3}$ (leuA-murC) and $\lambda^+ \lambda sep^{+46}$ (leuA-envA) (Fig 3.2) were induced and HFT's used to transduce strain GC2490$\lambda^+$ (leuA, tsl, sfiB114) to leu$^+$. In all, 90 leu$^+$ GC2490$\lambda^+$ colonies obtained from each HFT lysate were tested for complementation of sfiB114 by patch plating onto nutrient agar plates at 42°C and examined microscopically for filamentation. No phenotypically SfiB$^+$ colonies were obtained from lysates of either $\lambda sep^{+82}$ or $\lambda sep^{+3}$. In contrast, although the majority of the $\lambda sep^{+46}$ lysogens also failed to show complementation, 6 of the leu$^+$ colonies (7%) produced by this lysate did show extensive filamentation when grown at 42°C. However, when these six lysogens were induced, the $\lambda$ lysates produced were incapable of transducing a leuA strain to leu$^+$. It was concluded that in these six strains recombination had taken place between the leu-sfiB114 segment on the chromosome and the leu$^+$-sfiB$^+$ segment on the $\lambda sep^{+46}$ giving rise to a sfiB$^+$ derivative i.e. that marker rescue had taken place.

3.4 Discussion
In attempting to interpret the results of the $\lambda$-transducing data we had to look at the detailed organisation of the genes in the ftsQ-secA region (Fig 3.3). Five genes (ftsQ, ftsA, ftsZ, envA and secA) have been mapped and shown to be transcribed in a clockwise direction. In addition, V.Darby had previously shown (PhD thesis, 1981) that a promoter and open reading frame exists between envA and secA and that this
Figure 3.3
The detailed organisation of genes in the \textit{ftsQ-secA} region of the \textit{E.coli} chromosome. The gene labelled "U" is the open reading frame between \textit{envA} and \textit{secA} reported by Darby (1981) and Donachie (1984).
secA

ftsZ

ftsQ

envA

kb

U
also may be involved in cell division (Sullivan and Donachie, 1984) although its precise functional role is unknown. Since λ sep⁺46, but not λ sep⁺3, provided an opportunity for marker rescue of sfiB114 this implied that at least part of the sfiB gene was located between murC and secA (see Fig 3.3). If this is the case then either λ16-2 or λ DO2, which overlap this region should show complementation. One explanation for the lack of complementation shown by λ16-2 and/or λ DO2 could be that transcription from λ promoters prevented efficient expression of sfiB⁺ (Ward and Murray, 1979). However since these phages were capable of expressing other transduced markers, such an effect would have been anomalous. The reason for the observation of a high frequency of marker rescue (7%) when attempting to lysogenise with λ sep⁺46 was thought to be due to the very low lysogenisation frequency shown by all of these defective phages (Fletcher et al., 1978). In consequence, a relatively high proportion of the leu⁺ cells obtained from the use of such lysates might result from marker rescue.

Whilst these experiments were in progress evidence began to accumulate from other groups that at least some sfiB mutations were dominant. Gottesman (1981) isolated an sfiB mutation (sfiB367) which showed dominance using similar tests to those carried out by Huisman et al. (1980c), but using strains with different chromosomal backgrounds. Gottesman (1981) concluded that the recessive/dominance character of sfiB observed using partial diploid experiments could be affected greatly by the particular strain used. It was also claimed that strain CGSC4251, carrying F'104 (1-6 min.) and
used for determining the recessivity of sfiB mutations was in fact an Hfr strain (R.D'Ari, Personal Communication). This would explain the failure of V.Darby (1981) to repeat the work of Huisman et al. (1980c) on the recessivity of sfiB. At this point it was suspected, due to the lack of complementation of some of the specialised transducing phages that sfiB114 was also at least partially dominant. In view of this, it was decided to clone the ftsA-envA region from chromosomal DNA prepared from an sfiB114 strain (GC2490) and investigate this region for the presence of an at least partially dominant sfiB locus.
4.1 Introduction

Results presented in the previous chapter indicated that a) sfiB114 could be dominant, and b) sfiB114 is located between ftsA and secA in the 2 min region of the E.coli chromosome. We therefore wished to isolate this region from the DNA of a sfiB strain (GC2490) in order to test for the presence of a dominant sfiB locus. The strategy adopted involved selecting a clone harbouring the ftsA+ region from a bank of E.coli genes, by its ability to complement a recessive ftsA mutation enabling such a mutant to grow at 42°C.

4.2 Choice of cloning vector

In view of their convenience for use in gene expression systems, easy manipulation in-vitro and recovery via transformation, a plasmid vector was chosen rather than a vector based on bacteriophage λ. In addition the use of UV-irradiation and mutagens in the actual tests for the sfiB phenotype precludes the use of λ-vectors unless additional steps, often difficult, are taken to render such vectors non-inducible. A low copy number plasmid vector was required as multiple copies of cell division and envelope genes were expected to be disadvantageous or lethal. Indeed V.Darby (1981) had reported that recombinant plasmids carrying DNA from the envA region cloned into pSC101 (6-8 copies per cell) were highly unstable in certain chromosomal backgrounds. The vector chosen was a temperature sensitive copy number plasmid, pOU71 (Larson et al., 1984) (Fig. 4.1). This plasmid
Figure 4.1

A restriction and functional map of the vector pOU71. The physical distances around the circle are shown in kb. The large solid arrow represents the direction of transcription from $\lambda P_R$.  

B : BamHI  

H : HindIII  

E : EcoRI
has the copy number control region and DNA replication origin of plasmid R1 together with the bacteriophage \( \lambda \text{Cl}_{857} \) gene and \( p_R \) promoter, with \( \beta \)-lactamase as a selectable marker. At \( 30^\circ \text{C} \) the copy number of pOU71 is 1. As the growth temperature increases the repression on \( p_R \) by the thermolabile \( \text{Cl}_{857} \) protein decreases and convergent transcription from \( p_R \) causes copy control to become relaxed and so plasmid copy number increases linearly with temperature (above \( 37^\circ \text{C} \)) reaching up to 1000 copies per cell at \( 42^\circ \text{C} \). This effect on plasmid copy number control is recessive to the presence of wild type \( \text{Cl} \) repressor produced by a host \( \lambda^+ \) lysogen, such that in a \( \lambda^+ \) host pOU71 maintains a copy number of 1 at all temperatures.

4.3 Cloning strategy

By superimposing the previously published restriction maps of transducing phages \( \lambda \ 16-2 \) (murC-envA) and \( \lambda \text{D01} \) (secA), a restriction map of the whole \( \text{ftsA-secA} \) region implicated as the position of \( \text{sfiB} \) can be obtained. Inspection of Fig. 4.2 indicates that there is a BamH1 site to the left of the \( \text{ftsA} \) gene and no second BamH1 site until at least beyond \( \text{secA} \). Therefore it was assumed that a BamH1 digest of \( \text{E.coli} \) chromosomal DNA should yield a DNA fragment (8.5kb+) extending from \( \text{ftsA} \) to beyond \( \text{secA} \).

4.4 Preparation and screening of an \( \text{E.coli} \) gene bank in pOU71

Chromosomal DNA from strain GC2490 (tsl,sfiB114) was prepared, digested with BamH1 and concentrated by ethanol precipitation. pOU71 DNA was likewise digested with BamH1 and
Figure 4.2
Restriction maps of transducing phages $\lambda$16-2 and $\lambda$D011, superimposed to show the presumed restriction map of the whole murC-secA region. $ftsQ$ is excluded from the chromosomal diagram because when the restriction pattern of $\lambda$16-2 was first reported $ftsQ$ was unknown.
treated with calf intestinal phosphatase to reduce vector recirculation. Donor and vector DNA were ligated in the ratio of 4:1 and in a total volume of 10 μl. This ligation mix was used to transform 400 μl of competent cells of the ftsA strain TKF12λ+ prepared 24 h previously. After allowing 2-3 h for expression the cells were plated on 11 nutrient agar plates + ampicillin, 10 being incubated at 42°C and 1 at 30°C overnight. TKF12λ+ had been shown previously to form 4 x 10^-8 colonies at 42°C compared to 30°C on nutrient agar plates. Consequently only transformants containing ftsA+ DNA fragments in the vector were expected to form colonies at the restrictive temperature. At 30°C 110 transformants appeared on the 30°C transformation plate giving approximately 1100 transformants plated out at 42°C. Since the E.coli genome is approximately 4000 kb in size and the average size of a BamHI generated fragment is 4.096 kb then this corresponds to approximately 1 gene bank (assuming all colonies contained recombinant plasmids).

One colony was recovered from the 42°C plates which could be restreaked to give good growth at 42°C. Microscopic examination of the cells of this transformant showed a few long filaments, indicating that it was probably a partial diploid and not an ftsA+ revertant. In order to confirm this a small scale plasmid preparation from this transformant was carried out. This plasmid DNA was capable of retransforming TKF12λ+ to temperature resistance with high efficiency. Thus it was concluded that an E.coli DNA fragment had been cloned onto pOU71 which had the capacity to complement a recessive ftsA mutation. The resulting plasmid was called pLG550.
4.5 Cloning ftsA* into pOU71 from sfiB* chromosomal DNA

As a control in experiments to investigate the possible location of a dominant sfiB mutation on pLG550 the cloning procedure shown above was repeated with chromosomal DNA from an ftsA*, sfiB* strain (MC4100). In this case approximately 4600 TKF12λ⁺ transformants were obtained, corresponding to about 5 gene banks. In fact 5 temperature resistant λTKF12⁺ transformants were identified and plasmid DNA derived from each one of them was then shown to be capable of retransforming TKF12λ⁺ to temperature resistance. One of these recombinant plasmids (pLG551) was selected for further study.

4.6 Transformations with pLG550

In order to investigate the presence of a dominant sfiB mutation on pLG550, pLG550 plasmid DNA was transformed into a tsl strain and the effect of pLG550 on tsl-associated filamentation during growth at 42°C studied. To do this pLG550 DNA (and pOU71 DNA as a control) was used to transform competent cells of strains GC24901λ⁺(tsl), GC24901λ⁺ (tsl, sfiB114) and TKF12λ⁺ (ftsA). After allowing 2 h for expression, the transformation mixture was plated onto nutrient agar plates, incubated overnight at 30°C and 42°C and numbers of colonies counted. The results shown in Table 4.1 show the difference in colony forming ability at 42°C between a tsl strain and a tsl, sfiB114 strain (transformed with pOU71). They also show that pLG550 DNA prepared in TKF12⁺ cannot transform GC2490 or GC24901 efficiently whereas pOU71 (prepared in strain CSH26ΔF6) can transform all
<table>
<thead>
<tr>
<th>recipient strain</th>
<th>pLG550</th>
<th>pOU71</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>30°C</td>
<td>42°C</td>
</tr>
<tr>
<td>GC2490λ⁺(sfiB114)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>GC24901λ⁺(sfiB⁺)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>MC4100</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>TKF12λ⁺(ftsA)</td>
<td>10³</td>
<td>10³</td>
</tr>
</tbody>
</table>
three strains. Since DNA prepared in TKF12 \( \lambda^+ \) was apparently only able to transform TKF12 \( \lambda^+ \) an unreported DNA modification mutation in TKF12 was suspected. This was confirmed by inducing strain TKF12 \( \lambda^+ \) for \( \lambda \) using UV-irradiation and showing that the resulting phage lysate formed plaques with a \( 8 \times 10^3 \) lower efficiency on MC4100 than on TKF12. In order to prepare pLG550 and pLG551 DNA capable of transforming GC24901, plasmid DNA obtained from the TKF12 host was first transformed into strain 5K (\( \text{hsdR}^-,\text{hsdM}^+ \)) and then DNA prepared from this strain was used to transform other host strains.

4.7 Tests for \( \text{sfiB} \) on pLG550 and pLG551

Transformants of GC24901\( \lambda^+ \) (\( \text{tsl} \)) carrying pLG550 and pLG551 obtained at 30°C were streaked out on nutrient agar plates at 42°C where \( \text{SfiA} \) synthesis should be constitutive in order to test for the \( \text{SfiB}^- \) phenotype. The pLG550 transformant grew normally at 42°C with only a few long filaments visible, whereas the pLG551 transformant grew very poorly at 42°C and very extensive filamentation was observed. Therefore a \( \text{tsl} \) strain carrying pLG550 gave a \( \text{SfiB}^- \) phenotype whilst the same strain carrying pLG551 gave an \( \text{SfiB}^+ \) phenotype. In order to exclude the possibility that \( \text{sfiB}^{114} \) from the incoming plasmid had recombined into the chromosome, the temperature resistant transformant of GC24901\( ^+ \) carrying pLG550 was cured of the plasmid as described below and then retested for a \( \text{SfiB}^- \) phenotype.

4.8 Curing GC24901\( \lambda^+ \) of pLG550

pOU71 had been reported to be highly unstable in the absence
of selection with plasmid loss occurring at approximately 2% per generation (J. Light pers. comm.). Consequently GC24901λ^pLG550 was cured of plasmid pLG550 by growth in nutrient broth without ampicillin selection for 30 generations at 30°C and cells plated out onto nutrient agar. Then 45 colonies were patch plated onto nutrient agar + and - ampicillin and grown overnight at both 30°C and 42°C. As a result 41 colonies were found to be amp^s and temperature sensitive and 4 were amp^r and temperature resistant. Therefore the presence of pLG550 correlated exactly with a sfiB^- phenotype in an tsl host strain.

4.9 Effects of pLG550 and LGG551 on cell division in GC24901λ^+ 
In order to test the effects of pLG550 and pLG551 on cell division in GC24901λ^+ at the restrictive temperature, GC24901λ+ carrying pOU71, pLG550 or pLG551 were grown in nutrient broth at 30°C to A_450 0.2 and then shifted to 42°C. Samples were removed at 15 min intervals and A_450 and cell number measured (Fig. 4.3). GC24901λ^+ pLG550 showed normal cell division at 42°C, whereas strains carrying both pOU71 and pLG551 showed little or no increase in cell number at 42°C (although some recovery was observed after 90 min at 42°C for pLG551). After 2 h growth at 42°C samples were removed from cultures bearing the pLG550 and pLG551 plasmids and the cells examined microscopically. Considerable filamentation was observed in the pLG551 culture whereas a small number of long filaments were seen in the pLG550 culture.
Figure 4.3
Measurement of cell number (open circles) and cell mass (closed circles), as measured by O.D. $A_{450}$, for cultures of GC24901†(tsl) grown in nutrient broth at 42°C containing plasmids:

a) pOU71
b) pLG550
c) pLG551
In further tests cultures of GC24901λ⁺ carrying pOU71, pLG550 and pLG551 were also grown at 30°C in nutrient broth and serial dilutions plated out onto nutrient agar plates and incubated at 30°C and 42°C overnight. Survival figures are given in Table 4.2. Although pLG551 seems to confer increased resistance to tsl expression under these conditions, the colonies formed at 42°C were extremely small and microscopic examination showed extensive filamentation. Nevertheless the ability to form even very small colonies indicated a small protective effect on tsl induced filamentation by pLG551 carrying the wild type sfiB⁺ allele under these conditions. The recovery in cell division after shifting to 42°C shown in Fig. 4.3 by GC24901λ⁺ pLG551 also suggested such an effect. As a consequence of these findings it was suspected that the λcl⁺ gene on the GC24901λ⁺ chromosome, required to prevent runaway of plasmid copy number, was not completely repressing the transcription from pR on pOU71 at high temperature. This could lead to an increase in the copy number of pOU71, and its derivatives, particularly after prolonged incubation at 42°C. In addition, any 'escape' transcription from pR could affect the synthesis of other proteins encoded by cloned fragments in pOU71. Either of these consequences could result in the small protective effect on tsl induced filamentation observed in strains carrying pLG551 grown at high temperature.

4.10 Effect of pLG550 and pLG551 on filamentation in a lon strain

Having shown that pLG550 was capable of suppressing the Tsl phenotype we wished to test pLG550 for the suppression of the Lon phenotype (another property of sfi mutants).
<table>
<thead>
<tr>
<th>strain</th>
<th>30°C</th>
<th>42°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>GC2490λ^+ (sfiB114)</td>
<td>1</td>
<td>0.7</td>
</tr>
<tr>
<td>GC24901λ^+ (sfiB^+)</td>
<td>1</td>
<td>1.1 x 10^-4</td>
</tr>
<tr>
<td>GC24901λ^+ pOU71</td>
<td>1</td>
<td>1.6 x 10^-4</td>
</tr>
<tr>
<td>GC24901λ^+ pLG550</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>GC24901λ^+ pLG551</td>
<td>1</td>
<td>0.14</td>
</tr>
<tr>
<td>strain</td>
<td>-MMS</td>
<td>+MMS</td>
</tr>
<tr>
<td>------------------------------</td>
<td>------</td>
<td>-------</td>
</tr>
<tr>
<td>PAM162m+ (lon,sfiB26)</td>
<td>1</td>
<td>5.8 x 10^{-1}</td>
</tr>
<tr>
<td>PAMsfi^+ (lon,sfiB^+)</td>
<td>1</td>
<td>3.0 x 10^{-5}</td>
</tr>
<tr>
<td>PAMsfi^+ pOU71</td>
<td>1</td>
<td>5.0 x 10^{-5}</td>
</tr>
<tr>
<td>PAMsfi^+ pLG550</td>
<td>1</td>
<td>2.6 x 10^{-2}</td>
</tr>
<tr>
<td>PAMsfi^+ pLG551</td>
<td>1</td>
<td>1.7 x 10^{-6}</td>
</tr>
</tbody>
</table>
Transformants of PAMsfi\(^+\) (lon, sfiB\(^+\)) (see Chapter 3) carrying pOU71, pLG550 or pLG551 were grown in nutrient broth at 30\(^\circ\)C to \(A_{450} = 0.5\), plated out on nutrient agar plates + and - MMS, and grown at 30\(^\circ\)C to determine the numbers of survivors. The \(\lambda\) cI\(_{857}\) gene on pOU71 was derived from a ind\(^-\) bacteriophage \(\lambda\) and so MMS was not expected to affect the plasmid copy number or transcription from \(p_R\). The results presented in Table 4.3 clearly show the difference in MMS sensitivity between PAMsfi\(^+\) carrying pOU71, pLG550 and pLG551, and provide conclusive evidence for the presence of the sfiB114 on plasmid pLG550.

4.11 Conclusion

DNA fragments carrying the ftsA\(^+\) marker were successfully cloned from the chromosomal DNA of sfiB114 (GC2490) and sfiB\(^+\) (MC4100) strains into the BamHI site of plasmid vector pOU71. The cloned DNA was expected to carry ftsA and extend to a point beyond secA and consequently carry sfiB. The recombinant plasmid derived from the DNA of a sfiB114 mutant indeed was shown by several different tests to carry a dominant sfiB\(^-\) mutation rendering cells resistant to the effects of the SfiA division inhibitor. It was concluded that sfiB114 and sfiB\(^+\) genes had been cloned onto recombinant plasmids pLG550 and pLG551 respectively.
5.1 Introduction
In the previous chapter, the isolation of recombinant plasmids pLG550 and pLG551 was described. The results presented indicated that both plasmids carried wild type $ftsA^+$ genes, and that the plasmid derived from sfiB114 chromosomal DNA carried a dominant sfiB mutation. As a prerequisite to identifying the exact location of the sfiB locus, these recombinant plasmids were characterised by restriction endonuclease mapping. In addition, in order to more precisely locate sfiB and in an attempt to reduce the problems involved in using pOU71 as a cloning vector, restriction fragments from pLG550 and pLG551 were subcloned into low copy number plasmid vector, pLG339.

5.2 Preparation of pLG550 and pLG551 DNA
In order to prepare sufficient quantities of pLG550 and pLG551 DNA suitable for restriction mapping, both plasmids were prepared using strain 5K($\lambda^-$) as a host to allow amplification of plasmid copy number by growing exponentially growing plasmid bearing cultures at 42°C. Cultures of 5K pLG550 and 5K pLG551 were grown in nutrient broth at 30°C to $A_{450}$ 0.2 and shifted to 42°C for 1 h after which they were grown at 37°C for 6-8 h or overnight before harvesting and cells used in large scale plasmid preparations.
5.3 Restriction of pLG550 and pLG551 with BamH1

In order to confirm that pLG550 and pLG551 contained identically sized chromosomal DNA fragments, both were restricted with BamH1 and separated on a 0.8% agarose gel. As BamH1 was the restriction enzyme used to isolate the chromosomal inserts ligated into the vector pOU71, BamH1 digestion should yield DNA fragments corresponding to vector and inserts. Fig. 5.1 shows the results of this digestion and indicates that both pLG550 and pLG551 contains chromosomal inserts of approximately 13.5 kb. In addition to the band corresponding to vector DNA (6.2 kb) in the pLG551 digestion a further fragment of 3.8 kb in size can be seen. Thus it was concluded that the ftsA complementing region on both recombinant was contained on a 13.5 kb BamH1 fragment and that during the construction of pLG551 an additional BamH1 fragment had been ligated into pOU71. Because of the presence of this unwanted fragment on pLG551, initial restriction mapping was limited to pLG550.

5.4 Physical mapping of pLG550

In order to compare the restriction pattern of pLG550 with that of the λ-transducing phages covering the ftsA-secA region, pLG550 was mapped with respect to restriction enzymes BamH1, HindIII and EcoR1 using both single and double enzyme digestions. The restriction map deduced from these experiments is shown in Fig. 5.2. This agrees completely with published data on the restriction maps of λ 16-2 (murC-envA) and λ D01 (secA), see Fig. 4.2, and shows that the 13.5 kb chromosomal fragment continues for approximately 5 kb beyond
Figure 5.1

Agarose gel of pLG550 and pLG551 DNA digested with BamH1.

Tracks are:

S - λ HindIII standards
1 - pLG551 x BamH1
2 - pLG550 x BamH1
Figure 5.2
Restriction map of pLG550 with respect to restriction enzymes BamHI (B), EcoRI (E) and HindIII (H). The dark part of the circle represents the vector pLG339 and the numbers on the inside show size in kb.
secA. pLG551 was also provisionally mapped and although the analysis was complicated by the presence of the extraneous 3.8 kb fragment, the map obtained of its 13.5 kb insert was identical to that seen with pLG550. It was concluded that both pLG550 and pLG551 carried the ftsA-secA region implicated by P1 and λ-transductional mapping to be the location of sfiB.

5.5 Subcloning of pLG550 and pLG551 chromosomal fragments into vector pLG339

There were several reasons for wishing to subclone chromosomal fragments present in pLG550 and pLG551 into an alternative vector to pOU71.

a) The protective effect on ts1 induced filamentation at 42°C shown by pLG550 had been assumed to have been due to an amplification of plasmid copy number and/or effect on gene expression caused by the complex copy number control system of pOU71. This effect was expected to severely complicate further analysis of the location and function of sfiB by reducing the difference in phenotype between pOU71 derived plasmids that are genetically sfiB⁻ or sfiB⁺.

b) It was thought necessary to remove the extraneous DNA fragment carried by pLG551 to avoid complications when analysing the polypeptides encoded for by the sfiB⁻ and sfiB⁺ carrying plasmids.

c) pOU71 encodes resistance to ampicillin and therefore produces β-lactamase. This was possibly inconvenient as the size of β-lactamase protein (30 kD) is near to the size of one of the expected gene products of pLG550 and pLG551, EnvA (33 kD) making distinction between them difficult (Darby,
The vector chosen for this subcloning was pLG339 (Stoker et al., 1982) (Fig. 5.3), which is a low copy number plasmid vector (6–8 copies per cell) derived from pSC101 and carries resistance to tetracycline and kanamycin.

pLG550 and pLG551 DNA was digested with BamH1 and ligated to pLG339 DNA similarly digested with BamH1, and ligated DNA used to transform TKF12(ftsA), selecting for kanamycin resistance and temperature (42°C) resistance. Many ts<sup>r</sup> transformants were identified from both ligations. 12 colonies from each ligation were replated on nutrient agar plates at 30°C and 42°C, and all grew well at both temperatures although some filamentation could be seen at 42°C. One transformant from each ligation was selected and it was shown that plasmid DNA prepared from each was capable of retransforming TKF12 to temperature resistance. It was thus concluded that BamH1 restriction fragments had been cloned from pLG550 and pLG551 into pLG339 that were capable of complementing ftsA. These recombinant plasmids were named pLG552 and pLG554 respectively.

5.6 Properties of pLG552 and pLG554

Host cells carrying either pLG552 or pLG554 were found to grow very slowly in both nutrient broth and minimal-media + casamino acids. Microscopic examination of cells carrying pLG552 and pLG554 showed them to be very short, and this may be correlated with the fact that it was often difficult to efficiently harvest cells in culture by centrifugation.

This slow growth phenotype caused considerable problems
Figure 5.3
Restriction and functional map of plasmid vector pLG339. The position of the kanamycin and tetracycline resistance genes are shown together with the region required for plasmid replication.

H - HindIII
E - EcoRI
B - BamHI
X - XhoI
S - SalI
when attempting to prepare plasmid DNA from pLG552 and pLG554 carrying cells as fast growing cells arose frequently by the deletion of large segments of the chromosomal inserts. When preparing DNA of pLG552 and pLG554 a recA strain was therefore used as a host in an attempt to minimise plasmid instability. Despite this use of a recA strain, pLG552 and pLG554 plasmid DNA often contained significant amounts of deletion derivatives (Fig. 5.4). This instability of plasmids derived from the envA region had been reported previously (Darby, 1981). The instability of pLG552 and pLG554 was only thought to be a problem in large scale plasmid DNA preparations since this required growth through enough generations for any fast growing derivatives to constitute a major proportion of the plasmid molecules. Despite this plasmid instability enough uncontaminated pLG552 and pLG554 DNA was prepared to allow restriction mapping to proceed.

5.7 Restriction mapping of pLG552 and pLG554

To confirm that plasmids pLG552 and pLG554 carried the 13.5 kb chromosomal BamHI fragments from pLG550 and pLG551 respectively, DNA of each was prepared using large scale plasmid preparations and digested with BamHI. The results confirmed that the 13.5 kb BamHI fragments had been inserted into pLG339, and that in the construction of pLG554 from pLG551 the small extraneous 3.8 kb BamHI fragment had been lost. Thus we could conclude that pLG552 contained the 13.5 kb fragment from sfiB114 chromosomal DNA and pLG554 contained the same insert derived from an sfiB+ strain.

Digestion of pLG552 and pLG554 with HindIII showed the two BamHI fragments were inserted in opposite orientations.
Figure 5.4

Agarose gel analysis of three different preparations of pLG554 plasmid DNA (tracks 1, 2 and 3) showing the presence of deletion derivatives. Molecular weight standards (S) are λ DNA digested with HindIII.
into the BamHl site of pLG339 (Fig. 5.5). Further restriction mapping confirmed that the 13.5 kb BamHl fragments in pLG552 and pLG554 showed an identical restriction pattern to that of pLG550 and pLG551 (Fig 5.5).

### 5.8 Tests for sfiB114 on pLG552 and pLG554

To test for the presence of a dominant sfiB114 locus carried by pLG552, both pLG552 and pLG554 were transformed into strain GC24901 (tsl) and nutrient broth cultures of isolated transformants plated on nutrient agar plates and incubated at 30°C and 42°C. Results in Table 5.1 clearly show that pLG552 confers a SfiB− phenotype on tsl host cells whereas an otherwise almost identical recombinant plasmid obtained from sfiB+ chromosomal DNA (pLG554) does not. Results shown in Table 5.1 also show by the same criterion the presence of ftsA+ on both plasmids.

### 5.9 Discussion

In this chapter mapping of pLG550 and pLG551 with respect to restriction endonucleases has shown that both contain a 13.5 kb BamHl chromosomal insert covering the ftsA-secA region of the E.coli genome and extending approximately 6 kb beyond secA. After subcloning the 13.5 kb BamHl fragments from pLG550 and pLG551 into pLG339, the recombinant plasmid derived from sfiB114 chromosomal DNA (pLG552) gave a completely unambiguous sfiB phenotype when transformed into tsl cells. The identical fragment derived from sfiB+ chromosomal DNA, when inserted into pLG339 (pLG554) gave no such phenotype and thus by subcloning these fragments into a
<table>
<thead>
<tr>
<th>strain</th>
<th>relative survival</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>30°C</td>
</tr>
<tr>
<td>GC24901(tsl,sfiB&lt;sup&gt;+&lt;/sup&gt;) pLG339</td>
<td>1</td>
</tr>
<tr>
<td>GC24901 pLG552</td>
<td>1</td>
</tr>
<tr>
<td>GC24901 pLG554</td>
<td>1</td>
</tr>
<tr>
<td>GC24901 pLG553</td>
<td>1</td>
</tr>
<tr>
<td>TKF12(ftsA) pLG339</td>
<td>1</td>
</tr>
<tr>
<td>TKF12 pLG552</td>
<td>1</td>
</tr>
<tr>
<td>TKF12 pLG554</td>
<td>1</td>
</tr>
<tr>
<td>TKF12 pLG553</td>
<td>1</td>
</tr>
</tbody>
</table>
Figure 5.5

a) Agarose gel of pLG552 (track 1) and pLG554 (track 2) digested with HindIII.

b) Restriction maps of pLG552 and pLG554 derived from the HindIII digestion seen above showing the different orientation of the chromosomal inserts in pLG339.

B - BamHI
H - HindIII
plasmid with a low and constant copy number the ambiguities associated with the use of pOU71 were eliminated.

The origin of the slow growth of pLG552 and pLG554 carrying cells and consequent plasmid instability was thought to be possibly due to the effect of multiple copies of envA, or nearby genes as W. Donachie (personal communication) had reported that fragments of DNA carrying envA could not be cloned onto high copy number plasmids.

Having obtained and characterised plasmids carrying the sfiB114 and sfiB* genes, subcloning and transpositional mutagenesis could now be used to find the precise location and nature of sfiB.
CHAPTER 6

Mapping of sfiB114 by transpositional mutagenesis

6.1 Introduction

Having cloned a dominant sfiB114 locus onto recombinant plasmid pLG552, it became possible to identify the position of sfiB114 using transpositional mutagenesis. If transposons could be inserted randomly into pLG552, those having insertions into sfiB114 should be identifiable by loss of the SfiB phenotype (i.e. ability to suppress tsk induced filamentation). Mapping of the position of such insertions into pLG552 using restriction enzyme analysis combined with studying the effects of sfiB114 inactivating insertions on polypeptides produced by pLG552 in E.coli. gene expression systems should enable both the precise location and the gene product of sfiB114 to be identified. In addition Fig. 3.3 showed that much of the region implicated as the position of sfiB corresponds to known genes and so it was considered possible that the sfiB locus corresponded to an allele of a previously defined gene. If this was the case then transpositional mutagenesis of pLG552 was expected to aid in its identification, as inactivation of sfiB114 should also inactivate complementation by pLG552 of mutations in this gene.

6.2 Tn1000 mutagenesis of pLG552

A simple method of isolating transposition mutants has been developed using the Tn3-like transposon Tn1000 (Fig 6.1), also called 7 (Guyer, 1978). Tn1000 is carried by the
Figure 6.1
A restriction map of the transposon Tn1000 showing the sites of cleavage for the enzymes EcoRI (E), HindIII (H), XhoI (X) and BamHI (B).
conjugative plasmid F. A non conjugative plasmid in the same cell as F may be mobilised with high frequency provided it has an origin of transfer. Plasmids mobilised in this way are not altered in the transfer process. However, plasmids that are non-mobilisable can be transferred, at a much lower efficiency, by integration into the F plasmid. The non-mobilisable plasmid is transferred passively in this co-integrate form. Once within the recipient cell, the co-integrate is resolved, leaving the F factor and the non-mobilisable plasmid separated (Fig. 6.2). In over 99% of cases, co-integrate formation occurs by the transposition of Tn1000 such that almost all recipient cells will have non-mobilisable plasmid molecules with a single copy of Tn1000 inserted fairly randomly into their DNA. Since pLG339 is a non-mobilisable vector, Tn1000 mutagenesis was suitable for use with pLG552.

pLG552 was transformed into the F* strain RB308(recA) (co-integrate formation is recA independent). This strain was mated with GC24901 which is tsl, and also rpsL, and so transconjugant GC24901 cells carrying pLG552::Tn1000 plasmids were selected by plating the mating mixture onto nutrient agar plates containing kanamycin and streptomycin and the plates were incubated at 30°C. 270 transconjugants were patch tested onto nutrient agar at 30°C and 42°C to test for the possible inactivation by Tn1000, of the SfiB114 phenotype conferred by pLG552. 6 colonies were found to show extensive filamentation when grown at 42°C, and were therefore phenotypically SfiB+. Four of these colonies, numbered 62, 164, 165 and 174, were selected for further analysis. In
Fig. 6.2: Postulated mechanism of Tn1000 mutagenesis

A: The non-mobilisable plasmid to be mutagenised is introduced into a strain harbouring an F factor carrying Tn1000.

B: Transposition by Tn1000 into a copy of the non-mobilisable plasmid results in formation of a cointegrate plasmid.

C: The cointegrate plasmid is transferred to a recipient during conjugation.

D: Resolution of the cointegrate in the recipient cell, forming the F plasmid in its original state, and the non-mobilisable plasmid carrying a copy of Tn1000.

(Diagram from an original by N. G. Stoker)
order to confirm this apparent inactivation of SfiB114 on pLG552, cultures of GC24901 carrying pLG552::Tn1000 mutants 62, 164, 165 and 174 were spread on nutrient agar plates at 30°C and 42°C and relative survival figures determined. As controls, two colonies numbered 1 and 2 which did not show filamentation when patch tested at 42°C were included in this test. Results in Table 6.1 show that Tn1000 insertions 62, 164, 165 and 174 do indeed fail to show an sfiB phenotype compared to pLG552, pLG552::Tn1000-1 and pLG552::Tn1000-2.

6.3 Restriction site mapping of pLG552::Tn1000 plasmids

Plasmid DNA of pLG552::Tn1000 derivatives 1, 2, 62, 164, 165 and 174 was prepared by large scale plasmid preparation for the mapping of the Tn1000 insertions. Large scale preparations must be used with pLG339 derivatives as their copy number is too low to allow sufficient DNA to be obtained from small scale preparations. Tn1000 is 5.7 kb in size, and all six pLG552::Tn1000 plasmids showed an increase in plasmid size over that of pLG552, confirming the presence of an inserted sequence. All six plasmids were mapped with restriction enzymes XhoI, BamH1, HindIII and EcoRI and the position of the Tn1000 insertions found. The orientation of these inserts was ascertained from the BamH1 restriction digests as Tn1000 has a single assymmetric BamH1 site, 0.4 kb from one end (Fig. 6.1). The precise location of the Tn1000 insertions was determined by digesting pLG552::Tn1000 plasmids with HindIII and the sites of insertion mapped with respect to the HindIII site at the distal end of the ftsA gene (Fig. 6.3). The diagram shown in Fig 6.4 shows that all four SfiB114::Tn1000 insertions lie in the ftsA-ftsZ region,
<table>
<thead>
<tr>
<th>strain</th>
<th></th>
<th>relative survival</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>30°C</td>
<td>42°C</td>
</tr>
<tr>
<td>GC24901 (tsl, sfiB⁺) pLG552::Tn1000-1</td>
<td>1</td>
<td>0.58</td>
</tr>
<tr>
<td>GC24901 pLG552::Tn1000-2</td>
<td>1</td>
<td>1.85</td>
</tr>
<tr>
<td>GC24901 pLG552::Tn1000-62</td>
<td>1</td>
<td>$2.9 \times 10^{-3}$</td>
</tr>
<tr>
<td>GC24901 pLG552::Tn1000-164</td>
<td>1</td>
<td>$3.0 \times 10^{-4}$</td>
</tr>
<tr>
<td>GC24901 pLG552::Tn1000-165</td>
<td>1</td>
<td>$1.3 \times 10^{-4}$</td>
</tr>
<tr>
<td>GC24901 pLG552::Tn1000-174</td>
<td>1</td>
<td>$1.1 \times 10^{-4}$</td>
</tr>
</tbody>
</table>
Figure 6.3
An agarose gel of pLG339, pLG552 and pLG552::Tn1000 derivatives plasmids digested with HindIII. Tracks shown are:

S - λ HindIII standards.
1 - pLG339
2 - pLG552
3 - pLG552::Tn1000-1
4 - pLG552::Tn1000-2
5 - pLG552::Tn1000-62
6 - pLG552::Tn1000-164
7 - pLG552::Tn1000-165
8 - pLG552::Tn1000-174
Figure 6.4
A restriction and functional map of pLG552 showing the position of the Tn1000 insertions discussed in the text. The position of Tn1000 insertion 1 was not precisely ascertained and so a bar showing its approximate position is given.

B - BamHI
E - EcoRI
H - HindIII
whereas insertions 1 and 2 map in other regions of the plasmid (insertion 1 lying within the secA coding sequence). The arrangement of the coding portions of ftsA and ftsZ had previously been extensively investigated by Luktenhaus and Wu (1980) and from this data we could conclude that Tn1000 insertions 165, 62 and 174 lay in the structural gene for ftsA. Insertion 164 seemed to map distal to the ftsA coding region. Unfortunately the precise limits of ftsZ on the physical map were not previously established and so the significance of this insertion at this stage was unclear. This data indicated that the insertion of Tn1000 into ftsA and possibly ftsZ suppressed the SfiB114 phenotype.

6.4 Complementation of ftsA and ftsZ by pLG552::Tn1000 plasmids

Having shown that pLG552::Tn1000 insertions 62, 164, 165 and 174 inactivate SfiB114, the observation that three of these mutations mapped in one gene and one possibly in another was difficult to explain. To resolve this problem plasmids pLG552::Tn1000-62, pLG552::Tn1000-164, pLG552::Tn1000-165 and pLG552::Tn1000-174 were transformed into strains TKF12 (ftsA) and JFL100 (ftsZ), and transformants of each tested for complementation of the temperature sensitive mutations by determining relative survival values when the cultures of the transformants were plated on nutrient agar at 30°C and 42°C. Results in Table 6.2 show that plasmids with Tn1000 insertions which mapped in the ftsA sequence (i.e. numbers 62, 165 and 174) abolished ftsA complementation whereas insertion 164 did not.
<table>
<thead>
<tr>
<th>strain</th>
<th>30°C</th>
<th>42°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>JFL100(ftsZ) pLG339</td>
<td>1</td>
<td>$1.8 \times 10^{-8}$</td>
</tr>
<tr>
<td>JFL100 pLG552</td>
<td>1</td>
<td>$2.9 \times 10^{-3}$</td>
</tr>
<tr>
<td>JFL100 pLG552::Tnl000-62</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>JFL100 pLG552::Tnl000-164</td>
<td>1</td>
<td>$6.3 \times 10^{-6}$</td>
</tr>
<tr>
<td>JFL100 pLG552::Tnl000-165</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>JFL100 pLG552::Tnl000-174</td>
<td>1</td>
<td>$1.3 \times 10^{-5}$</td>
</tr>
<tr>
<td>TKF12(ftsA) pLG339</td>
<td>1</td>
<td>$1.2 \times 10^{-7}$</td>
</tr>
<tr>
<td>TKF12 pLG552</td>
<td>1</td>
<td>0.85</td>
</tr>
<tr>
<td>TKF12 pLG552::Tnl000-62</td>
<td>1</td>
<td>$3.4 \times 10^{-7}$</td>
</tr>
<tr>
<td>TKF12 pLG552::Tnl000-165</td>
<td>1</td>
<td>1.2</td>
</tr>
<tr>
<td>TKF12 pLG552::Tnl000-174</td>
<td>1</td>
<td>$9.2 \times 10^{-6}$</td>
</tr>
</tbody>
</table>
Complementation of \textit{ftsZ} even with pLG552 itself was not very efficient. Luktenhaus and Wu (1980) had reported that the addition of 0.4 \% NaCl to growth media resulted in full complementation of \textit{ftsZ} by lysogeny with \textit{ftsZ}+ λ-bacteriophages (even those expressing \textit{ftsZ} very poorly) but this was not observed using pLG552 or pLG554. We see from Table 6.2 that the insertions close to (174) or possibly within (164) \textit{ftsZ} significantly reduce the level of \textit{ftsZ} complementation. Insertions further upstream of \textit{ftsZ} also show reduced complementation and there seems to be a decrease in the level of inactivation of \textit{ftsZ}+ as insertions lie further upstream of the \textit{ftsZ} coding region. The observation that insertion 164 inactivates \textit{SfiB114} while not affecting \textit{ftsA} complementation indicated that \textit{SfiB114} was not an allele of \textit{ftsA}. To prove this, a restriction fragment containing \textit{ftsA} alone was subcloned from pLG550 and tested for the presence of a \textit{SfiB114} phenotype.

6.5 Subcloning of \textit{ftsA} from pLG550

Since Tn1000 insertions into \textit{ftsA} had been shown to inactivate \textit{SfiB114} it was possible that \textit{SfiB114} was an allele of \textit{ftsA}. However, one Tn1000 insertion, 164, inactivated \textit{SfiB114} without affecting \textit{ftsA}+. It was thought possible that Tn1000 insertion 164 was sufficiently near the terminus of the \textit{ftsA} gene to allow a slightly truncated protein to complement the \textit{ftsA} mutation in TKF12, yet prevent expression of a \textit{SfiB114} phenotype. In order to test this, a 2.5 kb BamH1-EcoR1 fragment was cloned from pLG550 into vector pLG339 (Fig 6.5). This fragment carries the whole of \textit{ftsA} and the proximal region of \textit{ftsZ}. This recombinant plasmid, named
Figure 6.5

A diagram showing the construction of pLG553 from pLG550 and pLG339.

B - BamHI
E - EcoRI
pLG553 was capable of complementing an ftsA mutation in TKF12 yet was unable to show a SfiB phenotype when transferred to GC24901 (tsl) (Table 6.2). This result demonstrated that sfiB114 is not an allele of ftsA.

6.6 Discussion

From the complementation data and restriction mapping of pLG552SfiB114::Tn1000 plasmids it was concluded that transposon insertion into both the distal region of ftsA or the proximal region of ftsZ inactivated sfiB114 carried by pLG552. Since almost all of the DNA in this area forms part of an already identified gene, it seemed very likely that sfiB114 was in fact an allele of one of these genes. Subcloning of a BamH1-EcoR1 fragment carrying ftsA alone from pLG550, showed that sfiB114 was not contained within ftsA. This led to the conclusion that one of the genes clockwise of the ftsA gene (e.g. ftsZ, envA) was the location of sfiB114 and that a segment within the ftsA was required for its full expression. ftsZ was considered to be the most likely candidate because of its well known involvement in cell division and the observed effect on ftsZ complementation by SfiB114::Tn1000 insertions. Lutkenhaus and Wu (1980) had previously shown that the 3.5 kb HindIII fragment carrying ftsZ was not sufficient by itself of showing full ftsZ complementation (under the growth conditions used) and concluded that an element within ftsA was required for full expression. This fits well with the proposal above.

In order to test this hypothesis, we sought to analyse the polypeptides encoded by pLG552 and pLG552SfiB114::Tn1000...
plasmids in order to study the effects of Tn1000 insertions on the production of the gene products of the plasmid pLG552.
Chapter 7

Analysis of polypeptides produced by pLG552 and pLG552::Tn1000

7.1 Introduction

In order to confirm that sfiB114 was an allele of ftsZ it was required to express pLG552 and pLG552SfiB114::Tn1000 plasmids in an E.coli gene expression system. The two in-vivo gene expression systems applicable to recombinant plasmids in common use are mini-cells (Fraser and Curtiss, 1975) and maxi-cells (Sancar et al., 1979). As described in Chapter 4, pLG552 carrying cells are very small and so the mini-cell system could not be used as it was found impossible to separate mini-cells from whole cells carrying pLG552.

The use of maxi-cells relies on the deficiency in DNA repair of recA cells. A recA strain (in this work Δ recA strain, CSH26AF6) carrying the plasmid of interest is UV-irradiated and incubated overnight to allow the breakdown of UV damaged chromosomal DNA whilst leaving undamaged plasmid DNA molecules intact. Cells are then washed in a medium containing a very low concentration of methionine and after pre-incubation, labelled with $^{35}$S-methionine and samples lysed in SDS sample buffer. SDS-PAGE and fluorography then allows the observation of the radioactive proteins produced.

7.2 Gene products of the ftsA-secA region

Previous studies by Luktenhaus and Wu (1980) and Oliver and Beckwith (1982) had identified the gene products of all of the well characterised genes in the ftsA-secA region. These
7.3 Analysis of polypeptides programmed by plasmids carrying SfiB114 and SfiB114::Tn1000

The maxi-cell strain CSH26ΔF6 was transformed with pLG552 and pLG552::Tn1000 derivatives numbered 1, 2, 62, 164, 165, and 174. Using the maxi-cell protocol, radioactive protein samples were obtained for each transformant and analysed using SDS-PAGE. An autoradiograph of a gel is shown in Fig. 7.1. In the pLG552, track bands are present corresponding to the expected products of all four genes *ftsA*, *ftsZ*, *envA* and *secA*, and a protein band previously shown to be associated with kanamycin resistance is also present. The actual sizes of the FtsA and FtsZ proteins were calculated to be 47 kD and 42 kD respectively compared to the previously reported sizes of 50 kD and 45 kD. This small difference was thought to be due to slight variations between the gel electrophoresis systems used.

In Fig. 7.1 the effects of the Tn1000 insertions into pLG552 on the polypeptide products can be observed. The profile for all four SfiB114::Tn1000 plasmids show considerable reduction or complete disappearance of the FtsZ band. In addition, plasmid pLG552::Tn1000-1, which had been shown by restriction mapping to have Tn1000 inserted within the secA gene, encodes a protein corresponding to a truncated SecA polypeptide.
Figure 7.1

Autoradiograph of maxi-cells labelled with $^{35}$S-methionine analysed using an 11% polyacrylamide gel. Tracks represent proteins encoded by:

1 - pLG552
2 - pLG552::Tn1000-1
3 - pLG552::Tn1000-2
4 - pLG552::Tn1000-62
5 - pLG552::Tn1000-164
7 - pLG552::Tn1000-165
8 - pLG552::Tn1000-174
In order to better correlate the effects of the Tn1000 insertions on polypeptide production from pLG552 with their physical position and phenotypic effects, the samples from Fig. 7.1 were re-analysed on an 11% SDS-PAGE gel (Fig. 7.2) in order of their position on the physical map (i.e. 164, 174, 62 and 165). In consequence, Fig 7.2 indicates that the further upstream of the \textit{ftsZ} coding sequence the Tn1000 insertions lie the greater the level of FtsZ synthesis which is observed. However, insertion 165, mapped approximately 0.5 kb away from the \textit{ftsZ} gene still has a dramatic effect on FtsZ synthesis. Thus it seems that the gradual reduction in the inactivating effect of SfiB114::Tn1000 insertions on the complementation properties of \textit{ftsZ} (chapter 6), which parallels their more distal position upstream of \textit{ftsZ}, is a direct consequence of increased \textit{ftsZ} synthesis.

A longer exposure of the autoradiograph shown in Fig. 7.2 is given in Fig 7.3 and this demonstrates the effects of the SfiB114::Tn1000 insertions on the weakly produced FtsA protein. Plasmid pLG552SfiB114::Tn1000-164 seems to encode a FtsA polypeptide of a similar size and abundance as the unmutagenised pLG552 plasmid. This is to be expected since this plasmid was shown to be capable of full complementation of \textit{ftsA} (chapter 6). However pLG552SfiB114::Tn1000 plasmids 174, 62 and 165, which fail to complement \textit{ftsA} do not encode proteins corresponding in size to FtsA. In fact, proteins apparently corresponding to truncated FtsA can be seen in the case of pLG552SfiB114::Tn1000-174 and pLG552SfiB114::Tn1000-62.

Therefore the analysis of the polypeptides produced by pLG552 and pLG552SfiB114::Tn1000 derivatives agreed with the
Figure 7.2

Autoradiograph of an 11% polyacrylamide gel loaded with samples used in Fig. 7.1. Tracks represent:

1 - pLG552
2 - pLG552::Tn1000-164
3 - pLG552::Tn1000-174
4 - pLG552::Tn1000-62
5 - pLG552::Tn1000-164
Figure 7.3
A longer exposure of the autoradiograph shown in Fig. 7.2.
Tracks represent.
1 - pLG552
2 - pLG552::Tn1000-164
3 - pLG552::Tn1000-174
4 - pLG552::Tn1000-62
5 - pLG552::Tn1000-165
complementation data presented in Chapter 6. In particular, the results confirmed that Tn1000 insertions into the distal area of the coding region of \textit{ftsA} and the proximal region of \textit{ftsZ} could inactivate the \textit{SfiB114} phenotype and eliminate or significantly reduce the synthesis of FtsZ protein.

7.4 Tests for a \textit{ftsZ} promoter on the 3.5 kb HindIII fragment

The results from the previous section left unexplained how insertions into \textit{ftsA} could prevent or reduce expression of \textit{ftsZ}. One possibility is that \textit{ftsZ} is expressed primarily from either the \textit{ftsA} promoter or from other promoters within the \textit{ftsA} coding sequence. Luktenhaus and Wu (1980) had observed that expression of \textit{ftsZ} from the 3.5 kb HindIII fragment carrying \textit{ftsZ} and \textit{envZ} (see Fig 6.3) was weak but they were unable to conclude whether or not \textit{ftsZ} possessed its own promoter. To answer this question it was decided to isolate the 3.5 kb HindIII DNA fragment and use this DNA to program the Zubay \textit{in-vitro} transcription translation system.

The Zubay \textit{coupled in-vitro} transcription translation system allows the analysis of the polypeptides encoded by specific DNA fragments (Pratt, 1984). This is done by incubating a purified DNA sample together with a preincubated \textit{E.coli} S30 extract + various enzymes and co-factors in the presence of $^{35}$S-methionine (Pratt, 1984). The radioactively labelled proteins produced can be visualised by SDS-PAGE and autoradiography. One of the advantages of this method is that if an S30 extract is prepared from an \textit{E.coli} recB strain (i.e. defective in the major \textit{E.coli} exonuclease, exonuclease V) its use avoids degradation of linear DNA restriction
fragments used as templates (Jackson et al., 1983).

The 3.5 kb HindIII restriction fragment carrying \texttt{ftsZ}
and \texttt{envA}, obtained from plasmid pLG552 DNA and therefore
carrying \texttt{sfiB114}, was purified from an 0.8\% agarose gel using
the DE81 procedure (see methods). After concentration by
ethanol precipitation and resuspension in TE buffer, this DNA
fragment was used to programme the Zubay system using a \texttt{recB}
S30 extract. The resulting \textsuperscript{35}S-methionine labelled sample was
run on an 11\% polyacrylamide gel alongside a maxi-cell sample
of pLG552 (Fig. 7.4). As shown in Fig 7.4 the 3.5 kb HindIII
does indeed express SfiB114 (FtsZ) strongly indicating that a
promoter capable of allowing transcription of \texttt{ftsZ} is present
on the 3.5 kb fragment.

7.5 Discussion

Results presented here have indicated that the \texttt{sfiB114}
mutation is an allele of the previously characterised
essential cell division gene \texttt{ftsZ} and that an element within
the \texttt{ftsA} coding sequence is required for efficient expression
of \texttt{ftsZ}. While this work was in progress Luktenhaus (1983)
published a paper in which two different \texttt{sfiB} alleles \texttt{sulB25}
(Johnson, 1977) and \texttt{sulB9} (Gayder et al., 1976) were shown to
be allelic to \texttt{ftsZ}. Luktenhaus (1983) also found that the
gene products of these alleles, SulB25 and SulB9, migrated
more slowly than the wild type FtsZ (SfiB\textsuperscript{+}) protein in SDS-
PAGE. This result indicated that the location of the \texttt{sfiB25}
and \texttt{sfiB9} mutations was within the \texttt{ftsZ} coding sequence
producing polypeptides with an altered size or mobility in
SDS-PAGE. These results confirmed the conclusion that \texttt{sfiB}
and \texttt{ftsZ} are allelic. The significance of the apparently
Figure 7.4
This shows an autoradiograph of an 11% acrylamide gel. Track 1 represents the results of using the 3.5 kb HindIII fragment from pLG552 in the Zubay \textit{in-vitro} transcription-translation system and Track 2 represents expression of pLG552 in maxi cells.
complex transcriptional regulation of the $sfiB$ gene will be discussed later. Having found the location of the $sfiB$ gene it remained to determine the mode of action of SOS induced division inhibition and the mechanism of suppression of $sfiB$ mutations lying within the $ftsZ$ gene.
CHAPTER 8

The design of a model system for studying sfiA-sfiB interactions

8.1 Introduction

Having identified the location and gene product of the sfiB gene, it remained to determine the role of sfiB in SOS mediated division inhibition. It has often been postulated that since the SfiA protein has the properties of a division inhibitor and sfiB is allelic to ftsZ, an apparently essential cell division gene, ftsZ could be a 'target' for the action of SfiA. The effects of sfiA upon sfiB might include reducing the synthesis of FtsZ, increasing its rate of breakdown or blocking its action. In this case sfiB mutations could be viewed as rendering the sfiB gene or the SfiB protein resistant to the action of SfiA. In order to test this theory a model system was designed based upon an E.coli gene expression system, to study the effects of the SfiA protein on the production or stability of FtsZ. This was to be done using recombinant plasmids carrying sfiA and either sfiB+ or sfiB114.

The sfiA locus had been cloned fortuitously by Henning et al. (1979) in studies of the adjacent ompA outer membrane gene. Two sfiA plasmids (Bremer et al., 1980) were available for use in this work: pTU101, consisting of a deletion derivative of a DNA fragment carrying sfiA and ompA ligated into the EcoR1 site of pSC101 (copy number 6-8) and pTU201 (Fig. 8.1) which has a 7.5 kb EcoR1 fragment carrying sfiA
Figure 8.1

A map of the plasmid pTU201 showing the position of the $\text{ompA}$ and $\text{sfiA}$ genes. The light line represents the vector pBR325. No restriction pattern for the pBR325 segment is shown because the orientation of the chromosomal insert is unknown.

E - EcoRI
B - BamHI
P - PvuII

Note that the PvuII site shown in the 1.78 kb BamHI fragment consists of two PvuII sites very close together.
and an amber ompA allele (ompA31) inserted into the vector pBR325 (copy number 15-40). These constructions therefore avoid the problem that wild type ompA^ is lethal in high copy number plasmids. Since pTU101 and pTU201 encode resistances to Tet, and Amp + Tet respectively and both the sfiB^ (pLG554) and sfiB114 (pLG552) plasmids encode resistance to Kan, both sfiA plasmids were suitable for use in a model gene expression system.

**8.2 Choice of a gene-expression system**

As described in chapter 7, there are two semi-in-vivo gene expression systems suitable for use with recombinant plasmids, mini-cells and maxi-cells. As a further alternative the Zubay in-vitro transcription-translation system was also available. The basic problem involved in using maxi-cells or minicells for setting up a sfiA/sfiB model system was to ensure a sufficiently high level of expression of sfiA. In fact severe problems were encountered with the use of all three gene expression systems with the initially available plasmids.

a) mini-cells: In order to achieve a suitable level of de-repression of SfiA synthesis, the mini-cell producing strain DS410 was transduced to tsl^−. This was achieved using a P1 stock grown on a strain (GC2302) having a Tn9 transposon inserted in the malB gene near tsl locus. Approximately 50% of the chloramphenicol resistant DS410 transductants were found to be tsl by patch plating on nutrient agar at 42°C and examining for filamentation using phase contrast microscopy. Unfortunately, the tsl mutation in the lexA gene produces a Tsl protein whose ability to repress SOS functions, including
SfiA synthesis is reduced even during growth at 30°C (Casaregola et al., 1982). Thus the high copy number sfiA plasmid pTU201 would not transform strain DS410tsl even at 30°C probably as a result of the poor repression of the Tsl protein on the multiple sfiA promoters, causing enough SfiA protein to be produced to block cell division. Strain DS410tsl could be transformed by the lower copy number, pTU101, but these transformants were highly filamentous when grown at 30°C. In order to obtain reliable information on sfiA/sfiB interactions it was considered necessary that the great majority of mini-cells should contain both sfiA and sfiB plasmids. Thus by using low copy number plasmid derivatives to transform DS410tsl it is considered likely that a relatively high proportion of mini-cells would contain only one of the two plasmids. This places severe limitations on the use of mini-cells as a model system for studying sfiA and sfiB interactions.

b) Maxi-cells: As with mini-cells, the use of maxi-cells in studying the effects of SfiA on sfiB requires a system which ensures that sfiA is expressed at a high level during the period of radioactive labelling of plasmid encoded products. The only way of ensuring this was to use a recA, ts1 strain as a host for maxi-cell experiments. Such a strain was obtained, GC2493. However, as described in the previous section the, ts1 allele does not fully repress the SOS functions (including DNA repair) even during growth at 30°C. Consequently GC4293 (ts1, recA) was found to be considerably more resistant to UV-irradiation than CSH26ΔF6 (recA) when grown in minimal medium + casamino acids at 30°C making the
use of this strain for maxi-cell experiments difficult.

c) Zubay in-vitro transcription translation system: The Zubay coupled in-vitro transcription-translation system was not considered to be a suitable model system to study sfiA/sfiB interactions as the concentration of proteins synthesised in the Zubay S30 system after addition of exogenous DNA is so low compared to the in-vivo concentration that no valid data was anticipated.

In conclusion, the plasmids available for the creation of a reliable model system to study sfiA/sfiB interactions were highly inconvenient. However, since the major problem involved in using the maxi-cell gene expression system was to ensure de-repression of sfiA without inducing SOS controlled DNA repair functions an alternative strategy was devised. Thus it was decided to subclone sfiA into a plasmid expression vector where sfiA would be under the control of a promoter whose activity could be easily regulated, allowing SfiA synthesis to be induced without altering the LexA repression of other SOS functions.

8.3 Choice of subcloning vehicle

The vector chosen for this work was a lac expression vector pPM60 (De Maeyer et al., 1982). This is a derivative of pAT153 (Twigg and Sherratt, 1980) containing the synthetic lacUV5 promoter with a BamH1 restriction site situated such that DNA fragments cloned into the BamH1 site are downstream of the UV5 promoter as indicated in Fig. 8.2 (Miller and Reznikoff, 1978). This plasmid has a high copy number and titration of the wild type LacI repressor leads to virtual constitutive expression from the UV5 promoter even in the
Figure 8.2
A map of the plasmid vector pPM60. The position of the synthetic lacUV5 promoter showing the direction of transcription is shown. The size in kb is indicated on the inside of the circle.

E - EcoRI
B - BamHI
S - SalI
absence of an inducer compound (such as IPTG). In order to prevent this all experiments involving pPM60 and derivatives were carried out using strains containing an F' carrying the mutant lacI<sup>q</sup> allele which codes for high level expression of the LacI repressor. In order to ensure maintenance of this F' the plasmid also carries the transposon Tn9 encoding Chl resistance. Using a derivative of pPM60 having the α1 human interferon gene cloned into the BamH1 site, De Maeyer et al., 1982 observed a 320 fold increase in α1 interferon expression upon addition of IPTG to a culture of E.coli carrying this recombinant plasmid (pIFS101) and the F'<lacI<sup>q</sup>.

8.4 Subcloning of sfiA into pPM60
The sfiA gene had previously been sequenced (Beck and Bremer, 1980) and found to lie on a 1.78 kb BamH1 fragment containing the proximal part of the ompA gene (the distal part of the ompA gene was contained on an adjacent 1.83 kb BamH1 fragment). This 1.78 kb BamH1 fragment (from pTU201, Fig 8.1) is convenient for cloning sfiA into the BamH1 site of pPM60 as there is only a small sequence (1036 base pairs) between the sfiA gene and the BamH1 site on the 1.78 kb fragment. This additional sequence would not be expected to affect expression of sfiA from the lacUV5 promoter when inserted into pPM60.

The 1.78 kb BamH1 fragment was purified from pTU201 DNA cut with BamH1. As the 1.78 kb and 1.82 kb DNA fragments were very similar in size the "freeze-squeeze" method (see Methods) was used to purify the 1.78 kb fragment from a 1.0% agarose gel. This method yielded a 1.78 kb fragment sample
with less contaminating 1.82 kb fragment than using the DE81-DEAE cellulose technique used in chapter 7. This purified \textit{sfiA} restriction fragment was ligated into the BamH1 site of pPM60 previously digested with restriction enzyme and the ligated DNA used to transform strain CSH26ΔF6 \textit{F'\textit{lacIQ}} to ampicillin resistance on nutrient agar plates (Fig 8.3). Two classes of transformant were obtained after growth at 37°C. One class was fast growing and showed normal cell morphology and the other grew very slowly and microscopic examination showed very extensive filamentation. Twelve colonies of each class were purified by streaking to single colonies on nutrient agar plates and then tested for sensitivity to IPTG (40 μg/ml) on nutrient agar. All fast growing transformants grew normally on IPTG containing media. However, seven of the slow growing, filamentous transformants would not grow on IPTG containing agar plates. As a result it was suspected that the fast growing transformants contained recircularised pPM60 vector molecules and the slow growing transformants contained pPM60 plasmids having the 1.78 kb BamH1 \textit{sfiA} fragment inserted into the BamH1 site. The IPTG sensitive transformants of this second class were expected to harbour plasmids carrying the \textit{sfiA} gene downstream of the lacUV5 promoter, transcription from the contiguous lacUV5 promoter being in the same direction as the \textit{sfiA} gene. In contrast the IPTG resistant transformants would carry recombinant plasmids having the \textit{sfiA} fragment in the opposite orientation. In order to confirm this, plasmid DNA was prepared from an IPTG resistant and an IPTG sensitive transformant (named pLG557 and pLG558 respectively). Both plasmids DNA samples were restricted with BamH1 to show that they contained the 1.78 kb
Figure 8.3
A diagram showing the construction of pLG557 and pLG558 from pPM60 and the 1.78 kb BamH1 fragment purified from pTU201 DNA digested with BamH1.

E - EcoR1
B - BamH1
P - PvuII
pPM60 x BamH1

1.78 kb BamH1
fragment from pTU201

ligate

pLG557

pLG558
BamH1 fragment and simultaneously digested with PvuII and EcoR1 to show the orientation of the insert. The 1.78 kb BamH1 fragment has 2 PvuII sites extremely close together at the extreme end (Fig 8.1) and so digestion with PvuII and EcoR1 allowed the orientation of the cloned region within the recombinant plasmid to be determined. The results of these restriction enzyme digestions confirmed the predicted structures of pLG557 and pLG558 (Fig 1.3). Thus it was concluded that in the recombinant plasmid pLG558 the sfiA gene is under the control of the lacUV5 promoter. However it must be noted that the sfiA gene on plasmid pLG558 still possessed its own LexA controlled promoter intact.

8.5 Properties of plasmids pLG557 and pLG558

In order to show that addition of IPTG to a strain carrying pLG558 was capable of blocking cell division as predicted from its structure, cultures of CSH26AF6F'\textsuperscript{lacI}q carrying pPM60, pLG557 or pLG558 were grown in minimal media + casamino acids at 37°C to $A_{450}^\text{max} = 0.1$ and IPTG was added to a final concentration of 40 $\mu$g/ml. Aliquots of each culture were removed at 15 min intervals and the $A_{450}$ and cell number measured. The results in Fig. 8.4 show that addition of IPTG to a growing culture of CSH26AF6F'\textsuperscript{lacI}q pLG558 causes a rapid cessation of cell division, an effect not seen with CSH26AF6F'\textsuperscript{lacI}q carrying pLG557 or pPM60. In addition, even in the absence of IPTG the mass per cell of CSH26AF6F'\textsuperscript{lacI}q pLG558 and pLG558 compared to pPM60. This increase in cell size in strains carrying pLG557 or pLG558 was thought to be due to
Figure 8.4
The effect on the cell number and cell mass of adding the lac inducer IPTG to cultures of CSH26ΔF6F'lacIq growing in minimal medium + casamino-acids carrying the plasmids:

a) pPM60
b) pLG557
c) pLG558
the synthesis of SfiA caused either by partial titration of LexA repressor by the multiple copies of the sfiA promoter or escape synthesis from the lacUV5 promoter by a similar mechanism involving LacI.

8.6 Tests for suppression of pLG558 induced filamentation by cloned and chromosomal sfiB114 mutations

In chapters 4 and 5 it was shown that sfiB114 cloned into either pOU71 (pLG550) or pLG339 (pLG552) could suppress tsl induced filamentation demonstrating that under these conditions sfiB114 is dominant. It was therefore interesting to test if a cloned or chromosomal sfiB114 mutation could suppress the filamentation induced by adding IPTG to a pLG558 bearing culture. Firstly pLG558 DNA was transformed into strain GC4276F'lacIq carrying a chromosomal sfiB114 allele. The effect on cell division of adding IPTG to a culture of this transformant was then tested as before. As shown in Fig. 8.5 the sfiB114 chromosomal mutation prevented the inhibition of cell division seen upon the induction of SfiA synthesis from pLG558 in a sfiB+ strain. Secondly, CSH26AF6F'lacIq was co-transformed with pLG558 + pLG552(sfiB114) and pLG558 + pLG554(sfiB+) plasmid DNA and the effect of adding IPTG to growing cultures of these transformants tested as before. The results given in Fig 8.5 surprisingly show that pLG552 (or pLG554) is not capable of full suppression of IPTG induced filamentation caused by pLG558. However, neither transformant showed the apparent decline in cell number after the addition of IPTG seen when pLG558 was present alone (this reduction is caused by the inefficiency of the coulter counter to count long filaments).
Figure 8.5
The effect on cell number and cell mass of adding IPTG to cultures growing in minimal media containing casamino-acids.

a) GC4276 (sfiB114) F'lacIq pLG558
b) CSH26∆F6 F'lacIq, pLG558, pLG552
c) CSH26∆F6 F'lacIq, pLG558, pLG554
and in fact a slight increase in cell number can be seen. In addition the mass per cell of these double transformants in the absence of IPTG is less than in the presence of pLG558 alone, indicating that higher levels of SfiA are being neutralised by increased levels of SfiB+ or SfiB114 under these conditions.

8.7 Discussion
In this chapter the design of a reliable model system for the study of sfiA/sfiB interactions has been described. This involved the construction of a plasmid which allowed the induction of SfiA synthesis without affecting the repression of other SOS functions. Such a plasmid, pLG558 was formed by the insertion of a 1.78 kb BamHI fragment carrying sfiA downstream of a lacUV5 promoter. Subsequently it was clearly demonstrated that induction of SfiA production by the addition of IPTG to pLG558 carrying cells blocks cell division. This result also confirms the hypothesis that SfiA protein is capable of blocking cell division in the absence of the induction of other SOS functions. While this work was in progress Huisman et al. (1984) described the construction and properties of a similar plasmid to pLG558, having sfiA downstream of a wild type lac promoter, although in this case the cloned sfiA had its own lexA controlled promoter removed. The results presented by Huisman et al. (1984) are completely consistent with those described here.

A sfiB114 chromosomal mutation was capable of fully suppressing the IPTG dependent filamentation shown by pLG558 carrying cells providing evidence for a direct interaction
between \textit{sfiA} and \textit{sfiB} as reported by Huiman \textit{et al.} (1984). However, quite surprisingly host cells with multiple copies of \textit{sfiB114} on a recombinant plasmid (but with \textit{sfiB}\textsuperscript{+} present on the chromosome) were almost completely sensitive to the effects of pLG558 in the presence of IPTG although in the absence of the \textit{lac} inducer filamentation was suppressed. This result is in contrast to the fact that pLG552 was shown to suppress \textit{tsl} induced filamentation (Chapter 5). However the levels of SfiA protein in cells containing pLG558 after induction with IPTG were expected to be significantly higher than after temperature induction of a \textit{tsl} mutant due to the high copy number of pLG558. The fact remains a puzzling one that the \textit{sfiB114} allele present on the chromosome did suppress the effect of IPTG on pLG558 carrying cells whereas when present on a recombinant plasmid (pLG552) it did not. This apparent anomaly and its relationship to FtsZ\textsuperscript{+} function will be discussed later.
Interactions between plasmids pLG558 and pLG552/pLG554 in maxi-cells

9.1 Introduction
In the previous chapter the construction of a plasmid (pLG558) was described from which SfiA production could be induced by the derepression of a lacUV5 promoter upstream of the sfiA gene. This permitted the study of interactions between cloned sfiA and sfiB genes in maxi-cells under conditions where SfiA production is induced.

9.2 Expression of pLG558 in maxi-cells
In order to ensure high level expression of sfiA from pLG558 in maxi-cells it was necessary to relieve repression on the lacUV5 promoter using the lac inducer IPTG. Maxi-cells were therefore prepared from CSH26 AF6 F'laclq pLG558 and IPTG added at the pre-incubation stage (see methods).

9.3 Interaction between pLG558 and pLG554/pLG552 in maxi-cells
In order to study any interaction between the gene products of pLG558 and pLG552 or pLG554 in maxi-cells, CSH26 AF6 F'laclq was transformed with pLG552, pLG554, pLG558 + pLG552 and pLG558 + pLG554. Maxi-cells were prepared for both single and double transformants and 35S-methionine labelled proteins analysed by SDS-PAGE and autoradiography. The amounts of each labelled protein was quantified by densitometric scanning of autoradiographs. A number of observations can be made on the
basis of the results presented in Fig. 9.1. Firstly, SfiB\(^\text{+}\) (FtsZ) seems to be expressed at a higher level (approximately 1.8 fold) than SfiB\(^{114}\), using EnvA and SecA as internal standards. Secondly the presence of pLG558 seems to actually increase the production of both SfiB\(^\text{+}\) and SfiB\(^{114}\) by a factor of 2.5 and 1.8 respectively over the level produced in the absence of pLG558 (again using EnvA and SecA as standards). Finally, the SfiA band is considerably stronger in the presence of pLG554 (sfiB\(^{+}\)) than with pLG552 (sfiB\(^{114}\)). Similar results were obtained in several different experiments indicating a specific effect on the abundance of SfiB\(^\text{+}\), SfiB\(^{114}\) and SfiA due to the presence of different plasmids.

There are two ways in which the abundance of a protein synthesised in maxi-cells can be altered, i.e. by a change in the rate of synthesis or a change in the rate of breakdown of the polypeptide. To test which of these was applicable to the variations seen in Fig. 9.1, plasmid bearing maxi-cells were pulse labelled with \(^{35}\)S-methionine to examine the stability of plasmid encoded products.

9.4 Pulse chase maxi-cell experiments with sfiA and sfiB carrying plasmids

In order to investigate the basis of the variation in relative abundance of SfiB\(^\text{+}\) and SfiB\(^{114}\) proteins, in the presence and absence of pLG558, maxi-cells containing either pLG552 or pLG554 were labelled with \(^{35}\)S-methionine for 2 min and then chased with an excess of cold methionine. Samples removed at increasing time intervals were fixed with TCA and analysed by SDS-PAGE. The results obtained are shown in Fig.
Figure 9.1
An autoradiograph of $^{35}$S-methionine labelled maxi-cells analysed using an 11% polyacrylamide gel. Tracks represent maxi-cells carrying:

1 - pLG554
2 - pLG552
3 - pLG554 + pLG558
4 - pLG552 + pLG558
9.2 (pLG552) and Fig. 9.3 (pLG554). These results show that both the SfiB\(^+\) and SfiB114 proteins are stable throughout the time course of the experiment. This would indicate that the difference in abundance between SfiB\(^+\) and SfiB114 and the increase in both SfiB\(^+\) and SfiB114 levels in the presence of pLG558 is due to differences in the rates of synthesis and not breakdown of the polypeptides.

Secondly, in order to investigate the basis of the apparent increase in the abundance of SfiA protein synthesised from pLG558 in the presence of pLG554, maxi-cells containing pLG558 alone, pLG558 + pLG552 and pLG558 + pLG554 were pulse labelled with \(^{35}\)S-methionine and then chased in the presence of cold methionine as above and the results are shown in Fig. 9.4 (pLG558), Fig. 9.5 (pLG558 + pLG552) and Fig. 9.6 (pLG558 + pLG554). The half-life of the SfiA protein, which as anticipated is extremely short (Mizusawa and Gottesman, 1983), is considerably increased in the presence of pLG554 but apparently not when pLG552 is present. The SfiA protein bands seen in Figs. 9.4, 9.5 and 9.6 were scanned densitometrically and the half-life of SfiA in each experiment calculated (Fig 9.7). This half-life was approximately 3 min for pLG558 alone and pLG558 + pLG552 but 10-14 min. for pLG558 + pLG554. Further inspection of Fig. 9.4-9.6 indicate the appearance of an additional polypeptide of about 17 kD under conditions where SfiA is unstable. The kinetics of appearance of this polypeptide suggest that this may be a preferred breakdown product of SfiA. These observations were based on the assumption that the unstable protein having a size of approximately 19 kD and encoded by pLG558 was in fact SfiA.
Figure 9.2
An autoradiogram of maxi-cells containing pLG552, pulse labelled with $^{35}$S-methionine and analysed using an 11% polyacrylamide gel.
Figure 9.3
An autoradiogram of maxi-cells containing pLG554, pulse labelled with $^{35}$S-methionine and analysed using an 11% polyacrylamide gel.
Figure 9.4
An autoradiogram of maxi-cells containing pLG558, pulse labelled with $^{35}\text{S}$-methionine and analysed using an 15% polyacrylamide gel.
Figure 9.5
An autoradiogram of maxi-cells containing pLG552 + pLG558, pulse labelled with $^{35}$S-methionine and analysed using an 15% polyacrylamide gel.
Mol. wt. (kD)

- 46 - SfiB 114
- 30 - EnvA
- 14.3 - SfiA

Time after chase (Minutes)

0  1  2  5  10  15  30
Figure 9.6
An autoradiogram of maxi-cells containing pLG554 + pLG558, pulse labelled with $^{35}$S-methionine and analysed using an 15% polyacrylamide gel.
Figure 9.7

A graph of the natural log of the percentage SfiA protein (as measured by densitometric scanning) seen in Figs. 9.5 and 9.6 as a fraction of the total labelled protein plotted against time. Closed circles represent pLG552 + pLG558 (Fig. 9.5) and open circles represent pLG554 + pLG558 (Fig. 9.6). The half-life of SfiA in each case was calculated as $-\ln \frac{2}{\text{gradient}}$. Similar calculations were done on Fig. 9.4 (pLG558 alone) (data not shown).
9.5 Programming of the Zubay system with DNA encoding sfiA

In order to confirm the identity of the supposed SfiA protein, a restriction enzyme fragment carrying sfiA was used to programme the Zubay coupled in-vitro transcription-translation system. From Fig 8.1 (based on the nucleotide sequence) it can be seen that the 1.78 Kb BamH1 restriction fragment from pLG558 encodes only sfiA and the proximal part of ompA. The ompA gene on pTU201 from which the 1.78 Kb fragment was derived is in fact a mutant amber ompA allele ompA31. Therefore if a Zubay S30 extract prepared from a sup° strain is used for the in-vitro transcription-translation of the BamH1 fragment then only the SfiA protein should be produced.

The 1.78 Kb BamH1 fragment was purified from pLG558 DNA cut with BamH1 using the DE81-DEAE cellulose method. This fragment and the whole pLG558 plasmid DNA was then used to programme the Zubay system using an extract prepared from strain N138 (recB-ts, sup°) (a gift from J.Pratt and M.Jackson) and ^35S-methionine labelled proteins analysed by SDS-PAGE (Fig. 9.8). The results in Fig. 9.8 show that the 1.78 Kb BamH1 fragment apparently encodes one major polypeptide of 19kD as expected for the SfiA protein, whereas the intact pLG558 plasmid DNA encodes both SfiA and -lactamase. The radioactively labelled SfiA protein synthesised from the isolated 1.78 Kb restriction fragment was therefore a suitable marker for identifying SfiA amongst the more complex protein profile obtained using pLG558 in maxi-cells.
Figure 9.8

An autoradiograph of $^{35}$S-methionine labelled proteins synthesised from DNA templates using the Zubay \textit{in-vitro} transcription-translation system. Templates were:

1 - No added DNA
2 - Purified 1.78 kb BamHI fragment from pLG558
3 - Intact plasmid pLG558
9.6 Confirmation of the identity of SfiA synthesised by pLG558 in maxi-cells

In order to show that the protein encoded by pLG558 and whose stability was affected by the presence of pLG554 was in fact SfiA, the samples in Fig. 9.4 (pLG558 alone) were re-analysed using a 15% polyacrylamide gel alongside the SfiA protein encoded by the 1.78 Kb BamH1 fragment *in-vitro*. An autoradiogram of this gel is presented in Fig 9.9 and shows that protein postulated to be SfiA was indeed the product of the *sfiA* gene. This was repeated with samples from Fig. 9.5 (pLG558 + pLG552) and Fig. 9.6 (pLG558 and pLG554) and again the identity of the SfiA protein was confirmed (data not shown).

9.7 Discussion

In this chapter results were presented in an attempt to demonstrate any interaction between the *sfiA* and *sfiB* genes and indicate the method of action of the *sfiB114* mutation. The "target" hypothesis of SfiA action on FtsZ (*SfiB*), has been described previously in Chapter 8 and postulated that induction of the synthesis of the SfiA protein would either block the synthesis of FtsZ, increase its breakdown or inactivate its function. It has been shown here that where *sfiA* and *sfiB* (ftsZ) are present together in maxi-cells, SfiA synthesis results in neither a reduction in synthesis nor an increase in breakdown of SfiB*.

The observation that the presence of pLG554 stabilised the SfiA protein in maxi-cells whereas the presence of pLG552, which only differs in the presence of the *sfiB114*
Figure 9.9

An autoradiogram of maxi-cells containing pLG558 pulse labelled with $^{35}$S-methionine and analysed using a 15% polyacrylamide gel. As a standard representing SfiA, a sample from the transcription-translation of the purified 1.78 kb BamHI DNA fragment seen in Fig. 9.8 was run alongside and indicates that the protein band previously identified as SfiA is in fact the product of the sfiA gene.
allele of \textit{ftsZ}, had no effect, is extremely important. Firstly, it shows that a locus is present on pLG554 that is capable of stabilising SfiA in maxi-cells and secondly, it shows that this locus is \textit{ftsZ(sfiB)}. This result provides evidence for an interaction between SfiA and SfiB\textsuperscript{+} proteins. Presumably this interaction resulted in some protection from the proteases that normally degrade SfiA. Since SfiB114 encoded by pLG552 could not stabilise SfiA it is possible that this was due to a reduction in the protection of SfiA against proteases. An alternative explanation for these observations is that under the conditions used, SfiB\textsuperscript{+} encoded by pLG554 non-specifically inhibits the action of proteases, including the Lon protein shown by Mizusawa and Gottesman (1983) to be involved in the degradation of SfiA. However, it is difficult to explain the suppression of SOS mediated division inhibition by \textit{sfiB} mutations \textit{in-vivo} in terms of an altered interaction of SfiB, an essential division protein, with the Lon gene product.

The apparent increase in the rate of synthesis of SfiB\textsuperscript{+} and SfiB114 in the presence of SfiA and indeed the difference in the rates of synthesis of SfiB\textsuperscript{+} and SfiB114 is intriguing. Although the difference in the rates of synthesis between SfiB\textsuperscript{+} and SfiB114 may imply that the location of the \textit{sfiB114} mutation is within the \textit{ftsZ} promoter region rather than the coding sequence this is not necessarily the case. Luktenhaus (1983) examined the gene products of two \textit{sulB} alleles, \textit{sulB9} and \textit{sulB25}, and found that both proteins were altered in size compared to the wild type FtsZ (SfiB\textsuperscript{+}) protein showing that both mutations are located within the coding region.
W. Donachie has claimed (personal communication) that \textit{ftsZ} is repressed by its own gene product. The \textit{sfiB114} mutation, if present within the \textit{ftsZ} coding sequence, might therefore alter the FtsZ protein reducing its efficiency to repress its own synthesis resulting in a higher level of \textit{sfiB114} expression compared to \textit{sfiB}$. Similarly the observed increase in the levels of synthesis of \textit{SfiB} and \textit{SfiB114} in maxi-cells in the presence of \textit{SfiA} may result from a reduced affinity of \textit{SfiB}, in association with \textit{SfiA}, for its own promoter region. This effect would seem to add more indirect evidence for the postulated \textit{SfiA-SfiB} interaction.

In interpreting the results of the maxi-cell experiments presented in this chapter it is important to recognise that the concentration of plasmid encoded products at the time of labelling proteins with $^{35}$S-methionine is far from physiological. The gene products of the \textit{envA}, \textit{secA} and \textit{sfiB}/\textit{sfiB114} genes are very stable and therefore after incubation overnight the UV-irradiated maxi-cells will contain very high levels of these plasmid encoded proteins. As stated in the text, the kinetics of appearance and disappearance of the protein band seen in Fig. 9.4-9.6 at a size of approximately 17 kD imply that this is a primary proteolytic breakdown product of \textit{SfiA}. Such a primary breakdown product was not observed by Mizusawa and Gottesman (1983) in $\lambda$-infection experiments used to measure the half-life of the \textit{SfiA} protein. This was possibly due to the relatively higher background seen using the $\lambda$-infection gene expression system (compared to maxi-cells) obscuring such a protein band. Alternatively, this breakdown product may be more rapidly degraded in $\lambda$-infected cells than in maxi-cells.
preventing its identification. Kowit and Goldberg (1977) showed that an intermediate fragment could be observed during the _lon_ dependent breakdown of a nonsense fragment of β-galactosidase. In this case the half-life of the intermediate fragment (30 min) was considerably longer than that of the original protein (5 min). However, Shoemaker _et al._, (1984) incubated an outer membrane fraction apparently containing SfiA with purified Lon protein and observed no degradation of SfiA and consequently suggested that the _lon_ gene controls the proteolysis of SfiA by a second protease.

Shoemaker _et al._, (1984) described experiments indicating that the SfiA protein co-fractionated with the outer membrane of _E.coli_. Clearly for the interaction between SfiA and SfiB demonstrated in this chapter to occur, the subcellular location of the proteins must be the same. In order to examine this directly, maxi-cells containing _sfiA_ and _sfiB_ carrying plasmids were separated into subcellular fractions and the location of the _sfiA_ and _sfiB_ gene products determined.
CHAPTER 10

Studies on the cellular location of SfiA and SfiB

10.1 Introduction

Having concluded that the SfiA and SfiB proteins interact in-vivo it was decided to determine their cellular location by fractionation into cytoplasmic, inner membrane and outer membrane fractions. The fractionation procedure was performed in the presence and absence of 10 mM Mg\(^{2+}\). Mg\(^{2+}\) stabilises protein-membrane binding and so fractionation in the presence or absence of Mg\(^{2+}\) allows the distinction between non-membrane binding, a specific family of membrane binding proteins (Salton, 1971) and integral membrane proteins.

10.2 Cellular fractionation of pLG554 containing maxi-cells

A 20 ml culture of CHS26AF6 pLG554 maxi-cells was UV-irradiated and plasmid encoded products labelled with \(^{35}\)S-methionine as described previously. Labelled maxi-cells were divided into two portions, one of which was resuspended in envelope buffer and another in envelope buffer + 10 mM MgCl\(_2\). Inner and outer membrane fractions were isolated by sonication of the cells followed by differential centrifugation. An excess of carrier membranes were added to the maxi-cell membrane fractions at this point since the yield of membranes from 10 ml of maxi-cells was inconveniently small. Inner and outer membranes were isolated by solubilisation with sarkosyl (inner membranes are sarkosyl soluble and outer membranes are not). Equivalent cell proportions of each fraction were loaded onto a polyacrylamide gel and analysed by SDS-PAGE and
autoradiography (Fig. 10.1). The efficiency of the fractionation procedure was monitored by staining the gel for protein using Coomassie blue (although of course this mainly analyses the efficiency of the fractionation of the carrier cells) before autoradiography. Results in Fig 10.1 show that both FtsZ and SecA display the properties of inner membrane binding proteins. Approximately 50% of each of these proteins fractionated with the inner membrane in the presence of Mg$^{2+}$ whilst other proteins such as EnvA and the protein associated with kanamycin resistance showed very little affinity with the inner membrane. The failure to find all of the labelled FtsZ and secA, which had previously been shown to be a membrane binding protein under these condition, could be ascribed to a number of factors. Following sonication a significant proportion (20-30%) of membrane proteins remain in the soluble fraction (Churchward and Holland, 1973; Boyd and Holland, 1976). In addition the Mg$^{2+}$ concentration used during the membrane fractionation may not have been optimal. The apparently intrinsic membrane protein seen in Fig. 10.1 located between EnvA and FtsZ is probably a fusion protein commencing at the Tet$^r$ (a known inner membrane protein) promoter on pLG339.

10.3 Localisation of SfiA and the effect of SfiA on the fractionation of SfiB$^+$

It was not found possible to derive useful data on the fractionation of SfiA protein from maxi-cells containing pLG558 alone. This was due to the short lifetime of SfiA which resulted in a very weak SfiA protein band observed in
An autoradiogram of maxi-cells containing pLG554, labelled with $^{35}$S-methionine, separated into subcellular fractions in the presence and absence of 10 mM Mg$^{2+}$ and analysed using an 11% polyacrylamide gel. Tracks represent:

1 - Cytoplasm + Mg$^{2+}$
2 - Cytoplasm - Mg$^{2+}$
3 - Inner membranes + Mg$^{2+}$
4 - Inner membranes - Mg$^{2+}$
5 - Outer membranes + Mg$^{2+}$
6 - Outer membranes - Mg$^{2+}$
fractionated pLG558 containing maxi-cells. However, maxi-cells containing both pLG558 and pLG554 were successfully fractionated and under these conditions the stabilisation of SfiA due to the presence of pLG554 produced a considerably stronger SfiA band (Fig. 10.2). Firstly the results in Fig. 10.2 show that SfiA also displays the properties of an inner membrane binding protein. Secondly the presence of SfiA does not seem to have affected the cellular fractionation of SfiB⁺.

10.4 Discussion
Work presented here has shown that both SfiA and SfiB proteins are associated with the inner membrane of E. coli. This result is apparently incompatible with the work of Shoemaker et al. (1984) who claimed that the SfiA protein fractionated with the outer membrane. There are however two possible explanations for this apparent anomaly. Firstly the cell fractionations of Shoemaker et al. (1984) were performed in the absence of Mg²⁺ and so the affinity of the SfiA protein for the inner membrane was severely reduced. Secondly, the SfiA protein has been reported to form aggregates during purification (S.Gottesman, personal communication). It is possible that the SfiA protein seen by Shoemaker et al. (1984) in the outer membrane fraction comprises aggregates of SfiA with the outer membrane. Moreover the procedure used by Shoemaker et al., (1984) to break up the cells (sonication) prior to separation of the membranes on sucrose membranes is not optimal for this purpose (Osborn et al., 1972). Inspection of the separation data of Shoemaker et al., (1984) indicates that quite atypical inner and outer
Figure 10.2
An autoradiogram of maxi-cells containing pLG554 + pLG558, labelled with $^{35}$S-methionine, separated into subcellular fractions in the presence and absence of 10 mM Mg$^{2+}$ and analysed using an 11% polyacrylamide gel. Tracks represent:

1 - Cytoplasm + Mg$^{2+}$
2 - Cytoplasm - Mg$^{2+}$
3 - Inner membranes + Mg$^{2+}$
4 - Inner membranes - Mg$^{2+}$
5 - Outer membranes + Mg$^{2+}$
6 - Outer membranes - Mg$^{2+}$
membrane profiles were obtained, casting further doubt upon the validity of their report.

Although both SfiA and SfiB associate with the inner membrane whether this association occurs on the cytoplasmic or peripasmic side of the inner membrane remains uncertain. Osmotic shock experiments on maxi-cells containing sfiA and sfiB encoding plasmids are in progress and should answer this question soon. Little work has been done to elucidate the cellular location of the gene products of other E.coli cell division genes (pbp3 being one exception). Experiments using maxi-cells containing the ftsA encoding plasmid pLG553 to find the cellular location of FtsA are in progress.
11.1 sfiB(ftsZ) and the control of cell division in E.coli

In this work it has been shown that sfiB is allelic to the essential cell division gene ftsZ, and evidence has been presented for an interaction between FtsZ and the SOS induced division inhibitor SfiA. A number of questions remain concerning the role of ftsZ in cell division both during normal cell growth and following the SOS response.

As stated in Chapter 1, on the basis of temperature shift experiments, the ftsZ gene has been proposed to be required at an early stage in the formation of the division septum. (Burdett and Murray, 1974; Walker et al., 1975). Likewise the discovery that the FtsZ protein is the target for the SfiA division inhibitor (and for the sfiC "inhibitor", D'Ari and Huisman, 1983) suggests that ftsZ plays a crucial role in the septation process. How then does the SfiA protein act to inactivate ftsZ function and what is the mode of action of a mutant sfiB? In this work it has been shown that SfiA neither decreases the synthesis nor increases the breakdown of FtsZ and so it was proposed (and subsequently demonstrated) that SfiA and FtsZ interact, presumably blocking the function of FtsZ in septum formation. sfiB mutations might therefore act via the formation of an altered FtsZ (SfiB) protein resistant to the action of SfiA or a protein which fails to bind SfiA.

Luktenhaus (1983) reported that an increase in ftsZ gene
dosage caused an increase in the amount of residual cell division after expression of \texttt{tsl}, suggesting that under these conditions more time was required before all FtsZ could be inactivated. Insight into the mechanism of FtsZ action came from recent work by Ward and Luktenhaus (1984) in which overproduction of a hybrid protein ("FtsZZ") consisting of part of LacZ fused to the distal region of the FtsZ protein caused inhibition of cell division. This inhibition was suppressed both by an increased \texttt{ftsZ}\textsuperscript{+} copy number or a chromosomal \texttt{sfiB} mutation. Ward and Luktenhaus (1984) put forward two alternative models of FtsZ function. Firstly, FtsZ may function as a multimer and so assembly of inactive \texttt{FtsZZ} into the multimer blocks its action. Secondly, FtsZ may have a specific site of action (e.g. a membrane complex) involved in septum formation and so production of \texttt{FtsZZ} may cause competition between FtsZ and inactive \texttt{FtsZZ} for this site resulting in inhibition of cell division. Although neither of these two models has been demonstrated experimentally, both go some way to explaining the anomalies in the literature concerning the dominance of \texttt{sfiB} as well as the intriguing diploid experiments presented in Chapter 8. For example, in Chapter 8 it was shown that although a chromosomal \texttt{sfiB114} mutation could suppress the IPTG sensitive filamentation caused by \texttt{pLG558}, \texttt{sfiB114} present on plasmid \texttt{pLG552} in a \texttt{sfiB}\textsuperscript{+} host strain could not. In this case either the inclusion of \texttt{FtsZ}\textsuperscript{+} into a \texttt{FtsZ}\textsuperscript{+}/\texttt{SfiB114} mixed multimer or competition between \texttt{FtsZ}\textsuperscript{+} and \texttt{SfiB114} for septation sites could create a situation where SfiA (via \texttt{FtsZ}\textsuperscript{+}) could inhibit cell division through \texttt{FtsZ}\textsuperscript{+} but not when the SfiA insensitive protein \texttt{SfiB114} was present alone.
An interesting alternative model for FtsZ action has been proposed by I.B.Holland. In this model FtsZ is an inhibitor of cell division whose action is modified during the normal cell cycle immediately before septation occurs. Presumably SfiA would act by "fixing" FtsZ into its inhibitor form, SfiB− protein again being resistant to SfiA action. A distinction between FtsZ being a promoter or inhibitor of division could be investigated by the construction of an amber mutation in \( \text{ftsZ} \) (in a strain containing a temperature sensitive suppressor) as has been accomplished with \( \text{ftsA} \) (Luktenhaus and Donachie, 1979).

Further indirect evidence for a central role of \( \text{ftsZ} \) in cell division control has come from the fact that despite intensive investigation, only one allele of \( \text{ftsZ} \) has been isolated (\( \text{ftsZ84} \)) compared to many for the adjacent \( \text{ftsA} \) and \( \text{ftsQ} \) genes (W.Donachie, personal communication). However, recently Belhumeur and Drapeau (1984) reported the isolation of a mutation which has many of the phenotypic traits associated with \( \text{lon} \) mutations (e.g. filamentation following UV-irradiation, poor lysogenisation and mucoidy). Moreover, this mutation was suppressed by multiple copies of \( \text{ftsZ} \) and was mapped to the 2 minute region of the \( E.\text{coli} \) chromosome. Belhumeur and Drapeau (1984) interpreted this as showing the isolation of an allele of \( \text{ftsZ} \) ("\( \text{ftsZ71} \)") which yielded a protein more sensitive to the action of SfiA. However, \( \text{ftsZ71} \) has not been shown to specifically map at the locus associated with \( \text{ftsZ} \) (G.Drapeau, personal communication) and its identity remains uncertain.
11.2 The division complex: A model for division control

An examination of Table 1.2 shows that there are several genes whose primary function appears to be concerned with cell division. How then, are the activities of these essential components of the septation machinery co-ordinated? I would like to propose the existence of a "division complex" of proteins involved in septum formation ("septalsome"). This division complex would comprise a number of proteins required for septation bound to the inner membrane to form a functional unit. Since the ftsZ gene is the only one whose function has been suggested to be involved in the early stages of septation it is tempting to suggest that FtsZ is crucial to the assembly of the septalsome.

One of the predictions of the division complex model is that if some mutations in septation genes block cell division by producing a protein no longer able to bind to, or function within the complex, extragenic suppressor mutations in other septation genes may be isolated. One example of an interaction between essential septation genes has been reported by Tormo et al. (1984) who showed that the growth of an ftsA mutant at the restrictive temperature caused a block in the binding of penicillin to PBP3. It was thus concluded that FtsA was required for the assembly or transport of PBP3 into the inner membrane.

The idea of a division complex makes no firm predictions about the control of cell division yet it does show how a controlling event in the cell cycle can trigger the activity of an already assembled septalsome to form a septa. The existence of a septalsome also makes it easier to envisage the co-ordination of activities of a multifunctional group of
proteins.

11.3 Gene regulation in the 2 minute region of the E.coli chromosome

In chapter 7 it was concluded that an element within the ftsA coding sequence was required for full expression of ftsZ. This has subsequently been confirmed using both gene fusion and DNA sequence analysis of the ftsQ-ftsZ region. Sullivan and Donachie (1984) created a range of galK fusions into the ftsQ-ftsZ region and showed that ftsZ possessed at least two promoters, PZ₁ immediately preceeding the ftsZ coding region and PZ₂ lying within the ftsA coding sequence. DNA sequence studies by Robinson et al. (1984) revealed areas of 2-fold symmetry corresponding to the positions of the promoters PZ₁ and PZ₂ of Sullivan and Donachie (1984). Donachie (personal communication) has recently shown that ftsA is also transcribed from at least 2 promoters, one of which lies within the ftsQ coding sequence. While the existence of genes with multiple promoters is not unknown in E.coli (for example, the uvrC is preceded by 3 promoters (Sharma et al., 1984)) the close proximity of genes with independent functions and apparently overlapping transcriptional units is intriguing.

Donachie (1984) has provided evidence for regulatory interactions between genes in the 2 minute region. Thus, Table 11.1 shows that transcription from PZ₂ is affected by mutations in ftsA, ftsI or ftsZ but not by the unlinked ftsE gene. Donachie (1984) has also measured the transcription from PZ₂ at different growth rates and found that the rate of
Table 11.1: The effect of chromosomal mutations on expression from PZg. This shows the ratios of β-galactosidase activities measured in mutant strains lysogenic for λ JFL100 (pftsZ::*lac) grown at 30°C and 42°C. In the absence of inducer, all β-galactosidase may be considered to originate from the ftsZ promoter. (From: Donachie et al., 1983).

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Ratio (42°C/30°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ftsE35(ts)</td>
<td>1.0</td>
</tr>
<tr>
<td>ftsA13(ts)</td>
<td>1.0</td>
</tr>
<tr>
<td><strong>ftsA16(am)</strong>*</td>
<td>5.0</td>
</tr>
<tr>
<td>ftsQ1(ts)</td>
<td>3.0</td>
</tr>
<tr>
<td>ftsZ84(ts)</td>
<td>1.7</td>
</tr>
<tr>
<td>ftsIkb(ts)</td>
<td>2.8</td>
</tr>
<tr>
<td>ftsIbs(ts)</td>
<td>3.1</td>
</tr>
<tr>
<td>envA</td>
<td>0.7</td>
</tr>
<tr>
<td>envK27</td>
<td>1.1</td>
</tr>
</tbody>
</table>

* This strain carried a temperature sensitive suppressor mutation.
transcription per septation is, in general, constant and independent of growth rate suggesting that the activity of the PZ₂ promoter is regulated in some way. Results presented in this work have also shown that ftsZ may be subject to autoregulatory control.

11.4 The SOS system and the heat shock response

Recent work has uncovered interesting connections between the SOS regulatory system and the heat shock response in _E. coli_ (Walker, 1984; Gottesman and Neidhardt, 1984).

The heat shock response in _E. coli_ consists of a range of polypeptides (at least 13) which are induced following a shift in growth temperature. This induction is under the control of the _htpR_ gene (Neidhardt and Van Bogelen, 1981). Krueger and Walker (1984) showed that the heat shock proteins GroEL and DnaK are induced by UV-irradiation and that this induction is dependent on _htpR⁺_. It has also been reported that both ethanol, coumermycin and chlorobiocin (Travers and Mace, 1982; Fairweather _et al._, 1981) can induce at least some heat shock proteins and so it has been suggested that the heat shock regulatory system is a general response to cell stress. A further link between the SOS and heat shock systems is that the Lon protein appears to be a heat shock protein, again under _htpR_ control (Phillips _et al._, 1984). It is interesting to note that a mutant sigma subunit of RNA polymerase which is degraded more slowly in _lon_ cells (Grossman _et al._, 1983), is broken down even more slowly in _htpR_ cells (Walker, 1984). It is tempting to suggest that Lon is induced by heat shock in order to more rapidly degrade damaged proteins. However _lon_ cells have not been reported to
be particularly temperature sensitive (i.e. defective in heat protective mechanisms). It is also possible that Lon is induced by DNA damage (as GroEL and DnaK are) to increase the degradation of SfiA but again htpR cells are not overly UV-sensitive (Krueger and Walker, 1984). A further complication is that Donachie (1984) has reported that temperature sensitive cell division mutations are frequently isolated in the dnaK gene. Moreover, several functions have already been ascribed to the dnaK gene; It is heat inducible (Tilley et al., 1983), required for λ replication (Georgopoulos and Hershowitz, 1971), has both ATP'ase and autophosphorylase activity (Zylicz et al., 1983) and is a highly conserved protein between E.coli and Man. However, insufficient data has been presented to explain the role of dnaK in cell division.

11.5 plasmid replication and cell division control
Recent work on the stability of mini-F plasmids has uncovered an interesting mechanism of coupling plasmid replication to cell division. When a thermosensitive replication defective plasmid carrying the 42.9-43.6 kb fragment from plasmid F, designated ccd (coupled cell division), is blocked for plasmid replication, copy number falls, cell division is inhibited and prophage λ is induced in λ+ strains (Ogura and Higara, 1983; Miki et al., 1984a,b). A genetic analysis of ccd has shown that two genes are present, ccdB(letD) which produces a protein that inhibits cell division and causes induction and ccdA(letA) whose product represses ccdB). The induction of prophage λ seen on expressing ccdB is dependent
on recA⁺ (Mori et al., 1984) however, whether the inhibition of cell division caused by CcdB induction is mediated through the SOS response is unclear. ccdB expression has been shown to be lethal to the cell in a recA⁻ strain (Mori et al., 1984) or in lexA, sfiA or sfiB strains (Miki et al., 1984b; Karoui et al., 1984). However, whilst Mori et al. (1984) reported that recA⁻ does not block ccdB induced division inhibition, Karoui et al. (1984) claimed that the lethality shown by lexA, sfiA and sfiB strains following ccdB expression is associated with only slight filamentation. It appears therefore, that the action of the ccd system may be operating both through recA dependent and recA independent pathways. The ccd system is currently being studied with regard to similarities between the co-ordination of plasmid DNA replication and cell division and the control mechanisms in the E.coli cell cycle.

11.6 Concluding remarks

In this discussion I have tried to indicate the relationship between ideas originating in this work and other concurrent studies on the E.coli cell cycle and the regulation of growth of this organism during stress. Overall our knowledge on the control of cell division in E.coli has yielded very little over the past three years. However, we are now in the position of being able to put forward models to explain mechanisms of septation control (particularly that following induction of the SOS response) and investigate these models using the powerful tools of modern technology and molecular biology.
REFERENCES


Castellazi, M., George, J. and Buttin, G. 1972b. Prophage
induction and cell division in E. coli II. Linked (recA, zab) and unlinked (lex) suppressors of tif-1 mediated induction and cell filamentation. Mol. Gen. Genet., 119, 153-174.


division inhibition associated with the SOS response in *Escherichia coli*. J. Bact., 156, 243-250.


Khachatorians, G.G., Clark, D.J., Adler, H.I. and Hardigree, A.A. Cell growth and division in Escherichia coli. A common genetic control involved in cell division and mini-cell


Miki, T., Chang, Z-T. and Horiuchi, T. 1984b. Control of cell division by sex factor F in Escherichia coli. II. Identification of genes for an inhibitor protein and trigger
protein on the 42.84-43.6 segment. J. Mol. Biol., 174, 627-646.


C.A. Jones and I.B. Holland*

Department of Genetics, University of Leicester, University Road, Leicester, LE1 7RH, UK

*To whom reprint requests should be sent

Communicated by R.H. Pritchard

A dominant sfiB allele has been cloned which renders partial diploids of an sfiB + Escherichia coli host resistant to division inhibition mediated by the SOS response. Transpositional mutagenesis was used to map the position of this sfi/B114 allele, carried by a plasmid pLG552, to an ~0.6-kb region overlapping the coding regions for ftsA and ftsZ, two genes essential for normal division. Most Tn1000 insertions which inactivated sfi/B114 also inactivated the ftsA function and caused the disappearance of both a 47-K polypeptide and reduced levels of a 42-K polypeptide in maxi-cells carrying pLG552. An additional insertion inactivating sfi/B114 was mapped to the right of ftsA and resulted in loss of the 42-K but not the 47-K polypeptide in maxi-cells. Moreover, a 2.1-kb BamHI-EcoRI DNA fragment was subcloned which carried ftsA and coded for a 47-K polypeptide but did not carry sfi/B114 and did not complement ftsZ. We conclude that sfi/B114 is located within ftsZ coding for a 42-K polypeptide. Nevertheless, insertions into ftsA coding the 47-K polypeptide suppress the sfi/B114 allele by substantially reducing the synthesis of the FtsZ (SfiB114) polypeptide. The level of residual FtsZ synthesis was minimal when Tn1000 was inserted closest to the distal end of sfiA, indicating the presence of a regulatory region essential for maximal expression of ftsZ.

Key words: SOS/u.v./division inhibition/sfi/B/E. coli

Introduction

U.V.-irradiation of Escherichia coli results in a complex DNA damage-repair response including inhibition of cell division (Witkin, 1967). Kinetic analysis of this effect in exponentially growing cultures has demonstrated that the onset of inhibition is extremely rapid, indicating that septum formation is blocked at a very late stage in the cell cycle (Burton and Holland, 1983). The period of division inhibition is proportional to the log of the irradiation dose, and restoration of division capacity follows the resumption of DNA synthesis (Burton, 1981; Darby and Holland, 1979). We have previously shown that the inhibition of division after u.v. is mediated by two independent pathways, the sfiA, sfiB dependent system and a second pathway which we have suggested results from failure to terminate rounds of DNA replication (Burton and Holland, 1983). The sfiA, sfiB pathway is regulated by the recA, lexA, so-called SOS repair system (Witkin, 1976).

The product of sfiA is apparently identical to the RAD1 product proposed by George et al. (1975) to be an inhibitor of division in cells containing damaged DNA. Huisman et al. (1980a) demonstrated that the synthesis of the sfiA gene product was induced by DNA damage and recently an 18-K polypeptide has been identified as the product of the sfiA (Mizusawa and Gottesman, 1983). Mizusawa and Gottesman (1983) have shown that the sfiA protein is very unstable with a half life of 1.2 min, which is consistent with its efficient removal from cells allowing the observed rapid resumption of division upon completion of DNA repair.

Certain mutations, (tsI) in the repressor gene, lexA, render the SOS system constitutive at 42°C, resulting in filamentation of the bacteria (Mount et al., 1973). Similar results are obtained with mutations, (tsI), in recA which lead to activation of the protease form of the recA protein at 42°C, and consequent cleavage of the lexA repressor (Little et al., 1980). Mutations in sfiA and a second locus sfiB suppress filamentation in both tsI and tsi mutants at 42°C (Witkin, 1976; Huisman et al., 1980b, 1980c). Similarly, mutations in another gene, lon, which block or delay recovery from u.v.-induced division inhibition are also suppressed by sfiA (sulA) and sfiB (sulB) mutations (George et al., 1975; Johnson, 1977; Gottesman et al., 1981). Mizusawa and Gottesman (1983) have also provided evidence that the lon protease normally degrades the sfiA protein.

Several groups have previously suggested that the sfiB gene product is essential for normal septum formation in E. coli and that it is the probable target for the proposed sfiA inhibitor. The sfiB locus was mapped by Johnson (1977) and by George et al. (1975) to the two minute region of the E. coli chromosome which contains a cluster of at least 12 essential genes involved in cell division or in the synthesis of surface components (Bachmann and Low, 1980). For example, mutations in the genes pbpB, ftsA and ftsZ cause the immediate cessation of cell division and consequent filamentation, at 42°C. Although the majority of genes in the region have now been accounted for (see e.g., Fletcher et al., 1978; Lutkenhaus et al., 1980), attempts to precisely locate sfiB have been unsuccessful. In this study, using initially P1 transduction and then specialised λ transducing phages and transpositional mutagenesis, we have mapped a dominant sfiB locus to an ~0.6-kb region in plasmid pLG552 which overlaps distal and proximal regions of ftsA and ftsZ, respectively, two genes essential for division in E. coli. Further subcloning and analysis of the polypeptides coded by different plasmids carrying Tn1000 insertions lead to the conclusion that sfi/B114 is located within ftsZ and that Tn1000 insertions in ftsA suppress the effects of sfi/B114 by substantially reducing the synthesis of the FtsZ (SfiB114) protein.

Results

Mapping of sfiB by P1-transduction

Many of the genes in the two minute region of the E. coli chromosome are only represented by difficult to score, temperature-sensitive mutations. In addition, strains with multiple cell surface/division mutations often show reduced viability, making three point crosses with P1 difficult to carry.
Table I. Co-transduction mapping of sfIB

<table>
<thead>
<tr>
<th>Donor</th>
<th>Recipient</th>
<th>Co-transduction frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>leuB(^{+}), (pbpB)</td>
<td>leuB(^{+}), (pbpB)</td>
<td>88%</td>
</tr>
<tr>
<td>(leuB^{+}), (ftsA^{+})</td>
<td>(leuB^{+}), (ftsA^{+})</td>
<td>59%</td>
</tr>
<tr>
<td>(leuA^{+}), (sfIB)</td>
<td>(leuA^{+}), (sfIB)</td>
<td>41%</td>
</tr>
<tr>
<td>(leuA^{+}), (envA)</td>
<td>(leuA^{+}), (envA)</td>
<td>41%</td>
</tr>
<tr>
<td>(leuA^{+}), (azC)</td>
<td>(leuA^{+}), (azC)</td>
<td>25%</td>
</tr>
</tbody>
</table>

The \(leuA^{+}\) marker was selected in each cross and the recipients scored for the unselected marker.

\[
\begin{align*}
\text{leuB} & \quad \text{leuA} \quad \text{pbpB} \quad \text{mur} \quad \\
\lambda sepB2 & \quad \lambda sep3 \quad \lambda sepA6 \quad \lambda 602
\end{align*}
\]

Fig. 1. A schematic diagram showing the organisation of known cell wall and division genes in the two minute region of the \(E.\ coli\) chromosome and the portions covered by the \(\lambda\)-transducing phages described in the text.

out. However, using \(leu^{+}\) as a primary selection it was possible by several two factor crosses to localise \(sfIB^{+}\) between \(ftsA^{+}\) and \(azC^{+}\) (Table I). In a three point cross involving \(leuA^{+}\), \(sfIB^{+}\) and \(envA^{+}\), \(sfIB^{+}\) could not be separated from \(envA^{+}\) (see Figure 1). For the reasons outlined above, it was not possible to carry out three factor crosses involving \(pbpB^{+}\) or \(ftsA^{+}\), \(sfIB^{+}\) and \(leuA^{+}\). Alternative methods involving \(\lambda\)-transducing phages were then employed in an attempt to position \(sfIB^{+}\) more precisely.

Mapping by \(\lambda\)-transducing phages

The two minute region is well covered by specialised \(\lambda\)-transducing phages (Fletcher et al., 1978; Lutkenhaus et al., 1980) and some of these were tested for the presence of \(sfIB^{+}\) in lysogens of a host strain carrying \(sfIB^{+}\). This \(sfIB^{+}\) allele was previously reported to be recessive in partial diploids (Huisman et al., 1980). Phages of interest were used to lysoginise strain GC2490 (\(tsl, sfIB^{114}\)) by selection for \(leu^{+}\), and the lysogens tested for the restoration of temperature-sensitive filamentation. Three of these phages, \(\lambda sepB2\), \(\lambda sep3\) and \(\lambda sepA6\) include \(E.\ coli\) DNA and \(leuA\) to \(pbpB\), \(murC\) and \(envA\) respectively (see Figure 1).

All three phages failed to complement \(sfIB^{114}\). However, \(sfIB^{+}\) was recovered by marker rescue from \(\lambda sepA6\)-infected cells after selection for \(leuA^{+}\), (see Materials and methods) indicating that at least part of the \(sfIB^{+}\) gene is present in this phage. Lysogens derived from \(\lambda x62\) (carrying \(murC\) to \(envA\)) also failed to show complementation of \(sfIB^{+}\) and although this phage, by comparison with \(\lambda sepA6\) should contain \(sfIB^{+}\), marker rescue was not observed. This may be simply explained by the very low lysogenisation frequency of \(\lambda sepA6\) and consequently the greater probability of detecting marker rescue.

Since \(\lambda DO2\), a phage carrying \(ftsA^{+}\), \(envA^{+}\) and \(secA^{+}\) (see Figure 1) also failed to complement \(sfIB^{114}\) (R.D'Ari, personal communication), despite the inferred presence of \(sfIB^{+}\), we concluded that \(sfIB^{114}\) must be a dominant muta-

Fig. 2. Restriction map of pLG550. The bold portion of the map indicates the vector pOU71 region. The positions and directions of transcription of the known genes are presented in the expanded section showing the organisation of the \(E.\ coli\) DNA insert. The direction of transcription of \(envA^{+}\) is deduced from our unpublished data (I.B.Holland and V.Darby). Numbered arrows show the positions of the \(sfIB^{114}:Tn1000\) insertions discussed in the text. Abbreviations used are \(B\), BamHI, \(E\), EcoRI and \(H\), HindIII.

Cloning sfIB114

Chromosomal DNA from an \(sfIB^{+}\) strain (MC4100) and an \(sfIB^{114}\) strain (GC2490) was prepared, cut with \(BamHI\) and ligated into the \(BamHI\) site of the vector pOU71. This vector was chosen initially to avoid possible problems with high copy numbers of genes coding surface components. With plasmid pOU71 the copy number can be maintained at a low level by performing experiments at \(30^\circ C\) or by using a \(\lambda^{+}\) lysogen as a host at \(42^\circ C\).

Re-ligated banks of \(BamHI\) fragments in pOU71 were used initially to transform TKF12 \(\lambda^{+}\) (\(ftsA^{+}\)) to temperature resistance, thus selecting for recombinants carrying \(ftsA^{+}\) fragments. These were obtained from both \(sfIB^{+}\) (pLG551) and \(sfIB^{114}\) (pLG550) gene banks and were found to carry inserts of \(\sim 13.5\) kb extending from \(ftsA^{+}\) to \(5\) kb beyond \(secA\) when the genetic and restriction enzyme maps were compared. The restriction maps of the plasmids (Figure 2), agreed with previously published data (Lutkenhaus et al., 1980; Lutkenhaus and Wu, 1980; Oliver and Beckwith, 1982). Both pLG550 and pLG551, by the criteria indicated in the Materials and methods section, showed complete complementation of the \(ftsA^{+}\) mutation and partial complementation of the \(ftsZ^{+}\) mutation (see Discussion).

To test the presence of \(sfIB^{+}\) alleles, a \(tsl, sfIB^{+}\), \(\lambda^{+}\) strain was transformed with both pLG550 and pLG551 and transformants were shifted to \(42^\circ C\) to test for suppression of \(tsl\)-induced filamentation. Plasmid pLG550 conferred almost complete suppression of filamentation whereas in the presence of pLG551 extensive filamentation was still observed (Figure 3).
Mutations at sfiB suppressed by inactivation of division genes

Fig. 3. Inhibition of cell division at 42°C in various tsl strains. Exponentially growing cultures of GC2490/1λ (tsd) cells bearing plasmids (a) pOU71, (b) pLG550 and (c) pLG551 were shifted to 42°C at time 0, cell mass (AΔΔG) and cell number (●) were monitored at intervals.

Table II. MMS-sensitivity of sfiB+ and sfiB114 strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Surviving fraction on MMS plates</th>
</tr>
</thead>
<tbody>
<tr>
<td>PAM162/1 (lon-, sfiB26)</td>
<td>5.8 x 10^-1</td>
</tr>
<tr>
<td>PAM660 (lon-, sfiB+)</td>
<td>3 x 10^-4</td>
</tr>
<tr>
<td>PAM660 (pOU71)</td>
<td>5 x 10^-4</td>
</tr>
<tr>
<td>PAM660 (pLG550)</td>
<td>2.6 x 10^-2</td>
</tr>
<tr>
<td>PAM660 (pLG551)</td>
<td>&lt;1.7 x 10^-4</td>
</tr>
</tbody>
</table>

Strains carrying sfiB+ or sfiB114 recombinant plasmids and a lon chromosomal mutation were plated on NA plates including 250 μL MMS at 30°C.

A lon- strain was also transformed with both plasmids, and survival of the transformants tested by plating out at 30°C on NA plates containing methyl methane sulphonate (MMS). The sfiB114 plasmid rendered the lon- strains significantly more resistant to MMS (Table II). Curing of the lon host strain to remove pLG550 led to restoration of MMS sensitivity (data not shown). These results indicated that the recombinant plasmid pLG550 carried the sfiB14 allele which was at least partially dominant over the chromosomal wild-type gene.

Mapping of sfiB114 by transpositional mutagenesis

In an attempt to map the position of sfiB114 more precisely, we sought to use Tn1000 insertional mutagenesis. For greater convenience in the isolation of these insertions the BamW fragment carrying sfiB114 was isolated from pLG550 in vitro and religated into the vector pLG339 to form pLG552 (see Materials and methods). pLG339 (Stoker et al., 1982) is present in ~8 copies at all temperatures, and carries the more convenient Kan+ marker for selection purposes in conjugational crosses.

Plasmid pLG552 was then transformed into the F+-donor strain, RB308. This strain was mated with GC2490/1 carrying chromosomal markers, ftsA-, sfiB+ and Tn1000 insertions selected by plating out for Kan+ recipients (see Materials and methods). Using this selection protocol we anticipated that GC2490/1 heterozygotes sfiB114/sfiB+ should not filament at 42°C due to the dominant sfiB114 allele, whilst Tn1000 insertions into the sfiB114 loci should restore filamentation in this host. The positions of various insertions were mapped from restriction enzyme digests and screened for other genetic markers present in the cloned DNA (Figure 2).

The results demonstrated that of four Tn1000 insertions which suppressed the effect of sfiB114, three inactivated the ftsA gene in functional tests and were located on the physical map at a position corresponding to that assigned to ftsA by Lutkenhaus and Wu (1980). A further insertion (164) did not inactivate ftsA (Figure 2). Unfortunately, the precise limits of ftsZ on the physical map were not previously established (Lutkenhaus and Wu, 1980). However, this insertion did abolish ftsZ in complementation tests, indicating that this insertion does lie within ftsZ. Other insertions into pLG552 did not suppress sfiB114 and mapped well outside the ftsA, Z region (data not shown).

The sfiB114 allele is not present within ftsA

The data in the previous section demonstrated that insertions of Tn1000 into ftsA and possibly ftsZ suppressed the sfiB114 phenotype. To resolve these possibilities, an attempt was made to clone from a plasmid carrying sfiB114, the chromosomal region coding for ftsA independently of ftsZ. Inspection of Figures 1 and 2 (see also Lutkenhaus and Wu, 1980) indicates that ftsA resides on an EcoRI-BamHI fragment which also carries only the proximal portion of ftsZ. Accordingly, a 2.1-kb EcoRI-BamHI fragment was cloned from pLG550 DNA into the plasmid vector pLG339. Appropriate transformants were then obtained and screening tests and restriction enzyme analyses were carried out to confirm that the expected fragment had been cloned (data not shown).
The results described above suggested that an intact \( \text{ftsA} \) gene was required for expression of the \( \text{sfB}/14 \) allele and we sought to confirm this by an analysis of the polypeptides programmed by various plasmids carrying \( \text{sfB}/14 \) and \( \text{sfB}/14::\text{Tnl}000 \) insertions. We also sought to confirm that insertion 164 specifically affected the synthesis of the \( \text{FtsZ} \) protein. The plasmid \( \text{pLG552} \) and various \( \text{Tnl}000 \) insertion derivatives were transformed into the maxi-cell strain, \( \text{Csh26} \) and polypeptides coded by the plasmids labelled with \( \text{[35S]} \text{methionine} \) and analysed by SDS-PAGE as described in the Materials and methods section.

The results (Figure 4a and b), clearly showed that the three insertions (62, 165, 174) into \( \text{ftsA} \) which also inactivated \( \text{sfB}/14 \) caused a dramatic reduction in the synthesis of a polypeptide of mol. wt. 42 K. A polypeptide of similar mol. wt. was also described by Lutkenhaus and Wu (1980) as a major polypeptide coded by this region and identified as the \( \text{ftsZ} \) gene product by these workers. The other major product seen in Figure 4a and b was identified as the 33-K EnvA protein (Lutkenhaus and Wu, 1980; Pratt et al., 1981). As expected, \( \text{pLG552} \) also coded for a 92-K polypeptide, apparently identical to the SecA polypeptide (Oliver and Beckwith, 1982) since inactivation by \( \text{Tnl}000 \) of this gene leads to truncation of this polypeptide (data not shown).

These results provided clear evidence that an intact \( \text{ftsA} \) was required for maximal expression of \( \text{ftsZ} \) and explained the suppression of \( \text{sfB}/14 \) by insertions into \( \text{ftsA} \). The \( \text{ftsA} \) gene product is normally expressed at very low levels (Lutkenhaus and Donachie, 1979) and a protein of similar mol. wt. to that reported previously was also consistently identified in this study (Figure 4) as a minor 47-K polypeptide coded by \( \text{pLG552} \). More significantly, \( \text{Tnl}000 \) insertions (62, 165, 174) which inactivated the \( \text{ftsA} \) function also resulted in the disappearance of this protein as well as loss or reduced expression of \( \text{ftsZ} \). Interestingly, we repeatedly observed that insertions 174 and 62 appeared to give rise to two new polypeptides (arrowed in Figure 4b), presumably truncated forms of FtsA. In contrast to the other insertions, 164 did not inactivate the \( \text{ftsA} \) function and failed to affect the synthesis of the 47-K polypeptide confirming that this is the \( \text{ftsA} \) product. Insertion 164 on the other hand completely blocked the synthesis of the 42-K, FtsZ protein and inactivated the \( \text{ftsZ} \) function and this confirmed our conclusion that this insertion lies within the \( \text{ftsZ} \) gene.

### Discussion

Our previous studies (Burton and Holland, 1983) have indicated that the SOS inhibitor of division must be able to act to block septum formation very late in the cell cycle. Similarly, Donachie and co-workers (1979) and Walker et al. (1975) have shown that both \( \text{ftsA} \) and \( \text{ftsZ} \) (see also Lutkenhaus et al., 1980 for definition of the \( \text{ftsA} \) and \( \text{ftsZ} \) loci) are required for division in the last few minutes of the cell cycle. The current findings that a dominant mutation at the \( \text{sfB} \) locus was suppressed in partial diploids by inactivation of either \( \text{ftsA} \) or \( \text{ftsZ} \) was therefore quite consistent with the hypothesis that either of the products of these genes is the target for the SOS (\( \text{sfIA} \)) inhibitor.

The \( \text{ftsA} \) gene product was previously demonstrated to be an \( \sim 50 \)-K polypeptide coded by a 2.1-kb \( \text{BamHI} \)-\( \text{EcoRI} \) restriction enzyme fragment (Lutkenhaus and Donachie, 1979; Lutkenhaus and Wu, 1980) and a similar polypeptide was identified in this study although in our gel system \( \text{FtsA} \) consistently ran at a position corresponding to 47 K. The \( \text{BamHI} \)-\( \text{EcoRI} \) fragment did not, however, carry the \( \text{sfB}/14 \) allele and failed completely to complement \( \text{ftsZ} \). Lutkenhaus and Wu (1980) also presented evidence that the region clockwise to \( \text{ftsA} \) on the \( \text{E. coli} \) map coded for the \( \text{ftsZ} \) locus. We
have shown in this study that this region codes for a 42-K polypeptide. Analysis of proteins synthesised by plasmids carrying sfbB14: Tn1000 insertions showed that insertions 62 and 174 appeared to produce truncated forms of the FtsA (47-K) protein. From the size of the apparently truncated form produced by insertion 174 and its map position, we conclude that this insertion must be located very close to the 3' terminus of ftsA. Interestingly, this insertion and insertions 165 and 62 all resulted in substantially reduced levels of the 42-K, FtsZ polypeptide in maxi-cells. This effect could be sufficient to explain the suppression of sfbB14 if it is located in ftsZ. Lutkenhaus and Wu (1980) have also observed that a region to the left of ftsZ was important for maximal expression of this gene consistent with our findings.

Concerning the mechanism of reduced synthesis of ftsZ by insertions into ftsA, it was striking that the more distal the insertion the greater the reduction in FtsZ synthesis (see Figure 4b). This result was confirmed in several experiments and the effect was observed independently of the orientation of Tn1000 (data not shown). Evidence has been presented previously (Lutkenhaus and Wu, 1980) that ftsZ is transcribed in the same clockwise direction as ftsA but from an independent promoter. Moreover, the 3.5-kb HindIII fragment (see Figure 3), which covers only the distal region of ftsA and the whole of ftsZ, has been isolated and was found to programme the synthesis of ftsZ in vitro (our unpublished data) with high efficiency. On the other hand, the data of Lutkenhaus and Wu (1980) appeared to show that the ftsZ promoter alone was insufficient in vivo to facilitate normal expression of ftsZ. Consequently we cannot rule out the possibility that in vivo expression of ftsZ is augmented by transcription from the ftsA promoter. In consequence the suppression of ftsZ and sfbB14 observed in this study by insertions into ftsA could simply be due to transcriptional polarity. However, it is difficult to conceive of any mechanism involving transcription emanating from the ftsA promoter, continuing through the large Tn1000 insertion, which would be so sensitive to the position of the insertion that we observe. Alternatively, we suggest that the increasing distally insertions of Tn1000 into ftsA separate the ftsZ promoter from an auxiliary control region located in the distal region of ftsA, necessary for high level expression of ftsZ. Since these effects on ftsZ expression were observed in host cells carrying a wild-type ftsZ + allele on the chromosome, any positive regulating effects of the FtsA protein itself on the ftsZ gene can be ruled out.

In marked contrast to insertions 62, 165 and 174, insertion 164, whilst inactivating sfbB14, had no effect on ftsA function and the 47-K protein was still synthesised. Moreover, we were able to show that the ftsZ function was also inactivated and the synthesis of a 42-K polypeptide was blocked by this insertion. This indicated that insertion 164 was within ftsZ. This together with the demonstration that the 2.1-kb BamHI-EcoRI fragment carries ftsA but not sfbB14 leads us to conclude that sfbB14 lies within ftsZ. Moreover, whilst this manuscript was in preparation, Lutkenhaus (1983) reported that another sfb (sulB25) mutation was, in fact, an ftsZ allele.

Finally, some comment is required on the previously reported recessivity of sfbB14 and other sfb alleles. In the case of sfbB14, the presence of a recessive allele in partial diploids appeared to be demonstrated unequivocally by subsequent P1 transduction analysis (Huisman et al., 1980).

<table>
<thead>
<tr>
<th>Table III. Bacterial strains</th>
</tr>
</thead>
<tbody>
<tr>
<td>Strains</td>
</tr>
<tr>
<td>--------</td>
</tr>
<tr>
<td>MC4100</td>
</tr>
<tr>
<td>PAM660</td>
</tr>
<tr>
<td>PAM162</td>
</tr>
<tr>
<td>PAM162/1</td>
</tr>
<tr>
<td>RB308</td>
</tr>
<tr>
<td>D22</td>
</tr>
<tr>
<td>TKF12</td>
</tr>
<tr>
<td>SP63</td>
</tr>
<tr>
<td>GC2490</td>
</tr>
<tr>
<td>GC2490/1</td>
</tr>
<tr>
<td>CHS2ΔF6</td>
</tr>
<tr>
<td>JFL100</td>
</tr>
</tbody>
</table>

Nevertheless, both in this study and that recently reported by Lutkenhaus (1983), sfb (sulB) alleles were clearly shown to be dominant. Dominant alleles of sfb have also been reported by Gottesman et al. (1981).

Materials and methods

Strains

Bacterial strains used in this study are shown in Table III.

Tests for unselected markers

Methyl methane sulphenonate (MMS) was used to distinguish sensitive strains (lon) from wild-type or lon strains also carrying sfb. MMS (250 μl/l) was incorporated into oxoid nutrient agar (NA) and strains patch-tested. Strains mutant at envA were distinguished by sensitivity to rifamycin, 2–10 μg/ml according to host strain, incorporated into NA plates. Mutant azi strains were resistant to 150 μg/ml sodium azide. Expression of temperature-sensitive, lethal mutations, was accomplished by shifting exponentially growing cultures to 42°C for 2 h and examining by phase contrast microscopy for filaments, or by plating on NA plates for viable colonies at 42°C compared to 30°C. In this case most temperature-sensitive mutants gave virtually a 100% cell survival at 42°C when partial diploids were constructed for complementation by the wild-type allele. In the case of the ftsZ mutant, survival at 42°C was < 10^−1, a maximum of 0.5% of partial diploids formed colonies at 42°C, indicating that the mutant allele was partially dominant under these conditions.

Cell number

Bacterial cells were counted using a Coulter Counter model ZBI with a 30 μm orifice.

Phase transduction

PI transducing lysates were prepared as described by Buxton and Holland (1973) and transductions carried out as described by Willits et al. (1989). Where necessary, recipients were grown in nutrient broth for 2 h to allow expression of specific markers before placing onto selective media. A transduction was carried out as described in Fletcher et al. (1978).

DNA preparation

E. coli chromosomal DNA was prepared by a modification of Chou et al. (1977). Plasmid DNA was prepared as described by Stougaard and Molin.
C.A. Jones and I.B. Holland

(1981), and an adaptation of the Birnboim and Doly method was used to prepare small quantities of DNA (Maniatis et al., 1982). Restrictions and ligations were carried out using manufacturers recommended buffers and where appropriate, BRL core restriction buffer for double enzyme digests. DNA fragments were analysed using 0.8% agarose gels as described by Broome-Smith (1980).

*Tn1000* mutagenesis

R1316 *F* + carrying pLG552 and the recipient strain GC4290 were grown in nutrient broth to an *A* of 0.2 at 30°C mixed in the ratio of 1:10 and mated for 6 – 10 h at 30°C. The culture was plated out on selective plates containing kanamycin (25 µg/ml) and streptomycin (100 µg/ml). The trans-conjugants, containing pLG552 (Kar*+) mobilised for transfer by insertion of Tn1000, were screened for the inactivation of *gfiB14* present in pLG552. This was achieved by patching out individual colonies at 42°C, *tsl* – *sfl* / *gfiB14* heterozygotes grew at 42°C whereas, *tsl* – *sfl* / *gfiB14* Tn1000 heterozygotes failed to grow.

Tn1000 insertions were mapped from analysis of the *BamH*I, EcoRI and *HindIII* restriction digests of the mutagenised plasmids. This allowed the insertions to be positioned within a previously ordered restriction fragment and the relative position determined from the size of the new fragments which appeared. The orientation of the Tn1000 insertion was deduced from the pattern of *BamH*I fragments since Tn1000 contains an asymmetric *BamH*I site.

**Synthesis of plasmid coded polypeptides in maxi-cells**

Plasmids of interest were transferred into the maxi-cell strain, CSH26AF6 and Marker rescue was confirmed by the inactivation of the plasmid encoded tetracycline resistance gene. Plasmid pLG553 was constructed similarly from a fragment from pLG550 cloned into pLG339 were isolated by transforming pLG553 cut with *EcoR*I and *BamH*I with the ligation mixture and screening for kanamycin-resistant, temperature-resistant recipients. Cloning into the *BamH*I site of pLG339 was confirmed by the inactivation of the plasmid encoded tetracycline resistance gene. Plasmid pLG553 was constructed similarly from a *BamH*I–EcoRI double restriction digest of plLG539 prior to ligation into pLG339 cut with EcoRI and *BamH*I.

**Marker rescue**

Lysates of the defective *λ* transducing phages Xsep82, Xsep3 and Xsep46 were used to transduce CC4290 to *lev* + transductants were tested for the presence of the *λ* transducing phages by their ability subsequently to yield a *lev* transducing lysate after u.v. irradiation. Those failing to produce such a lysate were presumed to have acquired *lev* + by marker rescue. In the case of Xsep46, where lysogens are recovered only rarely, many of these recombinants were shown to have acquired *gfiB14* +, presumably by marker rescue. The presence of the *gfiB14* allele was detected by screening individual clones of the *tsl* - *sfl* / *gfiB14* host for filamentation at 42°C by phase contrast microscopy.

**Acknowledgements**

C Jones gratefully acknowledges the receipt of a Science and Engineering Research Council Studentship. We wish to thank Dr Dick D’Ari for unpublished data and many useful discussions. We also thank Dr Julie Pratt and Dr Neil Stoker for their helpful discussions. We are also extremely grateful to Dr Sorin Miplin for providing plasmid pOU71.

**References**


Willetts, N.S., Clark, A.J. and Low, B. (1969) *J. Bacteriol.*, 97, 244-249.


**Received 16 January 1984**
ABSTRACT

Title SFIB AND THE CONTROL OF CELL DIVISION IN E.COLI

Christopher Andrew Jones

Mutations at two loci, sfiA and sfiB, suppress the filamentation seen on irradiation of lon mutants or expression of tsl(lexA(Ts)) or tif(recA441) mutations in Escherichia coli. The sfiA and sfiB genes have been assumed to be involved in the inhibition of cell division associated with the SOS response. The product of the sfiA gene has been shown to be a division inhibitor and the sfiB gene has been postulated to be the target for the action of such an inhibitor.

The sfiB gene was mapped to the ftsQ-secA region of the E.coli chromosome using Pl transduction and specialised λ-transducing phages. The sfiB+ and sfiB114 genes were cloned onto recombinant plasmids and sfiB114 found to be at least partially dominant in tsl and lon strains.

Using Tn1000 mutagenesis, it was found that the sfiB gene is allelic to the essential septation gene ftsZ and that an element within the preceeding and contiguous ftsA gene is required for full ftsZ(sfiB) expression.

Maxi-cells containing plasmids encoding sfiA and either sfiB+ or sfiB114 were used to demonstrate an interaction between SfiA and FtsZ. The presence of an ftsZ(sfiB+) carrying plasmid in maxi-cells increased the half-life of the unstable SfiA protein to 10-14 min (compared to approximately 3 min in the presence of a sfiB114 encoding plasmid or where a sfiA plasmid was present in maxi-cells alone).

Finally, maxi-cells containing sfiA and sfiB carrying plasmids were separated into subcellular fractions and it was found that both SfiA and SfiB(FtsZ) proteins bind to the E.coli inner membrane.