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

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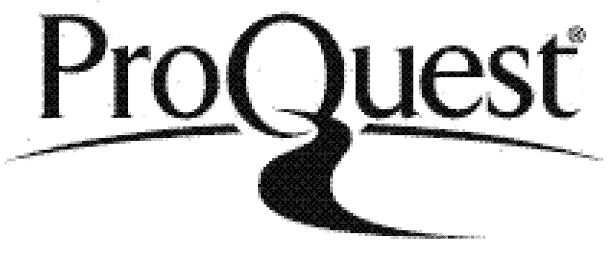

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DEDICATION

To all my family and friends.....especially Sclub.

ACKNOWLEDGMENTS

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ABBREVIATIONS

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

Genetic nomenclature

recA	gene
RecA	protein
RecA	phenotype

CONTENTS

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.,

1982; Vicente, 1984; Donachie, 1984).

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 τ, 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 continously with growth rate such that it approaches a constant value (approximately 37 min) at high growth rates. Where $\tau > C+D$ 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

cells (Cooper, 1979).

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 occured 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 intiation 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

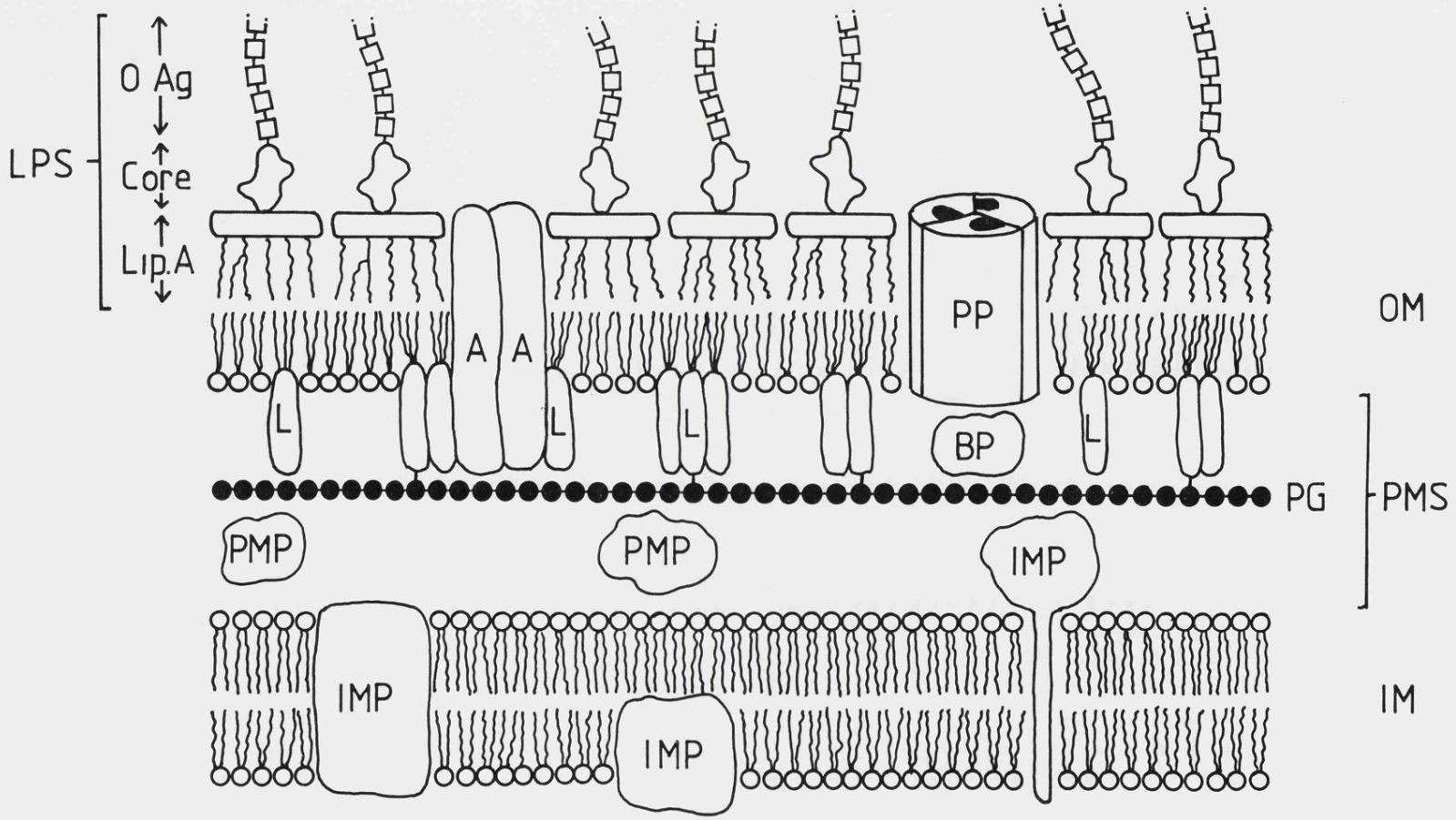

LPS
O Ag
Core
Lip.A
A
A
L
L
L
L
PP
BP
OM
PG
PMS
PMP
PMP
IMP
IMP
IMP
IM

Figure 1.2

The structure of part of the peptidoglycan of E.coli.

MurNac - N-acetyl muramic acid

GlcNac - N-acetyl glucosamine

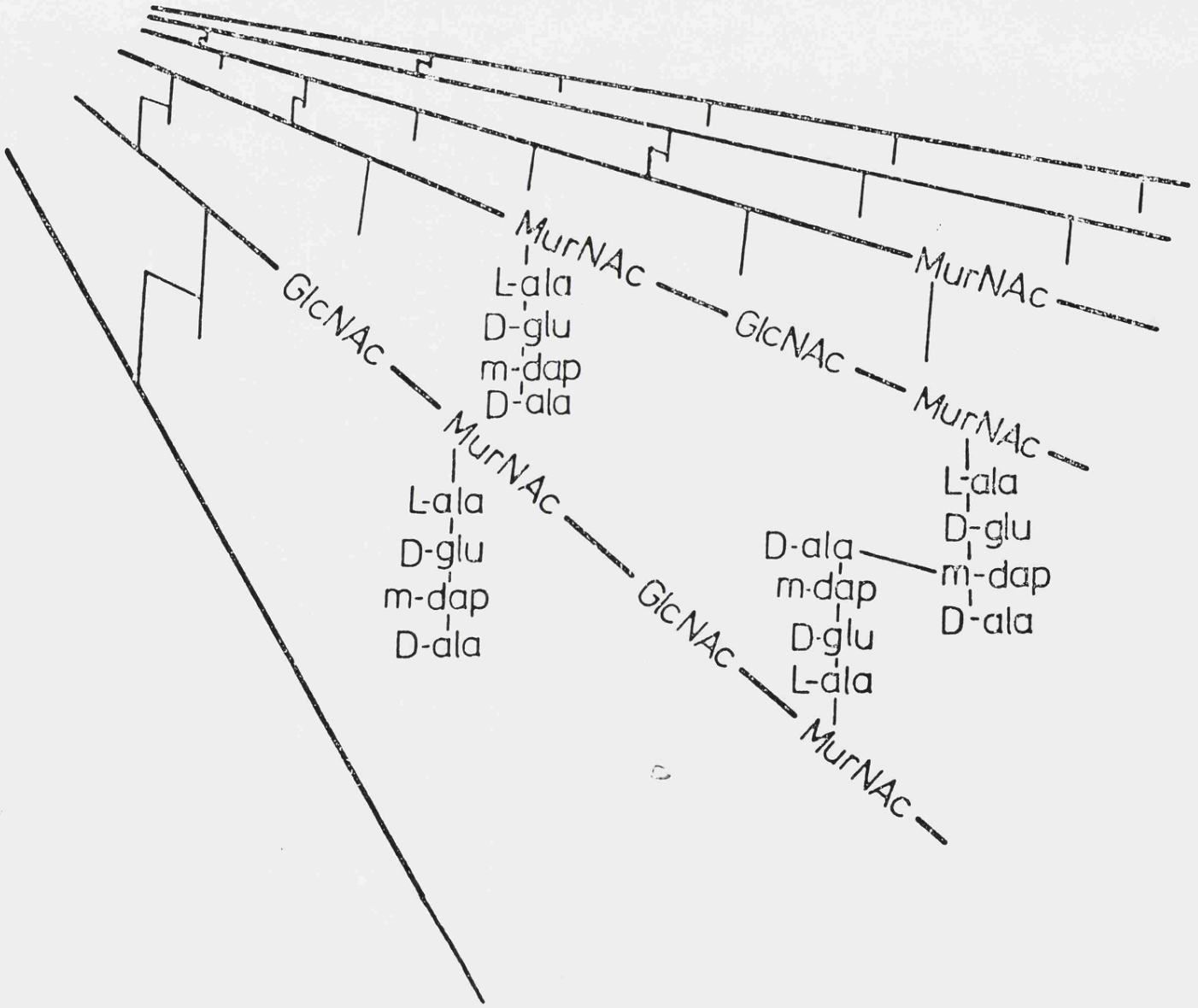
L-ala - L-alanine

D-glu - D-glutamate

m-dap - meso-diaminopimelate

D-ala - D- alanine

Taken from Stoker (1983).


MurNAc
L-ala
D-glu
m-dap
D-ala
GlcNAc
MurNAc
GlcNAc
MurNAc
L-ala
D-glu
m-dap
D-ala
GlcNAc
MurNAc
L-ala
D-glu
m-dap
D-ala
D-ala
m-dap
D-glu
L-ala
MurNAc

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 1.1 Properties of E.coli PBP's

PBP	MW	Molecules per cell	Enzymic activity	Function
1A	92,000	170	Transpeptidase/ Transglycosylase	Elongation
1B	90,000	60	Transpeptidase/ Transglycosylase	
2	66,000	20	Transpeptidase	Cell shape
3	60,000	50	Transpeptidase/ Transglycosylase	Cell division
4	49,000	110	DD-carboxypeptidase/ DD-endopeptidase	?
5	42,000	1800	DD-carboxypeptidase/ model transpeptidase	?
6	40,000	570	DD-carboxypeptidase/ model transpeptidase	?

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

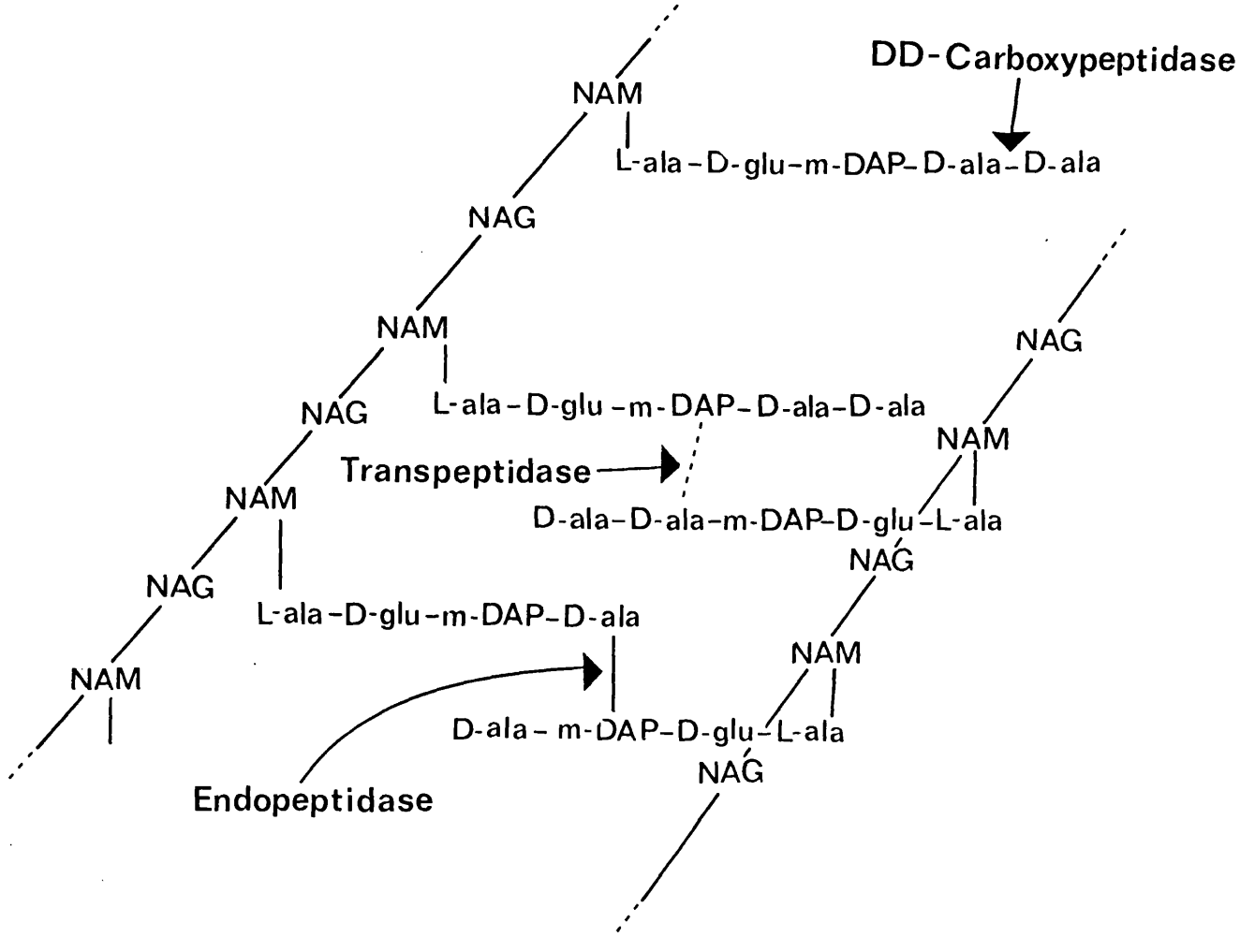
D-glu - D-glutamate

m-dap - meso-diaminopimelate

D-ala - D- alanine

Taken from Stoker (1983).

PEPTIDOGLYCAN.



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 (Buchannan, 1981).

A different set of antibiotics (e.g. cephalexin 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^{o}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 cephalexin, 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)

Class I : Genes required for net peptidoglycan (PG) biosynthesis.

Class II : Genes for outer membrane proteins involved in morphogenesis.

Class III : Genes required for cell elongation.

Class IV : Genes linking DNA replication with cell division.

Class V : Genes required for nucleoid segregation and septum localisation.

Class VI : Genes required for septum initiation.

Class VII : Genes required for septum formation.

Class VIII : Genes required for septum separation (cell division).

Class IX : Genes required for the inactivation of sites of septation.

Table 1.2(cont)

Genes involved in cell morphology in E.coli

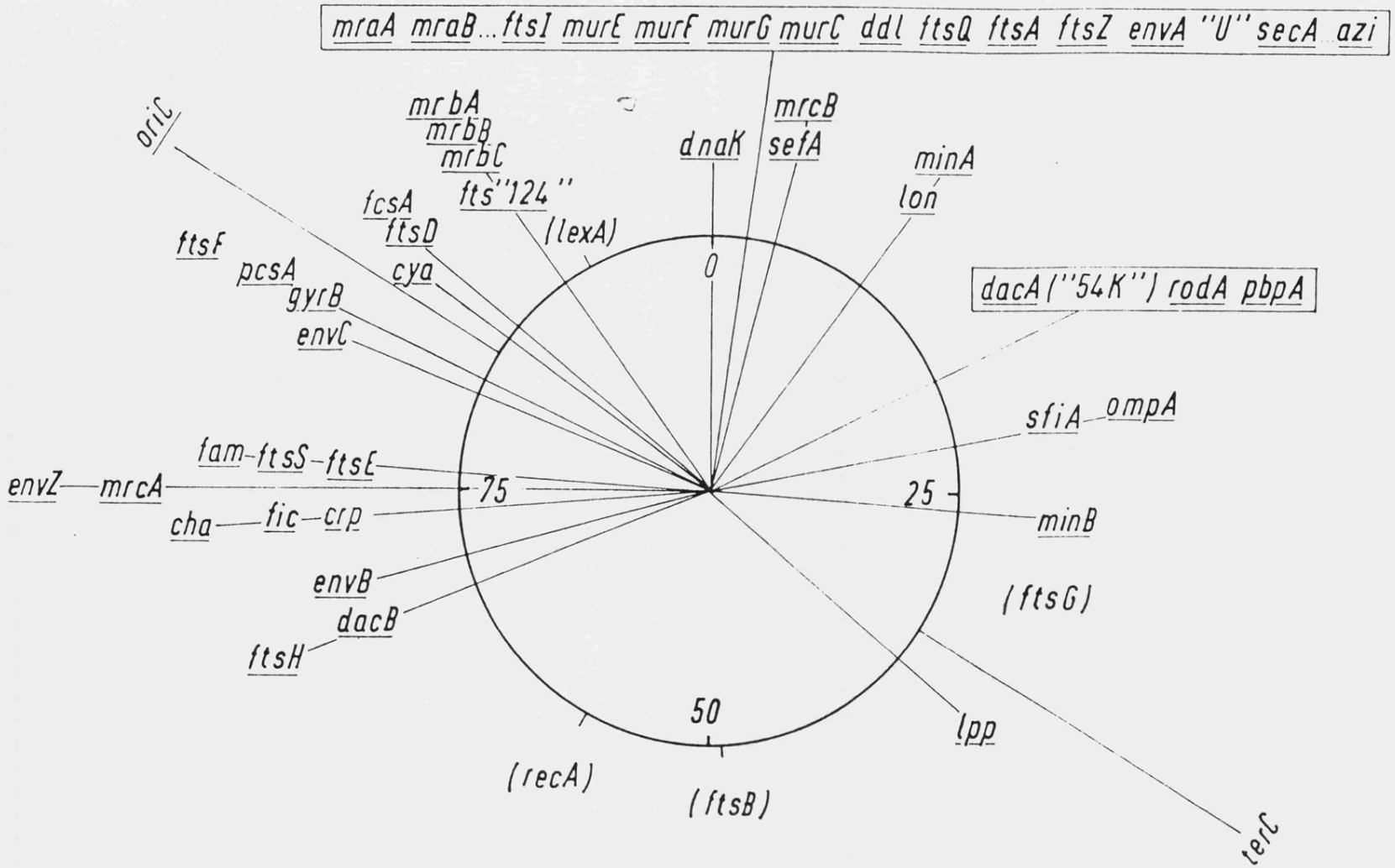
Location (min)	Gene	Function	class
0	dnaK	septation	VI/VII
2	mraA	D-alanine carboxypeptidase	I
2	mraB	D-alanine requirement	I
2	ftsI	PBP3, septation	VI+VII
2	murE	meso-diaminopimelate adding enzyme	I
2	murF	D-alanyl:alanine adding enzyme	I
2	murG	PG biosynthesis	I
2	murC	L-alanine adding enzyme	I
2	ddl	D-alanine:D-alanine ligase	I
2	ftsQ	septation	VI+VII
2	ftsA	septation	VII
2	ftsZ	septation	VI+VII
2	envA	division	VIII
2	secA	secretion	(VI/VII)
2	azi	septation (azi^r)	VI/VII
4	mrcB	PBP1B	I
4	sefA	septation	VII
10	lon	inactivation of SfiA	IV
10	minA	inactivation of septal sites	IX
15	dacA	PBP5, D-alanine carboxypeptidase	III?
15	rodA	cell shape	III
15	pbpA	PBP2	III
22	ompA	outer membrane protein (II^*)	II
22	sfiA	inhibitor of cell division	IV
26	minB	inactivation of septal sites	IX
29-31	ftsG	septation	VI/VII?

36	lpp	murein lipoprotein	II
49	ftsB	septation	VI/VII?
69	dacB	PBP4	I?/III?
69	ftsH	septation	VI
71	envB	cell shape	III
74	crp	CAP protein	III
74	fic	$cAMP^S$ septation step	VI/VII
74	cha	division, cell shape	VI/VII/II
75	mrcA	PBP1A	I
75	envZ	regulation of outer membrane protein assembly	II?
76	ftsE	septation	VI+VII
76	ftsS	septation	VI+VII
76	fam	control of murein lipoprotein	VI/VII
81	envC	septation	VI/VII
82	pcsA	chromosome segregation	V?
82	gyrB	DNA-gyrase B, chromosome segregation	V
82-83	ftsF	septation	(VI/VII)?
85	cya	adenyl cyclase, cell shape	III
86	ftsD	septation	(VI/VII)
86	fcsA	septation	(VI/VII)
90	mrbA	PG biosynthesis	I
90	mrbB	PG biosynthesis	I
90	mrbC	PG biosynthesis	I
90	ftsH	septation	VI+VII

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
oriC
mrbA
mrbB
mrbC
fts "124"
(lexA)
fcsA
ftsQ
cya
ftsF
pcsA
gyrB
envC
dnaK
mrcB
sefA
minA
lon
dacA ("54K") rodA pbpA
sfiA
ompA
minB
(ftsG)
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25
50
75
fam-ftsS-ftsE
envZ
mrcA
cha
fic
crp
envB
dacB
ftsH
(recA)
(ftsB)
lpp
terC

other ftsA alleles and renamed it ftsZ. In addition there are instances of two different genes being given the same name, for example, ftsH (Santos and D'Almeida, 1975; Holland and Darby, 1976) and ftsS (Dwek 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 et al. (1979) and studies have mainly concentrated on elucidating the following :

a) The possible osmoreversibility of the conditional cell division phenotype, in anticipation that envelope proteins might respond to this treatment.

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.

c) The effect of filamentation on peptidoglycan metabolism (Pages et al., 1975).

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.

1.10 Spherical mutants of E.coli

In Section 1.6 temperature sensitive alleles of the pbpA gene were described which produced spherical cells at high temperature. In addition, mutations in two other genes, 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$^-$) or in cAMP receptor protein (crp$^-$) 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).

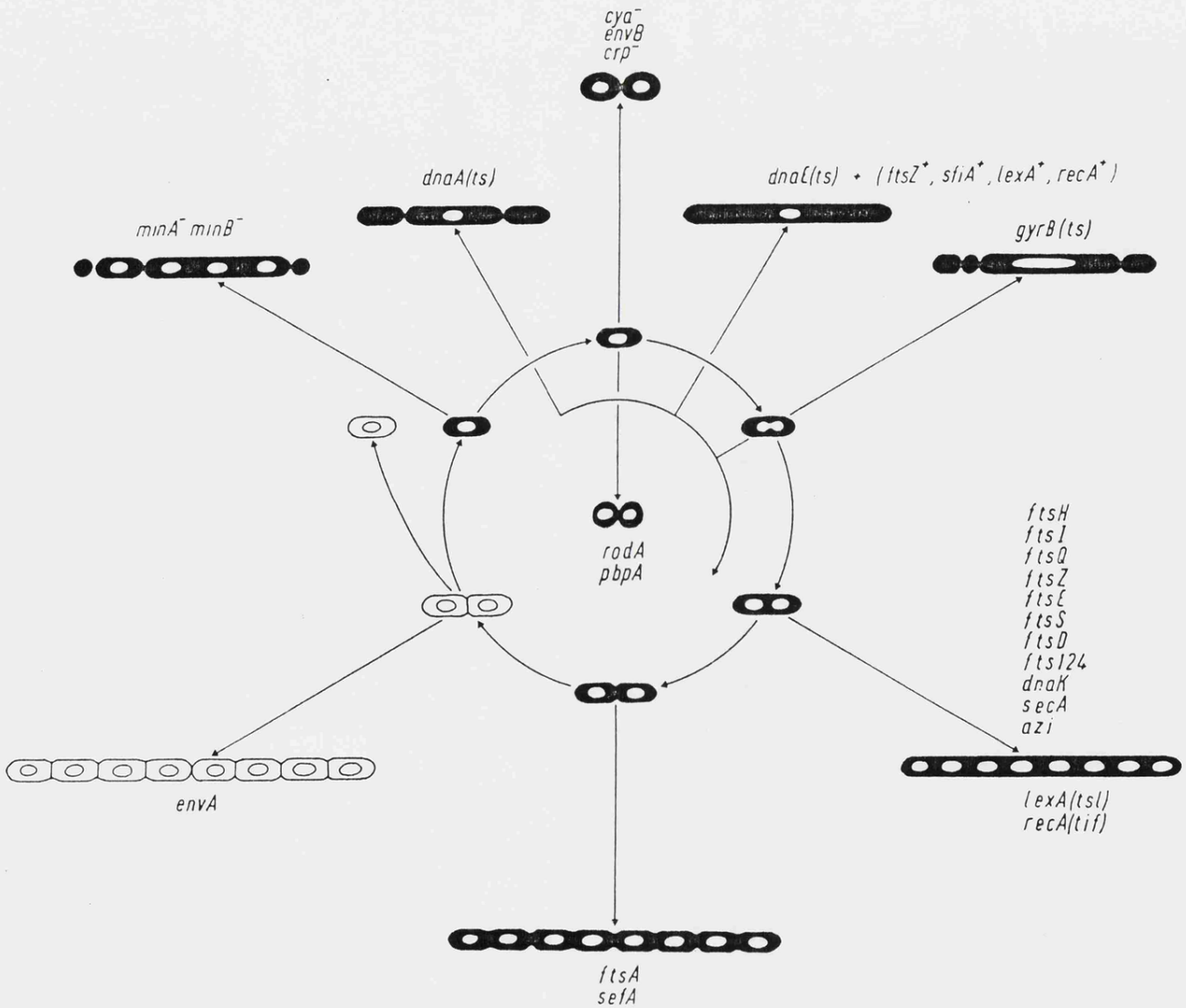
The cAMP-CRP complex is in some way involved in cell morphology. Donachie (1984) suggests that the cAMP-CRP complex exerts negative regulation on septal peptidoglycan synthesis. This is supported by the isolation of mutants, fic, that filament when treated with exogenous cAMP (Utsumi, 1982).

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

Figure 1.5

A schematic diagram (from Donachie, 1984) showing a temporal map, within the cell cycle, of the phenotypes produced by mutations affecting cell morphology.


cya⁻
envB
crp⁻
dnaA(ts)
dnaC(ts) + (ftsZ⁺, sfiA⁺, lexA⁺, recA⁺)
minA⁻ minB⁻
gyrB(ts)
rodA
pbpA
ftsH
ftsI
ftsQ
ftsZ
ftsE
ftsS
ftsD
fts124
dnaK
secA
azi
envA
lexA(tsl)
recA(tif)
ftsA
sefA

activity.

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 λ 16-2, 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 atudies (Burdett and Murray, 1974) and temperaure 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 existance 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).

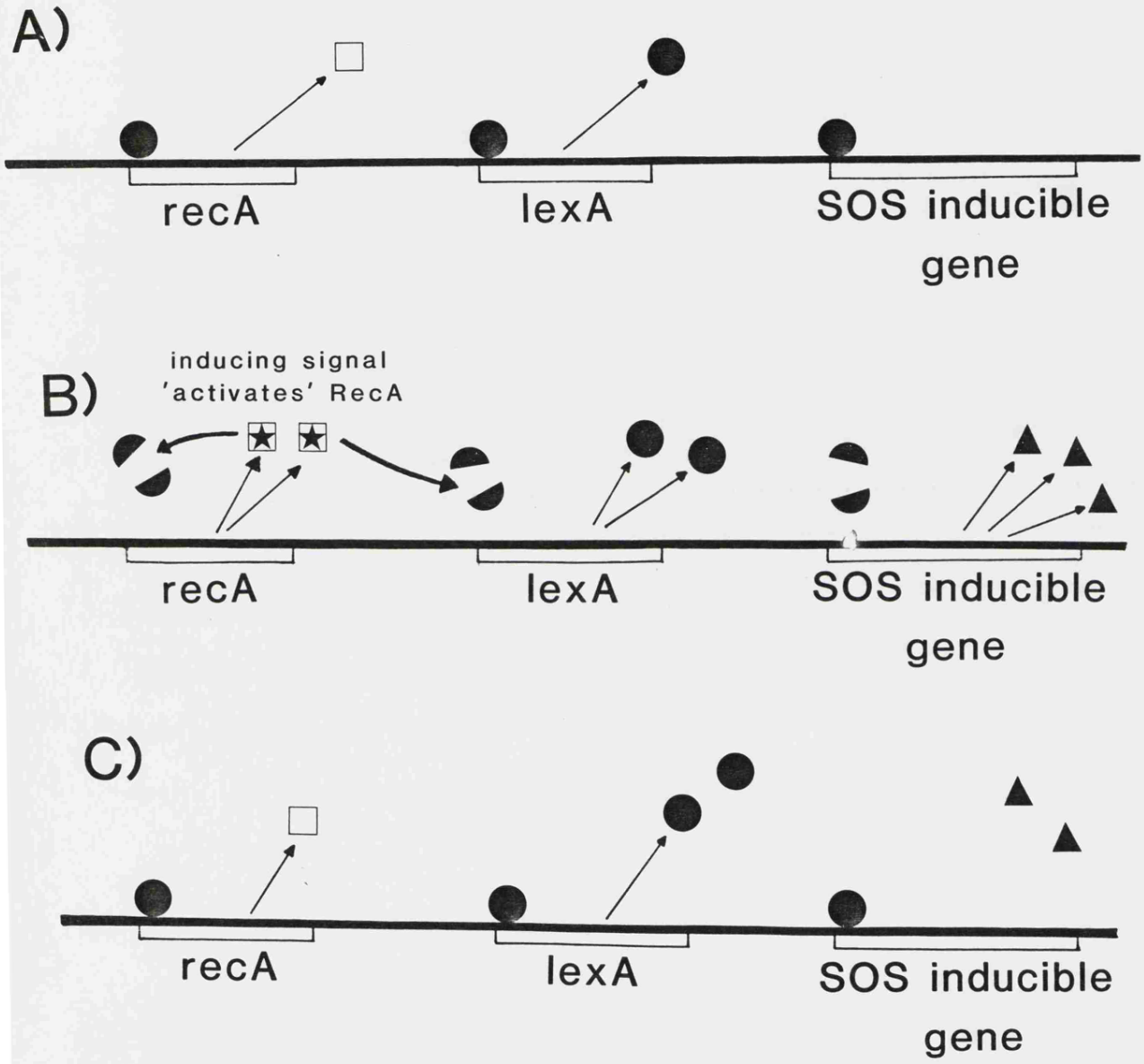
Figure 1.6

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 respones.

C) Following the disappearance of the SOS inducible signal, RecA becomes "de-activated" and LexA levels rapidly rise to repress SOS inducible genes.


A)
recA
lexA
SOS inducible
gene
inducing signal
'activates' RecA
B)
recA
lexA
SOS inducible
gene
C)
recA
lexA
SOS inducible
gene

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$^-$, (lexA(Ind$^-$)) 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$^-$); 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$^-$ 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 1.5

Mutations in the recA and lexA genes

Allele	Phenotype	Biochemical change
lexA alleles		
lexA(Ind$^-$) (*lexA*$^-$)	Dominant, no SOS induction	Protease resistant repressor
lexA(Ts) (*tsl*)	Recessive, filamentation and *recA* induction at 42^{o}C	Thermosensitive repressor
lexA(Def) (*spr*)	Recessive, constitutive expression of target genes	Defective repressor
recA alleles		
recA	Cannot induce SOS functions	Defective protein
recA441 (*tif*)	Spontaneous SOS expression at 42^{o}C	Protease thermoactivated
recA430	Cannot induce SOS system but recombination proficient	Specific defect in protease
recAo	Constitutive *recA* expression	Operator defect

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 λ cI 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 *lexA*(Def) cells or cells carrying multiple copies of *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 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 *lexA*.

In the identification of SOS inducible genes the most fruitful approach has involved the use of Mud1(Ap, *lac*) bacteriophage. The Mud1(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 Mud1(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 Mud1(Ap, lac) β-galactosidase gene fused to the promoters of SOS inducible genes. These SOS regulated genes were named din (damage inducible).

The Mud1(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 Mud1(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 1.6

lexA target genes

Gene	location (min)	Function	References
recA	58	Recombination	McPartland et al. (1980), Little et al. (1981)
lexA	91	SOS repressor	Brent and Ptashne (1980, 1981), Little et al. (1981)
uvrA	92	Excision repair	Kenyon and Walker (1981)
uvrB	17	Excision repair	Fogliano and Schendel (1981)
umuDC	25	Mutagenesis	Bagg et al. (1981)
himA	38	Site-specific recombination	Miller et al. (1981)
dinA	2	Unknown	Kenyon and Walker (1980)
dinB	8	Unknown	Kenyon and Walker (1980)
dinD	80-85	Unknown	Kenyon and Walker (1980)
dinF	91	Unknown	Kenyon and Walker (1980)
sfiA	22	Division inhibition	Huisman and D'Ari (1981)
recN	57	RecF dependent recombination	Lloyd et al. (1983), Lovett and Clark (1983)
ssb	92	Single strand binding protein	Brandsma et al. (1983)
ruv	41	UV resistance	Shurvington and Lloyd (1982)
uvrD	85	Excission repair (DNA helicase II)	Seigel (1983)
rpsU-dnaG-rpoD	67	See below	Lupski et al. (1983)

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 δ 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.

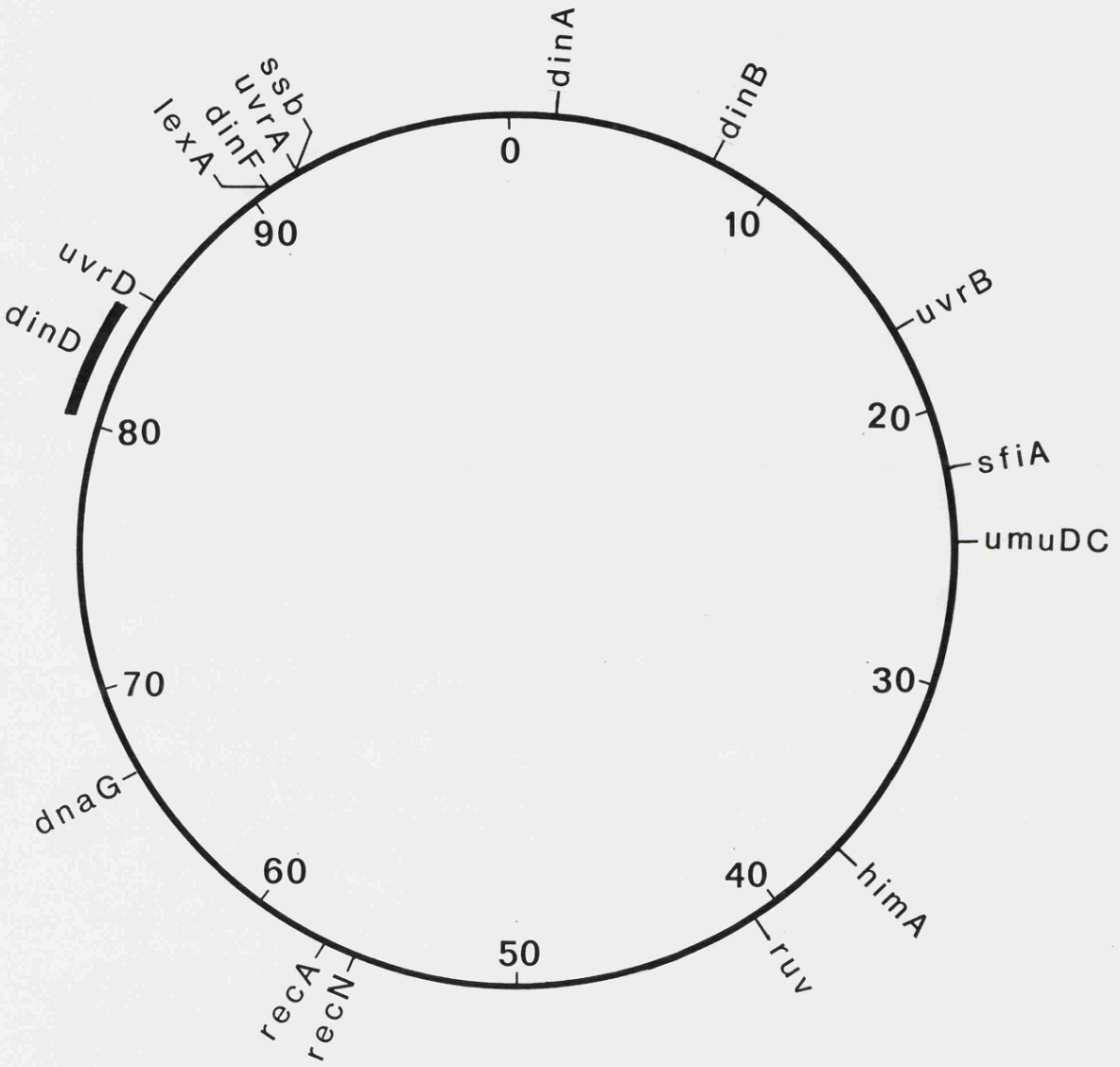
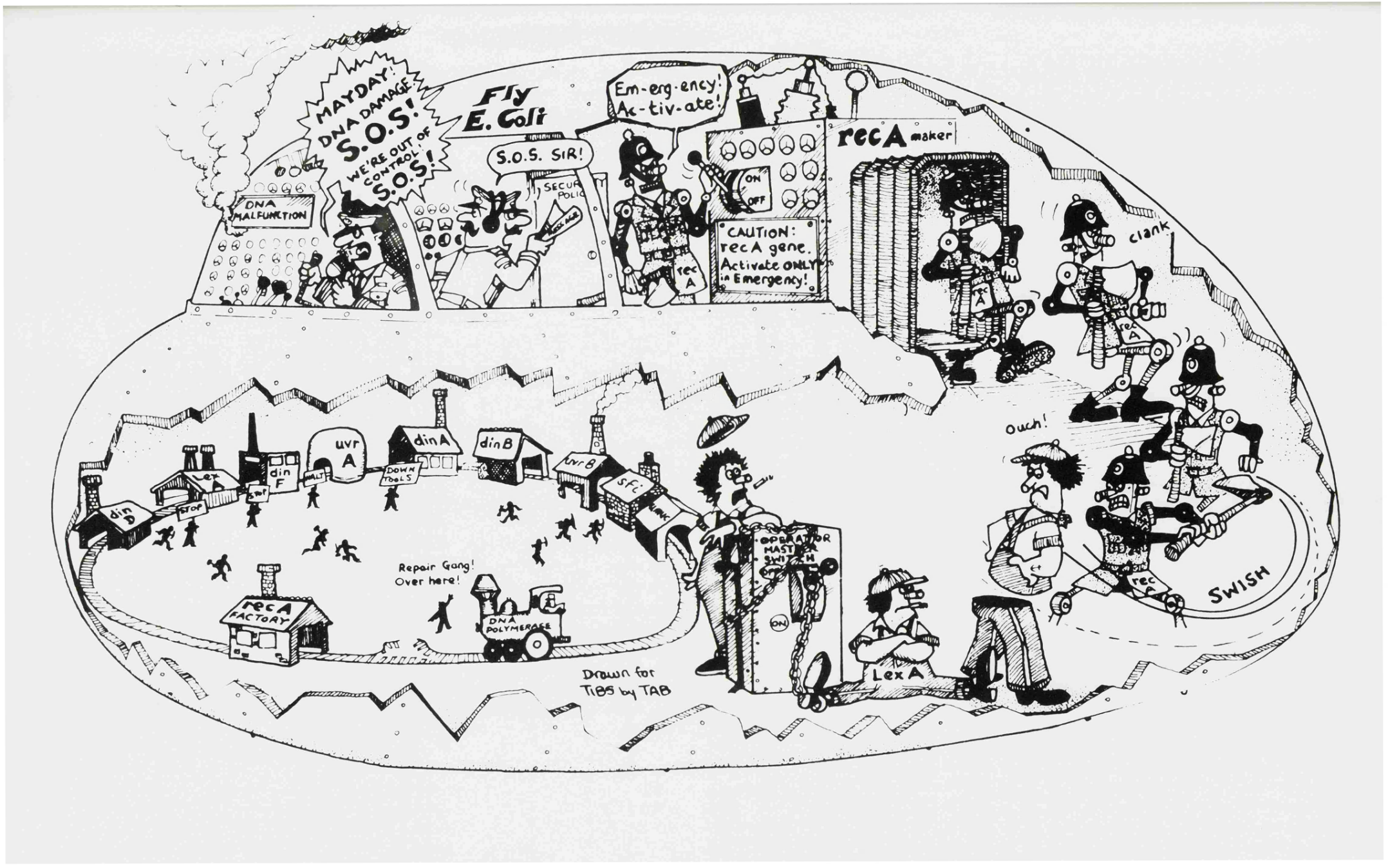

dinA
dinB
uvrB
sfiA
umuDC
himA
ruv
recN
recA
dnaG
uvrD
dinD
lexA
dinF
uvrA
ssb
0
10
20
30
40
50
60
70
80
90

Figure 1.8

A diagram of the processes occuring during the induction of the SOS system as seen by Kenyon (1983).


MAYDAY!
DNA DAMAGE
S.O.S!
WE'RE OUT OF CONTROL
S.O.S!
DNA MALFUNCTION
Fly E. Coli
S.O.S. SIR!
Em-erg-ency!
Ac-tiv-ate!
ON
OFF
CAUTION: recA gene. Activate ONLY in Emergency!
recA maker
clank
ouch!
SWISH
uvr A
dinA
dinB
uvr B
DOWN TOOLS
HALT
Repair Gang! Over here!
recA FACTORY
DNA POLYMERASE
OPERATOR MASTER SWITCH
LexA
Drawn for TIBS by TAB

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 dinE 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 colE1 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

Gene	Sequence	Reference
recA	TACTGTATGAGCATACAGTA	Brent and Ptashne (1981)
uvrA	TACTGTATATTCATTCAGGT	Sancar et al. (1982a)
uvrB	AACTGTTTTTTTATCCAGTA	Sancar et al. (1982b)
sfiA	TACTGTACATCCATACAGTA	Cole (1983)
uvrD	ATCTGTATATATACCCAGCT	Walker (1984)
lexA-1	TGCTGTATATACTCACACGA	Horii et al. (1981b)
lexA-2	AACTGTATATACACCCAGGG	Horii et al. (1981b)
cle1-1	TGCTGTATATACTCACACGA	Ebina et al. (1981)
cle1-2	CAGTGGTTATATGTACAGTA	Ebina et al. (1981)
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$^-$ and lexA(Ind$^-$) 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 (recA441) : The tif (recA441) 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$^-$ mutation blocked tif induced filamentation but not prophage λ 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 2.4×10^{-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 polysacharide (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) demonstated that the level of λcI 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 42^0C 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 tsl 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 lon 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 plausable model of cell division inhibition following induction of the SOS system.

Huisman and D'Ari (1981) isolated a Mud1(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 Mud1(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 lon 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

Figure 1.9

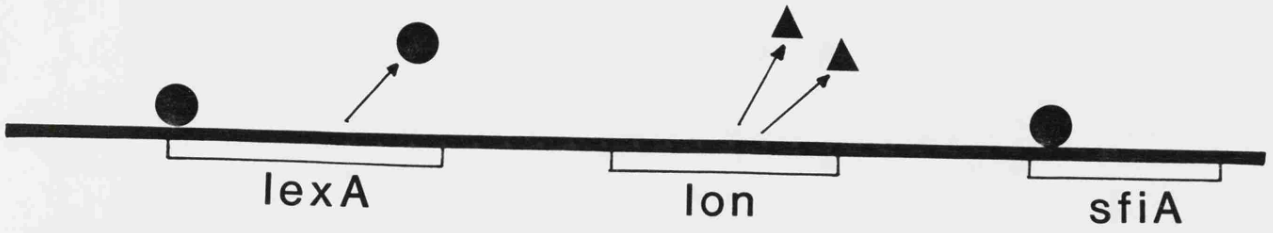
A schematic representation of the control of *sfiA* during the SOS response.

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.

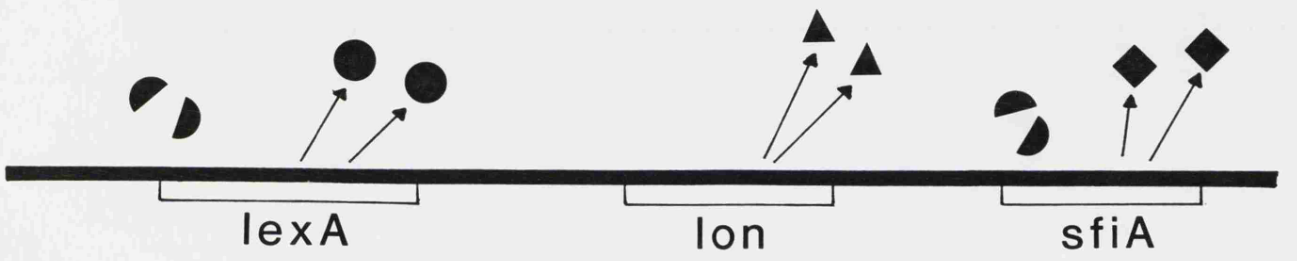
Recovery - LexA repression on the *sfiA* gene resumes and either the product of the *lon* gene or a protease under *lon* contol degrades SfiA and cell division can continue.

Rest

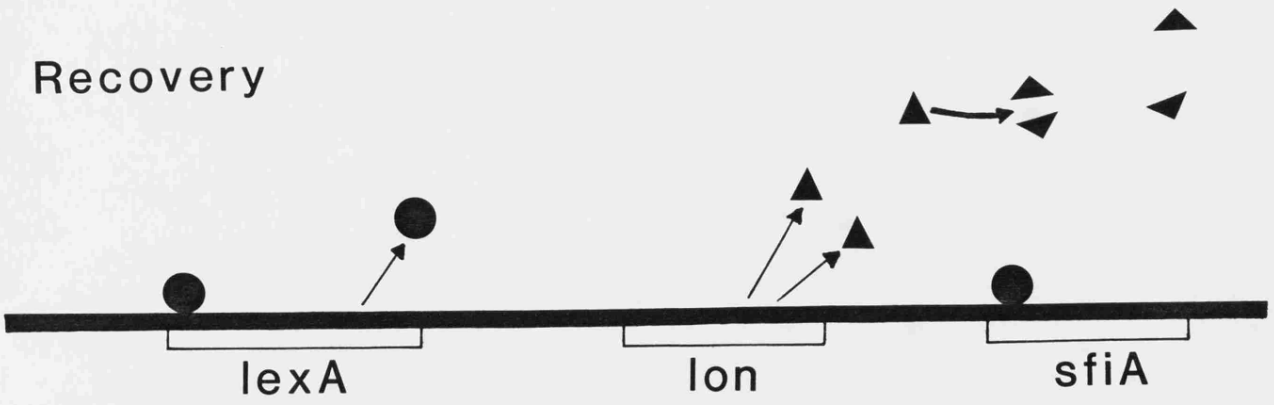


SOS induction

SfiA blocks cell division



Recovery



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

1.24 The aims of this project

The aim of this work was to investigate the location of the 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

P1 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 Na_2HPO_4, 20 mM KH_2PO_4, 8mM NaCl, 20 mM NH_4Cl, 1 mM $CaCl_2$, 10 mM $MgSO_4$.

Added as required : glucose (0.4%), maltose (0.2%),L-amino acids (50μg/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 2.1

Bacterial strains

Name	Genotype	Source
MC4100	araD, Δ lacIPOZYA, rpsL,thi	P.Bassford
PAM162m+	thi, thr, leuB6, proA, his, lon, lacY, galK, ara, xyl, mtl, rpsL, tsx, supE, sfiB26	
PAM162/1	thi, thr, leuB6, proA, his, lon, lacY, galK, ara, xyl, mtl, rpsL, tsx, supE, sfiB26 azi	
PAMsfi$^+$	thi, thr, leuB6, proA, his, lon, lacY, galK, ara, xyl, mtl, rpsL, tsx, supE, sfiB$^+$	
RB308recA	F$^+$, deoC, thyA, lacY, recA srl::Tn10	R.Buxton
D22	trp, pro, his, rpsL, ampA, envA	A.Salem
TKF12	thr, leu, thi, pyrF, thyA, ilv, ftsA12, his, arg, lac tonA	W.Donachie
SP63	trp(am), tyr(am), ilv, pbpB63	B.Spratt
GC2490	thr, pro, his, lac, gal leuA, tsl, rpsL, sfiB114	R.D'Ari
GC24901	thr, pro, his, lac, gal leuA, tsl, rpsL, sfiB$^+$	
GC4293	tsl, recA-99, rpsL	R.D'Ari
CSH26ΔF6	ara, Δ(lac, pro), Δ(recA,srl)F6, rpsL, thi	D.Oliver
JFL100	ilv, his, thyA, deo, ara(am)$_{ts}$ lac(am), galU(am), tyrT,supF^{ts}, ftsZ84	J.Lutkenhaus
MM52	araD$_{ts}$ Δ lacIPOZYA, rpsL,thi secA^{ts}	J.Beckwith
5K	hsdR, thr, leu, thi tonA	W.J.Brammar

P1 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.

P1 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_2PO_4, 50 mM Na_2HPO_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_2HPO_4 and 10 mM NaH_2PO_4 titrated together to pH 7.2.

2.4. Growth of bacterial cultures

Bacterial liquid cultures were shaken in a New Brunswick Gyrotatory 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 P1vir

(a) Preparation of P1 lysates

Donor strains were grown in nutrient broth to A_{450} 0.5 and $CaCl_2$ added to 2.5 mM. 0.3 ml of cells were mixed with 0.1 ml of a P1 lysate at a range of dilutions and left for 15 min at 37°C to allow the phage to adsorb. 3.5 ml of molten P1 top layer agar was then added to each tube and the contents poured onto P1 bottom layer agar plates and incubated overnight. The plates on which P1 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°C for 15 min after which the tubes were centrifuged and the supernatant taken off and stored at 4°C over chloroform. Yields were usually 2-10 x 10^{10} phage/ml.

(b) P1 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 P1 lysate (titre approximately $5x10^9$ phage/ml) and 0.5 ml of prewarmed Luria broth made 30 mM with respect to $MgCl_2$ and 15 mM with respect to $CaCl_2$ and the tubes incubated for 15 min at 37°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 necesary 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 relevent 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 $MgCl_2$ 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 λ cI^{857} and derivatives

Lysogens of λcI^{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 occured. Chloroform was added and the culture centrifuged to separate any bacterial debris from the phage lysate.

(c) UV induction of lysogens

λ 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 λ 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. γδ (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 20μg/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 H_2O.

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

Tris-sucrose	:50 mM Tris-HCl pH8.0 25% sucrose (w/v)
TE buffer	:10 mM Tris-HCl pH7.5 1 mM EDTA
TES buffer	:50 mM Tris-HCl pH8.0 5 mM EDTA 50 mM NaCl
TEG buffer	:25 mM Tris-HCl pH8 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 H_2O
Triton lysis mix	:2% Triton X-100 (v/v) 50 mM Tris-HCl pH8.0
PEG-NaCl	:25% polyethylene glycol 6000 1.25 M NaCl
CsCl-EtBr mix	:80g 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 $CHCl_3$ 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 sulution 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 elecrophoresis, 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 pH8.0. 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 disgarded and the pellet resuspended in 0.5 ml TE buffer containing 2 mM $ZnCl_2$. This was dialysed against 200 ml TE containing 0.1 mM $ZnCl_2$ 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 (pH8.0), 5 µl of 10 x CIP buffer (Table 2.3) and 48 µl of H_2O 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)

Enzyme	Tris/Cl	pH	$MgCl_2$	NaCl	KCl	DTT	SH	EDTA
EcoRI	500	7.4	50	250	-	2.5	-	-
BamHl	30	7.4	30	250	-	-	30	-
HindIII	50	7.4	50	250	-	2.5	-	-
core buffer	50	8.0	10	50	-	-	-	-

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_2O	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 H_2O, 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 acheived. 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 pH8.0, 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

volume of TE buffer.

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 $MgCl_2$ and 1/10 vol of acetic acid before precipitation of the DNA by ethanol. The isolated DNA fragment was washed in 70% ethanol before resuspention 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 H_2O. Ligation buffer (10 x ,Table 2.3) and T4 DNA ligase were added and the mixture incubated at 14^oC 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 A_{450} 0.4-0.5 and chilled in ice. 10 ml of cells were pelleted and

resuspended in 5 ml of cold 0.1 M $MgCl_2$ (all centrifugation was done at 4°C). Cells were again pelleted and resuspended in 5 ml of cold 0.1 M $CaCl_2$ and left on ice. After 20 min the cells were harvested and resuspended in 0.6 ml cold 0.1 M $CaCl_2$. Cells stored at 4°C remained competant for up to 7 days but highest frequencies of transformation were acheived with cells prepared 24 h before use.

(b) Transformation

200 µl of competant 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 A_{450} 0.5 and 5 ml of

cells UV-irradiated with 3.75 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 (25μg/ml) or cycloserine (250μg/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°C 2 μl of 35S-methionine was added to label the plasmid encoded proteins and incubation continued, normally for 30-60 min after which 40μl of cold methionine (44mg/ml) was added. The bacteria were then pelleted in an eppendorf centrifuge tube and resuspended in 40μl SDS-PAGE buffer A and 40 μl SDS-PAGE sample buffer and heated to 100°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 (*recB*^{ts}). Transcription-translation incubations were carried out at 37°C and contained:

7.5μl	low molecular weight mix
2.0μl	^{35}S -methionine (25 Ci)
3.5μl	0.1M magnesium acetate
5.0μl	S30 extract (strain indicated in text)
2 - 5μg	DNA in 10mM Tris/Cl 1mM EDTA pH 7
	10mM Tris/acetate pH 7 to 30μl

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 ^{14}C - 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 flourographed, 1nCi per slot.

Table 2.4: SDS PAGE solutions, buffers and gel recipes

Gel composition

		15%	11%
Separating gel:	Buffer A	13.5	13.5ml
	Acrylamide	9.2	6.8ml
	H_2O	3.6	6.0ml
	Ammonium persulphate (APS) freshly made, 10mg/ml	1.0	1.0ml
	N,N,N',N'-tetramethyl ethylenediamine (TEMED)	75	75µl

		7%
Stacking gel:	Buffer B	10.0ml
	Acrylamide	3.3ml
	H_2O	6.7ml
	APS	0.5ml
	TEMED	40µl

TEMED was always added immediately before the gel was poured

Buffer A	Tris/Cl pH 8.8	0.75M
	SDS	0.2% w/v
Buffer B	Tris/Cl pH 6.8	0.25M
	SDS	0.2% w/v
Acrylamide	Acrylamide	44% w/v
	N,N'-methylene-bis-acrylamide (bis)	0.8% w/v
Electrophoresis buffer	Trisma base	0.125 M
	Glycine	0.192M
	SDS	0.1% w/v
Sample buffer	Tris/Cl pH 6.8	0.125M
	Glycerol	20% v/v
	β-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^{o}C during exposure.

d)Densitometric scanning of autoradiographs

Autoradiographs were densitometrically scanned using an LKB 2202 Ultroscan 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 phoshate 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 phoshate 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 150µl 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. ^{35}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:

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

CHAPTER 3

Investigations into the location of sfiB

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 persistant 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$^+$ 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$^+$ to SfiB114. On the basis of these observations a cloned sfiB$^+$ 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$^+$ 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*$^-$ cells compared to *sfiB*$^+$ cells even at 37^{o}C. In addition, PAM162m$^+$ (*lon*, *sulB26*) showed highly abnormal cell morphology when grown in nutrient broth or on nutrient agar plates at 44^{o}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$^+$ was grown for 30 generations in nutrient broth at 44^{o}C and plated on nutrient agar plates, after overnight incubation at 44^{o}C large and small colonies were obtained. Large colonies were phenotypically *SfiB*$^+$ (i.e. normal cell morphology when grown at 44^{o}C and MMS^s) and were therefore assumed to be *sfiB*$^+$ revertants. One such revertant, PAM162sfi$^+$ (*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.

Figure 3.1

leuB leuA pbp3 murE F C ddl ftsQ ftsA ftsZ envA secA azi

Table 3.1

gene	phenotype of mutant
leuA	leucine prototrophy
pbp3	filaments when grown at high temperature
murE, murF, murC, ddl	lyse when grown at high temperature
ftsQ, ftsA, ftsZ	filament when grown at high temperature
envA	resistance to antibiotics and forms chains during growth
secA	lethal defect in secretory mechanism when grown at high temperature
azi	resistance to azide and cells filament when grown at high temperature.

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 under represented 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 λ -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 λ -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

Donor	Recipient	Co-transduction
leuB$^+$, *pbp3*$^-$	*leuB*$^-$, *pbp3*$^+$	88% (198/225)
leuB$^+$, *ftsA*$^+$	*leuB*$^-$, *ftsA*$^+$	59% (159/270)
leuA$^+$, *sfiB*$^+$	*leuA*$^-$, *sfiB114*	41% (111/270)
leuA$^+$, *envA*$^-$	*leuA*$^-$, *envA*$^+$	41% (92/225)
leuB$^+$, *azi*$^+$	*leuB*$^-$, *azi*$^-$	25% (56/225)

always carried out in a tsl chromosomal background. The tsl mutation is expressed at 42°C and leads to induction of all LexA repressed SOS functions (including division inhibition) but without inducing λ-prophage.

a) λDO2 and λ16-2

Specialised transducing phages λ DO2 (Oliver and Beckwith, 1982) and λ16-2 (Luktenhaus and Donachie, 1979) carrying ftsA-secA and murC-envA regions respectivly (Fig. 3.2) were used to lysogenise strain GC2490 (tsl, sfiB114) and lysogens tested by growth at 42°C on nutrient agar plates for complementation of sfiB114 (i.e. restoration of filamentation at 42°C). Neither lysogen class showed such filamentation when examined microscopically, and did not therefore show complementation of sfiB114, although the same lysates of λ DO2 and λ16-2 used to lysogenise GC2490 were able to complement temperature sensitive mutations in secA (MM52) and ftsA (TKF12) respectivly.

b) λsep$^+$82, λ sep$^+$3 and λsep$^+$46

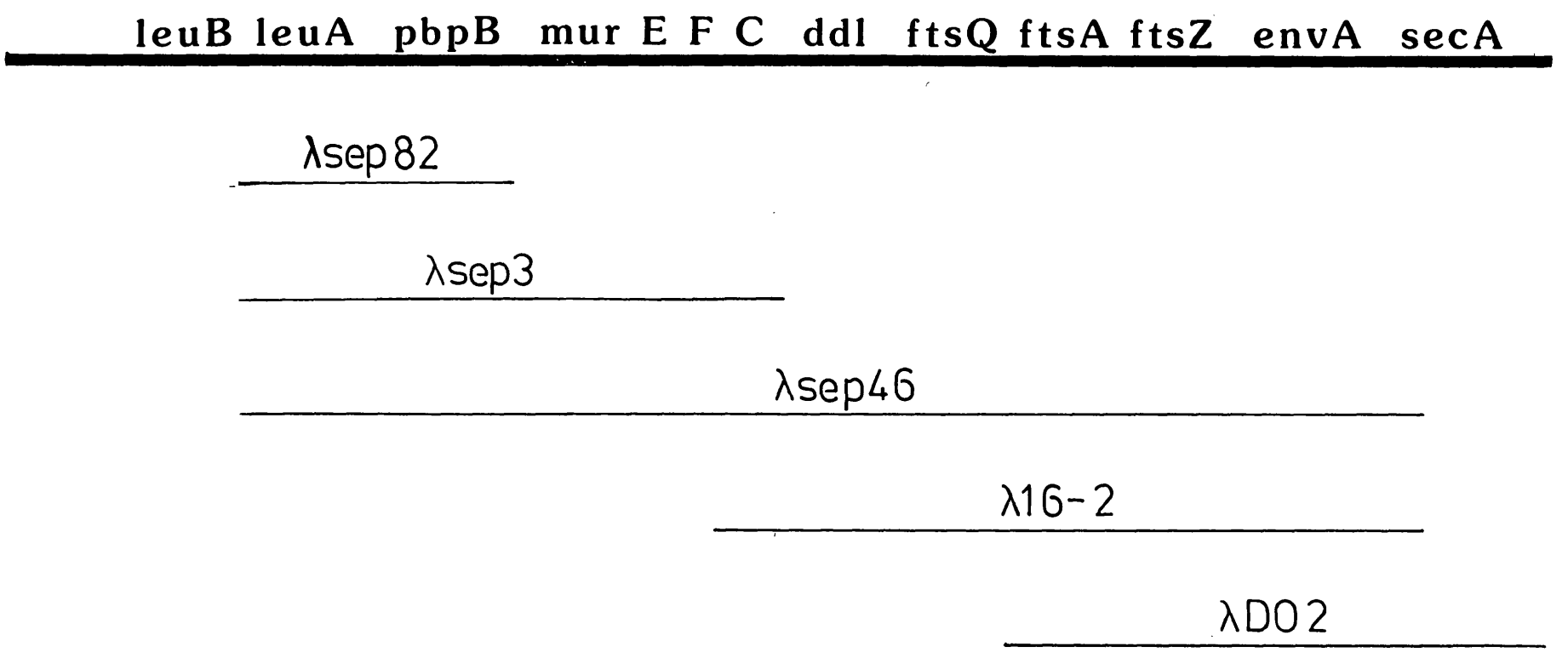
Fletcher et al. (1978) described a set of defective λ-transducing phages carrying carrying E.coli chromosomal segments extending from leuA in a clockwise direction towards secA. These phages were obtained from the induction of a lysogen having λcI^{857} integrated into the leu operon. Such a lysate provided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

Table 3.3

Phage	Complementation ability							
	leuA	pbp3	murE	murF	murC	ddl	ftsA	envA
λleuA13	+	-	-	-	-	-	-	-
λsep$^+$82	+	+	-	-	-	-	-	-
λsep$^+$69	+	+	+	-	-	-	-	-
λsep$^+$27	+	+	+	+	-	-	-	-
λsep$^+$3	+	+	+	+	+	-	-	-
λsep$^+$24	+	+	+	+	+	+	-	-
λmurF$^+$121	+	+	+	+	+	+	+	-
λsep$^+$46	+	+	+	+	+	+	+	+

Figure 3.2

A schematic diagram of the chromosomal DNA carried by transducing phages λsep$^+$82, λsep$^+$3, λsep$^+$46, λ16-2 and λDO2.


leuB leuA pbpB mur E F C ddl ftsQ ftsA ftsZ envA secA
λsep82
λsep3
λsep46
λ16-2
λD02

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.

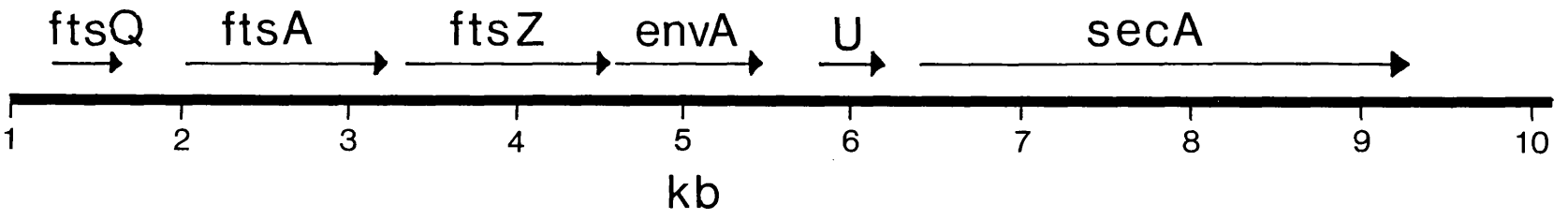
Lysogens carrying phages $\lambda cI^{857}\lambda sep^{+}82$ (leuA-pbp3), $\lambda^{+}\lambda sep^{+}3$ (leuA-murC) and $\lambda^{+}\lambda sep^{+}46$ (leuA-envA) (Fig 3.2) were induced and HFT's used to transduce strain GC2490λ^{+} (leuA, tsl, sfiB114) to leu^{+}. In all, 90 leu^{+} GC2490λ^{+} colonies 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 λ 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 λ-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 ftsQ-secA region of the E.coli chromosome. The gene labelled "U" is the open reading frame between envA and secA reported by Darby (1981) and Donachie (1984).


ftsQ
ftsA
ftsZ
envA
U
secA
1
2
3
4
5
6
7
8
9
10
kb

also may be involved in cell division (Sullivan and Donachie, 1984) although its precise functional role is unknown. Since λ sep$^+$46, but not λsep$^+$3, provided an opportunity for marker rescue of sfiB114 this implied that at least part of the sfiB gene was located between murC and secA (see Fig 3.3). If this is the case then either λ 16-2 or λ DO2, which overlap this region should show complementation. One explanation for the lack of complementation shown by λ16-2 and/or λ DO2 could be that transcription from λ promoters prevented efficient expression of sfiB$^+$ (Ward and Murray, 1979). However since these phages were capable of expressing other transduced markers, such an effect would have been anomolous. 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 experimants 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.

CHAPTER 4

Cloning of sfiB114 and sfiB$^+$ genes from chromosomal DNA

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^{o}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

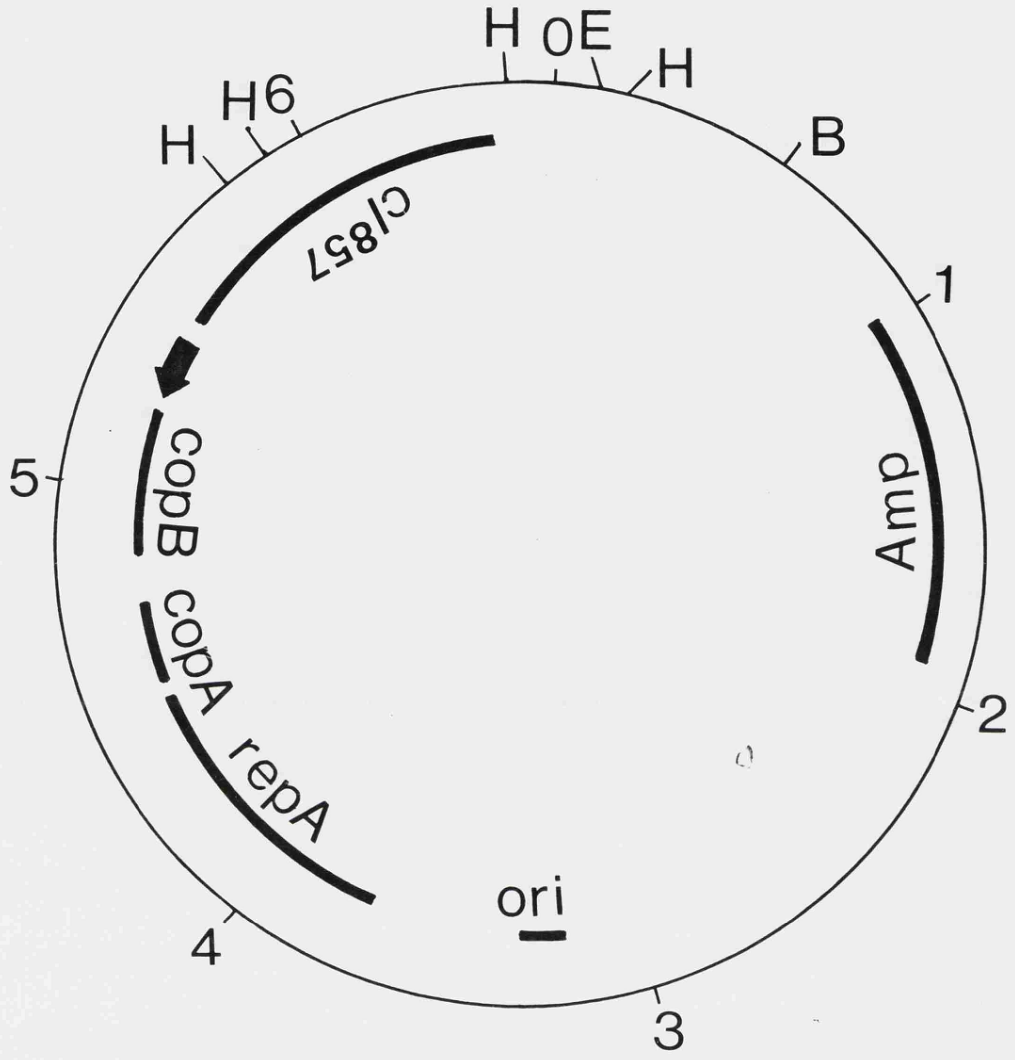
Figure 4.1

A restriction and functional map of the vector pOU71. Th physical distances around the circle are shown in kb. Th large solid arrow represents the direction of transcriptio from λP_R.

B : BamH1

H : HindIII

E : EcoR1


H
0E
H
B
1
Amp
2
3
ori
4
repA
copA
copB
5
cI857
H
H6

has the copy number control region and DNA replication origin of plasmid R1 together with the bacteriophage λcI_{857} gene and p_R promoter, with β-lactamase as a selectable marker. At 30°C the copy number of pOU71 is 1. As the growth temperature increases the repression on p_R by the thermolabile cI_{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°C) reaching up to 1000 copies per cell at 42°C . This effect on plasmid copy number control is recessive to the presence of wild type cI repressor produced by a host λ^+lysogen, such that in a λ^+ host pOU71 maintains a copy number of 1 at all temperatures.

4.3 Cloning strategy

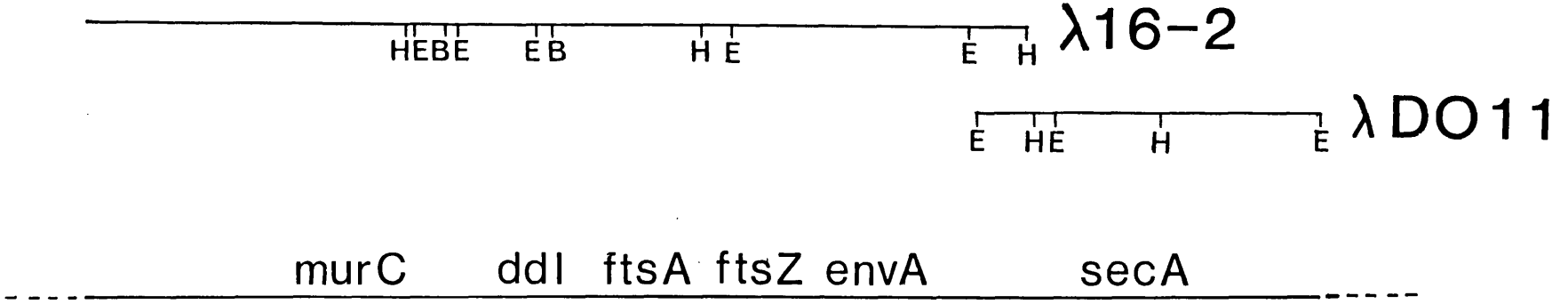
By superimposing the previously published restriction maps of transducing phages λ 16-2 (murC-envA) and λ DO11 (secA), a restriction map of the whole ftsA-secA region implicated as the position of sfiB can be obtained. Inspection of Fig. 4.2 indicates that there is a BamH1 site to the left of the ftsA gene and no second BamH1 site until at least beyond secA. Therefore it was assumed that a BamH1 digest of E.coli chromosomal DNA should yield a DNA fragment (8.5kb+) extending from ftsA to beyond secA.

4.4 Preparation and screening of an 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 λ16-2 and λ DO11, 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 λ16-2 was first reported ftsQ was unknown.


HEBE EB H E E H λ16-2
E HE H E λDO11
murC ddl ftsA ftsZ envA secA

treated with calf intestinal phosphatase to reduce vector recirculisation. 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 competant 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 BamH1 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.

4.5Cloning 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^{o}C studied. To do this pLG550 DNA (and pOU71 DNA as a control) was used to transform competant 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, incobated overnight at 30^{o}C and 42^{o}C and numbers of colonies counted. The results shown in Table 4.1 show the difference in colony forming ability at 42^{o}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 4.1

	pLG550		pOU71	
recipient strain	30°C	42°C	30°C	42°C
GC2490λ⁺(*sfiB114*)	0	0	10^3	10^3
GC24901λ⁺(*sfiB*⁺)	0	0	10^3	52
MC4100	0	0	10^3	10^3
TKF12λ⁺(*ftsA*)	10^3	10^3	10^3	0

three strains. Since DNA prepared in TKF12 λ^+ was apparently only able to transform TKF12 λ^+ an unreported DNA modification mutation in TKF12 was suspected. This was confirmed by inducing strain TKF12 λ^+ for λ using UV-irradiation and showing that the resulting phage lysate formed plaques with a 8×10^3 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 (hsdR$^-$,hsdM$^+$) and then DNA prepared from this strain was used to transform other host strains.

4.7 Tests for sfiB on pLG550 and pLG551

Transformants of GC24901λ^+ (tsl) carrying pLG550 and pLG551 obtained at 30°C were streaked out on nutrient agar plates at 42°C where SfiA synthesis should be constitutive in order to test for the 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 tsl strain carrying pLG550 gave a SfiB$^-$ phenotype whilst the same strain carrying pLG551 gave an SfiB$^+$ phenotype. In order to exclude the possibility that sfiB114 from the incoming plasmid had recombined into the chromosome, the temperature resistant transformant of GC24901 $^+$ carrying pLG550 was cured of the plasmid as decribed below and then retested for a SfiB$^-$ phenotype.

4.8 Curing GC24901λ^+ of pLG550

pOU71 had been reported to be highly unstable in the absence

of selection with plasmid loss occuring 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 <u>sfiB</u>$^-$ phenotype in an <u>tsl</u> 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.

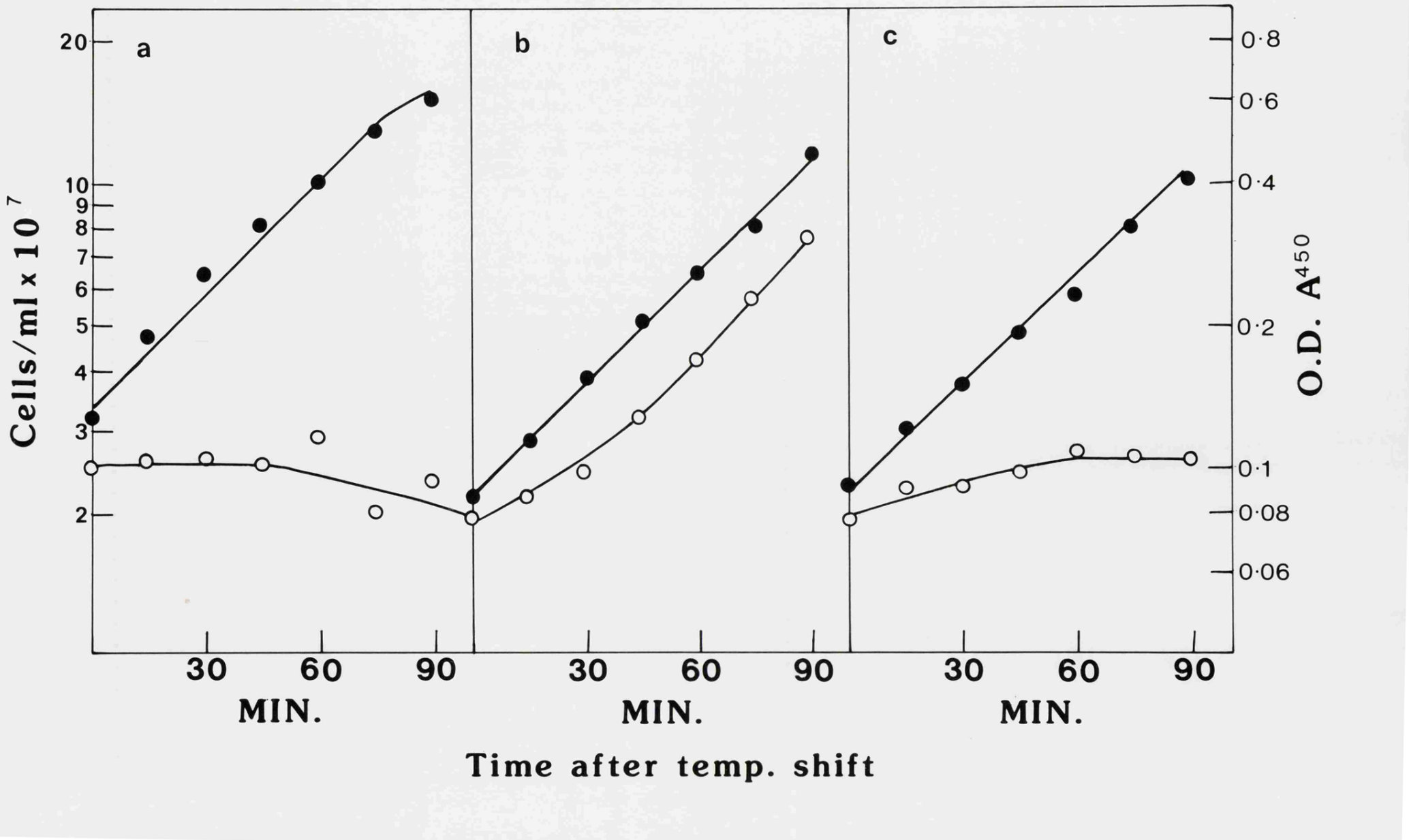
Figure 4.3

Measurement of cell number (open circles) and cell mass (closed circles), as measured by O.D. A_{450}, for cultures of GC24901λ^{+}(tsl) grown in nutrient broth at $42^{o}C$ containing plasmids :

a) pOU71

b) pLG550

c) pLG551


a
b
c
Cells/ml x 10^7
20
10
9
8
7
6
5
4
3
2
O.D. A450
0·8
0·6
0·4
0·2
0·1
0·08
0·06
30
60
90
MIN.
Time after temp. shift

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 GC24901 λ^+ pLG551 also suggested such an effect. As a consequence of these findings it was suspected that the λ cI$^+$ gene on the GC24901 λ^+ chromosome, required to prevent runaway of plasmid copy number, was not completely repressing the transcription from p_R 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 p_R 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 4.2

strain	relative survival 30°C	42°C
GC2490 λ^+ (*sfiB114*)	1	0.7
GC24901 λ^+ (*sfiB*$^+$)	1	1.1×10^{-4}
GC24901 λ^+ pOU71	1	1.6×10^{-4}
Gc24901 λ^+ pLG550	1	1
GC24901 λ^+ pLG551	1	0.14

Table 4.3

strain	relative survival -MMS	relative survival +MMS
PAM162m+ (*lon*,*sfiB26*)	1	5.8×10^{-1}
PAMsfi^{+} (*lon*,*sfiB*$^{+}$)	1	3.0×10^{-5}
PAMsfi^{+} pOU71	1	5.0×10^{-5}
PAMsfi^{+} pLG550	1	2.6×10^{-2}
PAMsfi^{+} pLG551	1	1.7×10^{-6}

Transformants of PAMsfi$^+$ (lon,sfiB$^+$) (see Chapter 3) carrying pOU71, pLG550 or pLG551 were grown in nutrient broth at 30^{o}C to A_{450} 0.5, plated out on nutient agar plates + and - MMS, and grown at 30^{o}C to determine the numbers of survivors. The λ cI_{857} gene on pOU71 was derived from a ind$^-$ bacteriophage λ 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 succesfully cloned from the chromosomal DNA of sfiB114 (GC2490) and sfiB$^+$ (MC4100) strains into the BamH1 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 respectivly.

CHAPTER 5

Characterisation and sub-cloning of plasmids pLG550 and pLG551

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(λ^-) as a host to allow amplification of plasmid copy number by growing exponentially growing plasmid bearing cultures at 42^{o}C. Cultures of 5K pLG550 and 5K pLG551 were grown in nutrient broth at 30^{o}C to A_{450} 0.2 and shifted to 42^{o}C for 1 h after which they were grown at 37^{o}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 DO11 (*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

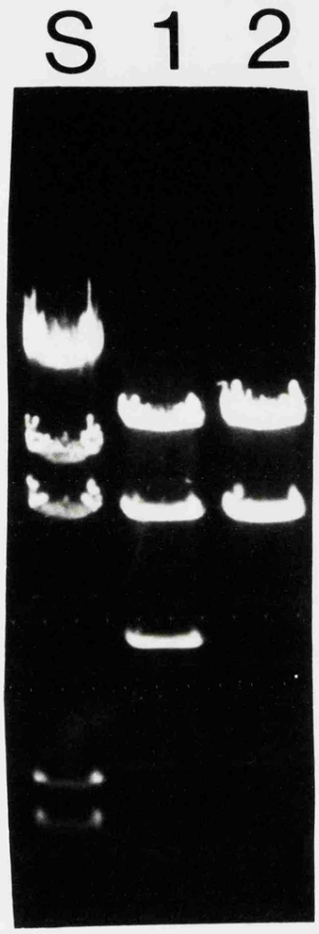
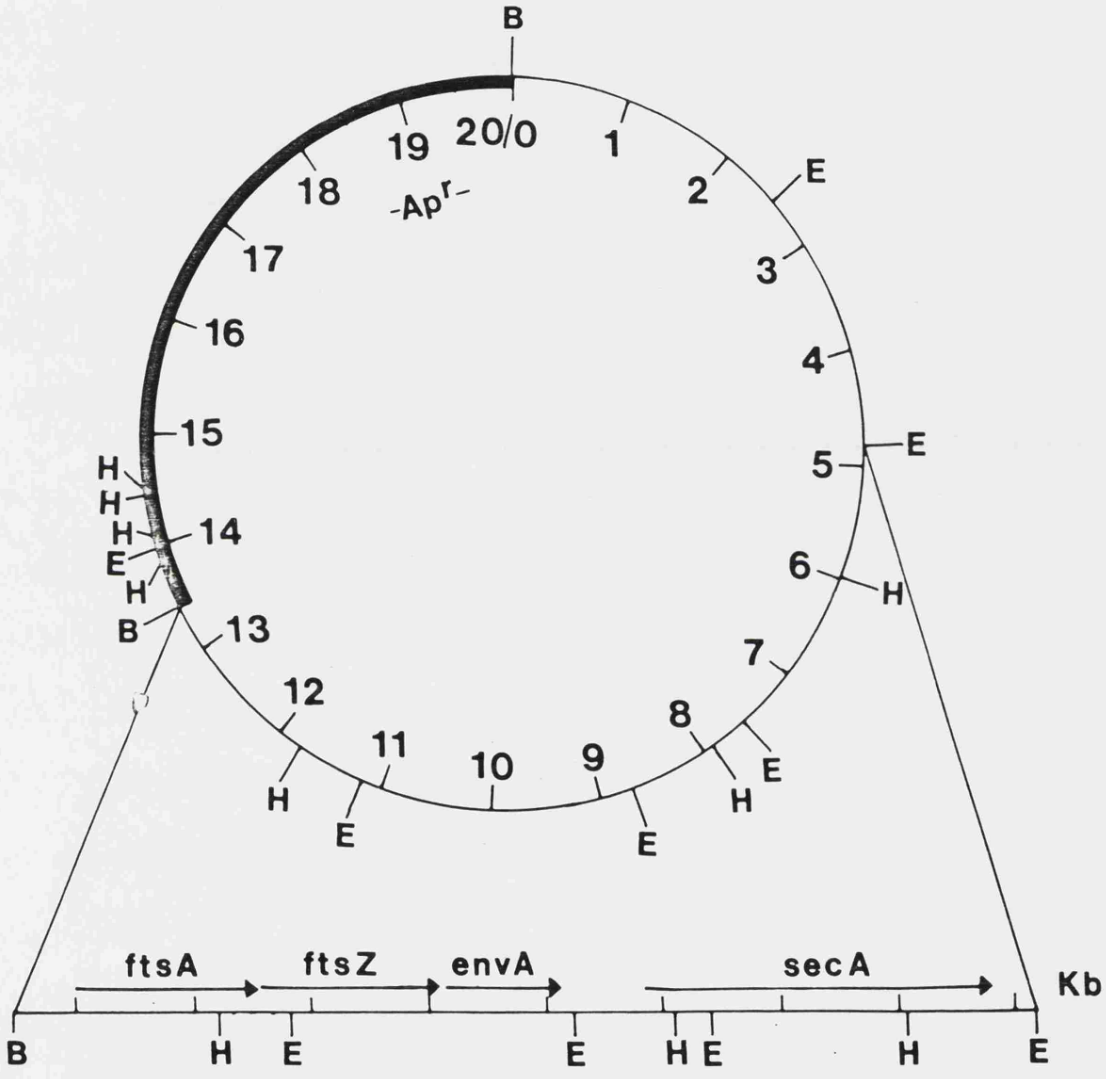

S 1 2

Figure 5.2

Restriction map of pLG550 with respect to restriction enzymes BamH1 (B), EcoR1 (E) and HindIII (H). The dark part of the circle represents the vector pLG339 and the numbers on the inside show size in kb.


B
20/0
1
2
E
3
4
5
E
6
H
7
8
E
H
9
E
10
11
H
E
12
13
B
H
H
H
E
H
14
15
16
17
18
19
-Ap^r-
ftsA
ftsZ
envA
secA
Kb
B
H
E
E
H
E
H
E

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 tsl 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 inconvienient 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,

1981).

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^r transformants were identified from both ligations. 12 colonies from each ligation were replated on nutient 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 respectivly.

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.

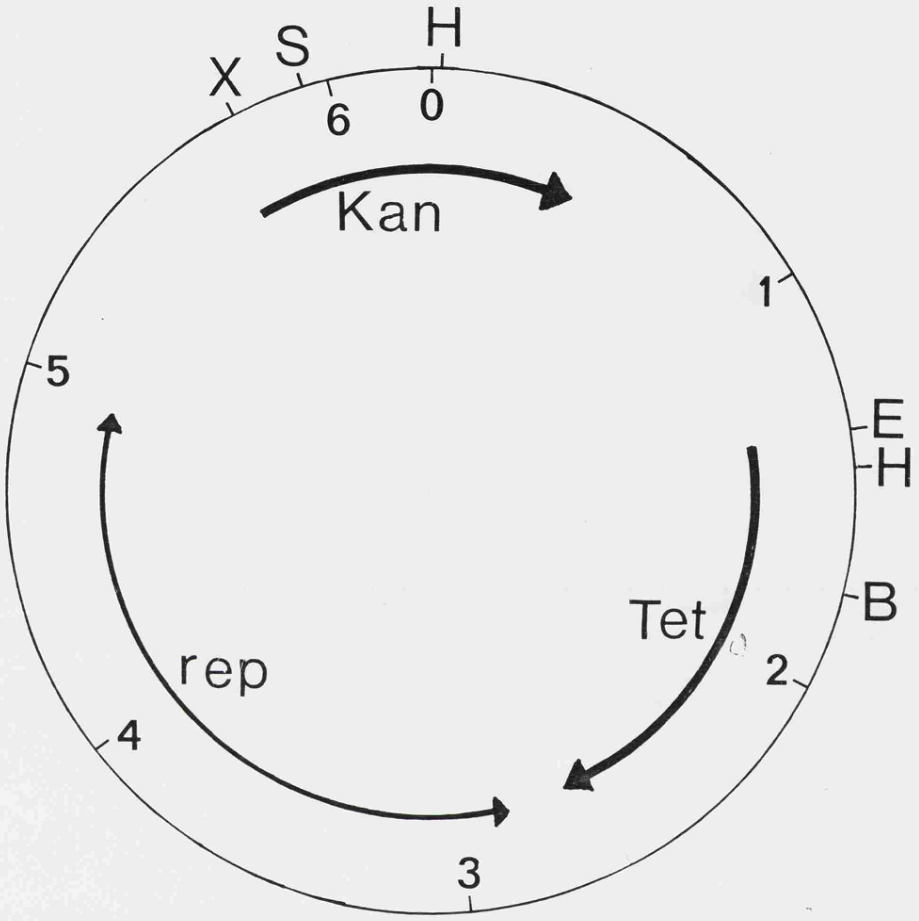
H - HindIII

E - EcoR1

B - BamH1

X - Xho1

S - Sal1


H
S
X
0
6
Kan
1
5
E
H
B
Tet
rep
2
4
3

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.

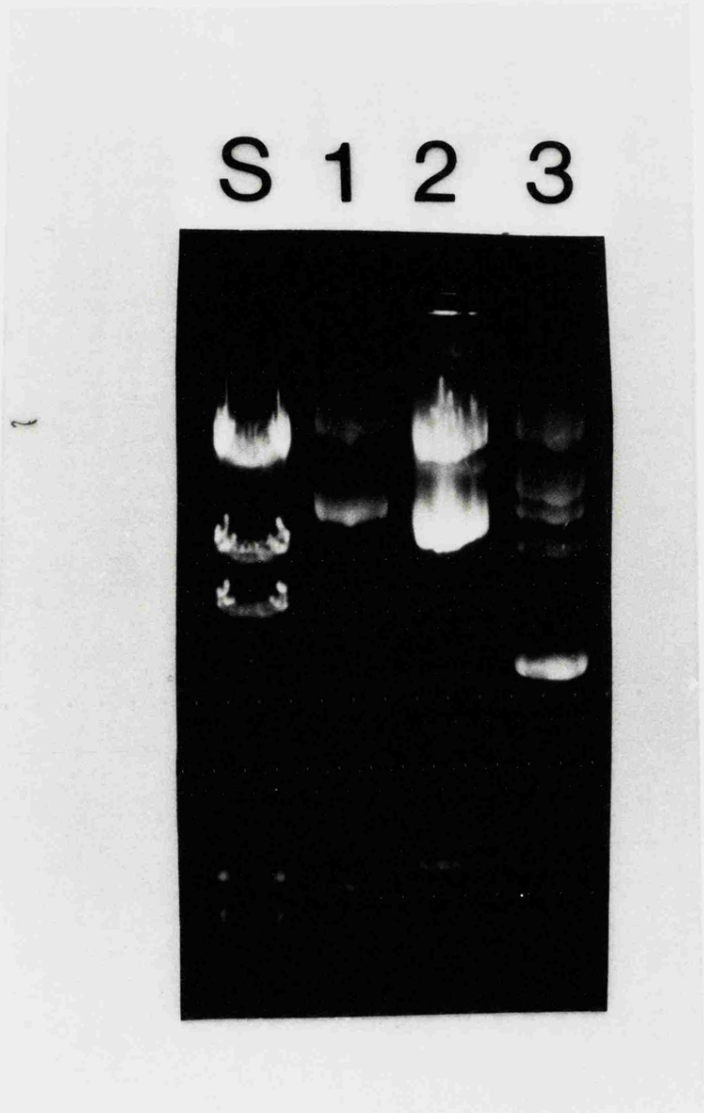
5.7 Restriction mapping of pLG552 and pLG554

To confirm that plasmids pLG552 and pLG554 carried the 13.5 kb chromosomal BamH1 fragments from pLG550 and pLG551 respectively, DNA of each was prepared using large scale plasmid preparations and digested with BamH1. The results confirmed that the 13.5 kb BamH1 fragments had been inserted into pLG339, and that in the construction of pLG554 from pLG551 the small extraneous 3.8 kb BamH1 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 BamH1 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.


S 1 2 3

into the BamH1 site of pLG339 (Fig. 5.5). Further restriction mapping confirmed that the 13.5 kb BamH1 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 incubted 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 repect to restriction endonucleases has shown that both contain a 13.5 kb BamH1 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 BamH1 fragments from pLG550 and pLG551 into pLG339, the recombinant plasmid derived from *sfiB114* chromosomal DNA (pLG552) gave a completely unambiguous *sfiB* phenotpye 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 5.1

strain	relative survival 30°C	42°C
GC24901(*tsl*,*sfiB*$^+$) pLG339	1	2.1×10^{-4}
GC24901 pLG552	1	0.85
GC24901 pLG554	1	6.8×10^{-5}
GC24901 pLG553	1	1.5×10^{-4}
TKF12(*ftsA*) pLG339	1	1.2×10^{-7}
TKF12 pLG552	1	0.85
TKF12 pLG554	1	1.07
TKF12 pLG553	1	1.25

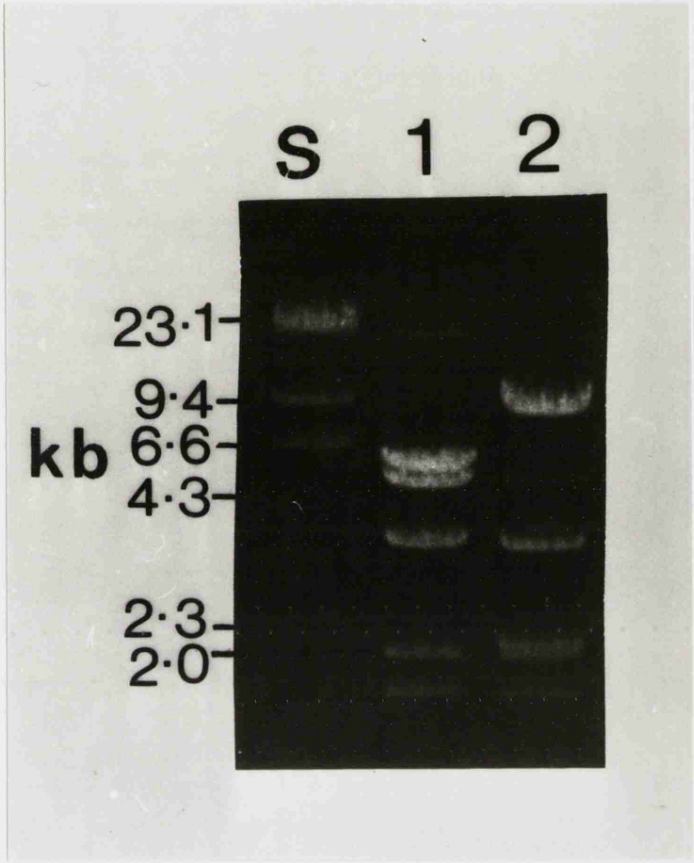
Figure 5.5

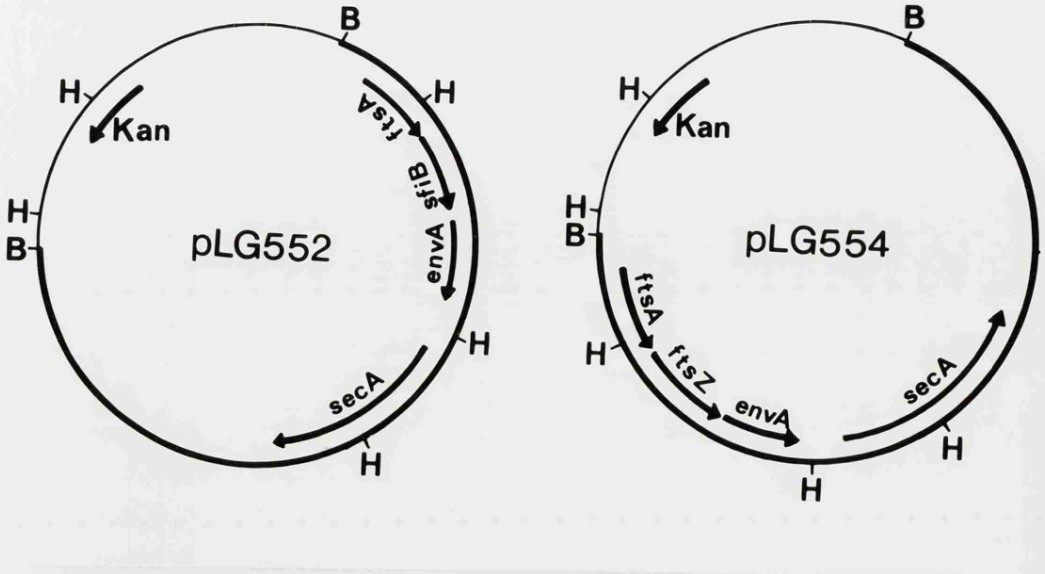
a) Agarose gel of pLG552 (track 1) and pLG554 (track 2) digested with HindIII.

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 - BamH1

H - HindIII


S
1
2
kb
23·1
9·4
6·6
4·3
2·3
2·0


B
H
Kan
ftsA
ftsZ
envA
secA
pLG552
pLG554

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

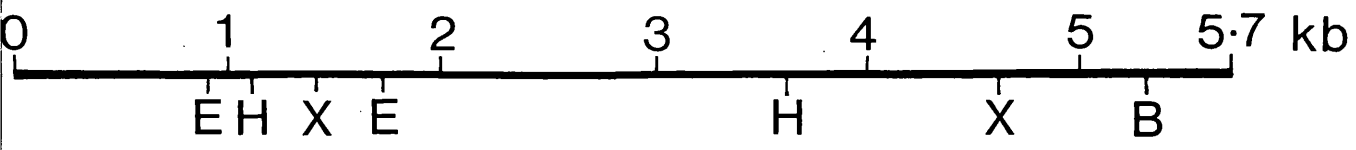
Having cloned a dominant sfiB114 locus onto recombinant plasmid pLG552, it became possible to identify the position of sfiB114 using transpositional mutagenesis. If transposons could be inserted randomly into pLG552, those having insertions into sfiB114 should be identifiable by loss of the SfiB phenotype (i.e. ability to suppress tsl 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 $\gamma\delta$ (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 EcoR1 (E), HindIII (H), Xho1 (X) and BamH1 (B).


0
1
2
3
4
5
5·7 kb
E H X E
H
X
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

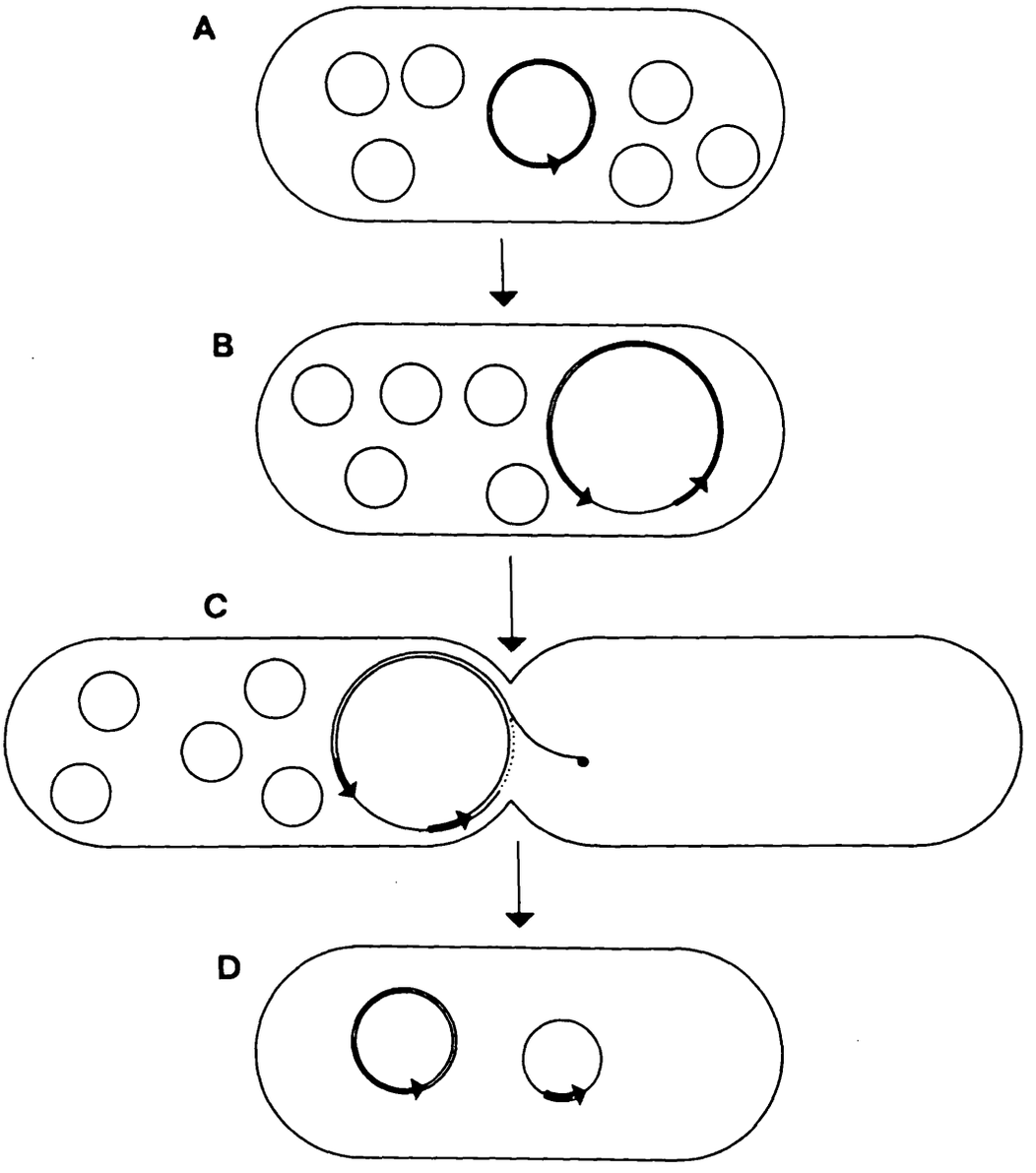
A: The non-mobilisable plasmid to be mutagenised is introduced into a strain harbouring an F factor carrying Tn1000.

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)


A
B
C
D

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 assymetric 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 6.1

strain	relative survival 30°C	42°C
GC24901(*tsl*,*sfiB*$^+$)pLG552::Tn1000-1	1	0.58
GC24901 pLG552::Tn1000-2	1	1.85
GC24901 pLG552::Tn1000-62	1	2.9×10^{-3}
GC24901 pLG552::Tn1000-164	1	3.0×10^{-4}
GC24901 pLG552::Tn1000-165	1	1.3×10^{-4}
GC24901 pLG552::Tn1000-174	1	1.1×10^{-4}

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

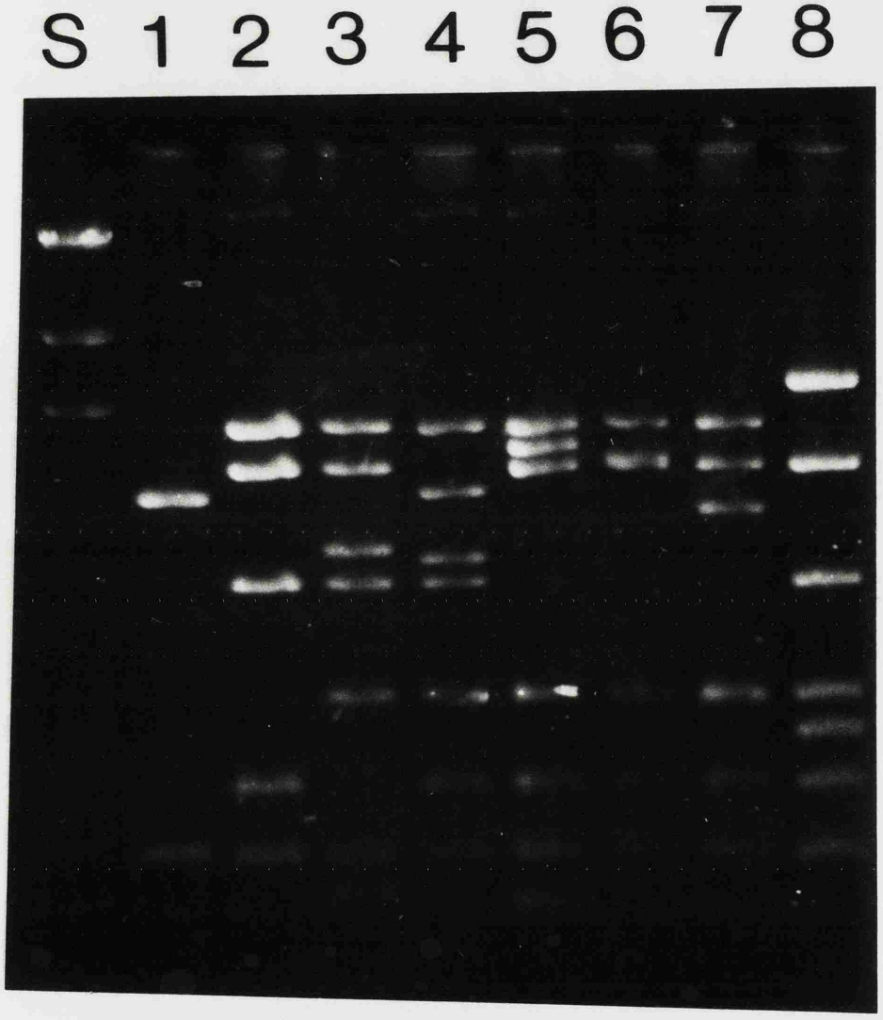

S 1 2 3 4 5 6 7 8

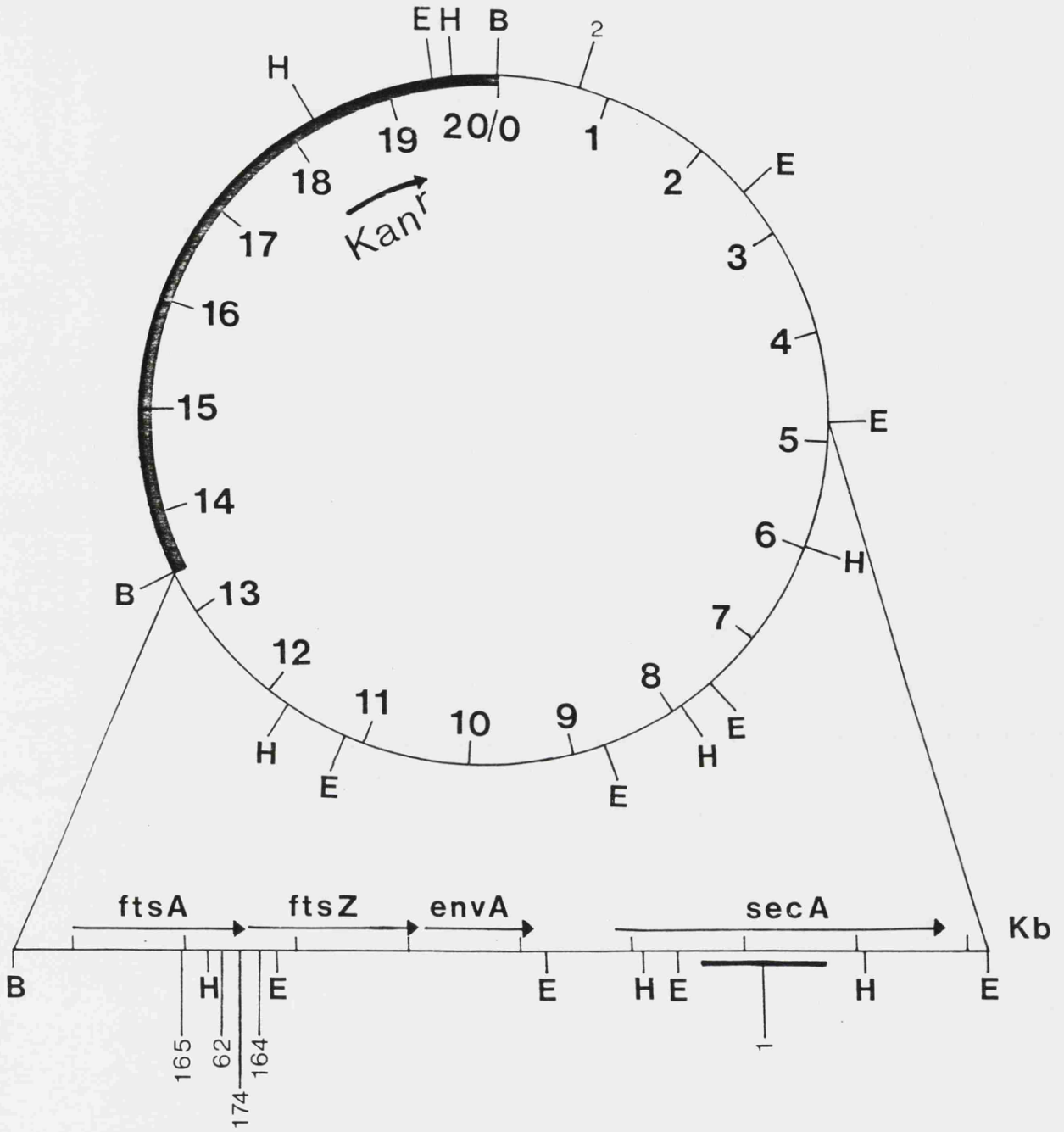
Figure 6.4

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 - BamH1

E - EcoR1

H - HindIII


E H B
2
H
19
20/0
1
18
Kan^r
2
E
17
3
16
4
15
E
5
14
6
H
B
13
7
12
8
11
10
9
E
H
H
E
E
ftsA
ftsZ
envA
secA
Kb
B
H
E
E
H E
H
E
165
62
174
164
1

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 6.2

strain	relative survival 30°C	42°C
JFL100(*ftsZ*) pLG339	1	1.8×10^{-8}
JFL100 pLG552	1	2.9×10^{-3}
JFL100 pLG552::Tn1000-62	-	-
JFL100 pLG552::Tn1000-164	1	6.3×10^{-6}
JFL100 pLG552::Tn1000-165	-	-
JFL100 pLG552::Tn1000-174	1	1.3×10^{-5}
TKF12(*ftsA*) pLG339	1	1.2×10^{-7}
TKF12 pLG552	1	0.85
TKF12 pLG552::Tn1000-62	1	3.4×10^{-7}
TKF12 pLG552::Tn1000-164	1	1.2
TKF12 pLG552::Tn1000-165	1	1.7×10^{-7}
TKF12 pLG552::Tn1000-174	1	9.2×10^{-6}

Complementation of 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 ftsZ by lysogeny with ftsZ+ λ- bacteriophages (even those expressing 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) ftsZ significantly reduce the level of ftsZ complementation. Insertions further upstream of ftsZ also show reduced complementation and there seems to be a decrease in the level of inactivation of ftsZ+ as insertions lie further upstream of the ftsZ coding region. The observation that insertion 164 inactivates SfiB114 while not affecting ftsA complementation indicated that sfiB114 was not an allele of ftsA. To prove this, a restriction fragment containing ftsA alone was subcloned from pLG550 and tested for the presence of a SfiB114 phenotype.

6.5 Subcloning of ftsA from pLG550

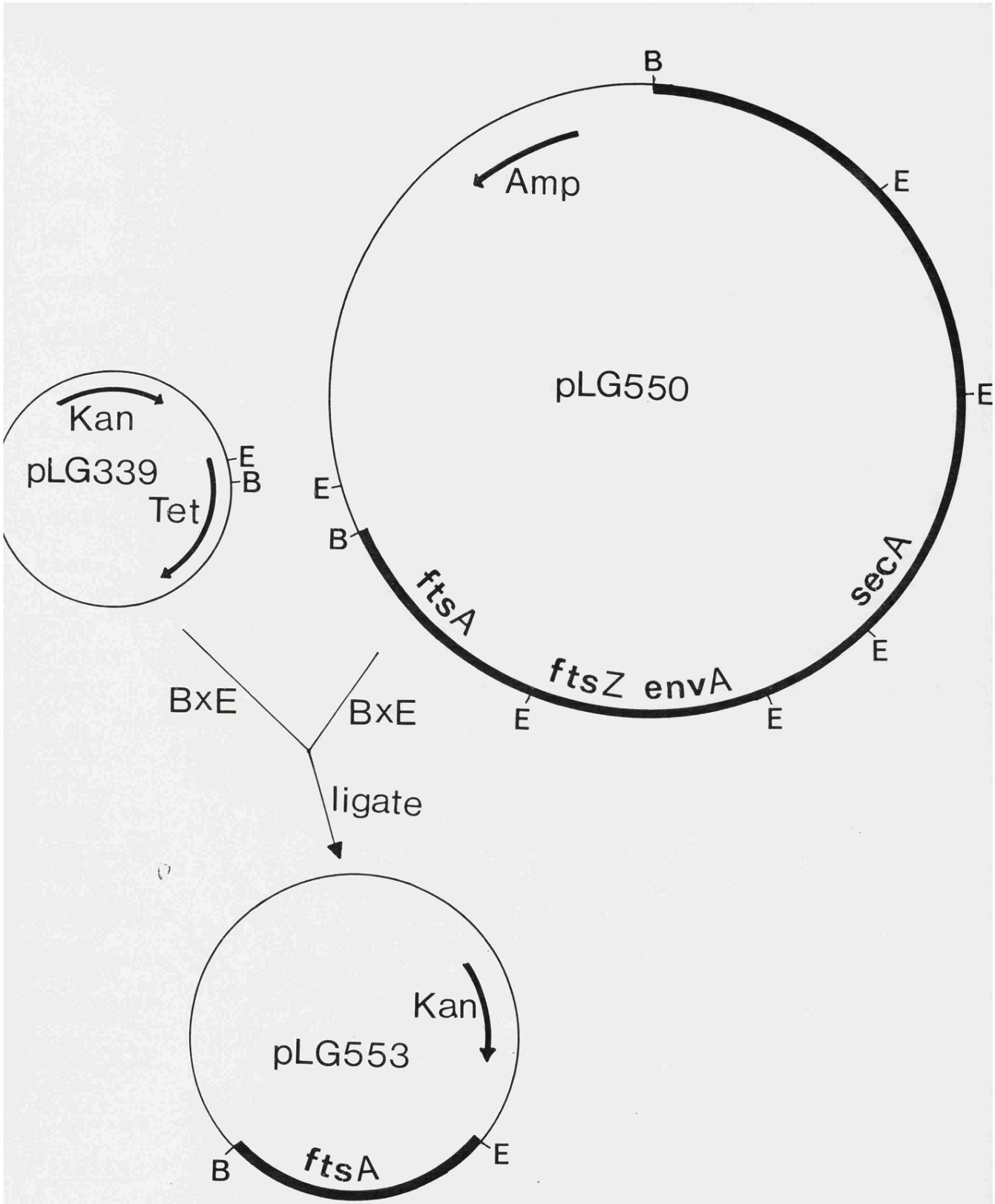
Since Tn1000 insertions into ftsA had been shown to inactivate sfiB114 it was possible that sfiB114 was an allele of ftsA. However, one Tn1000 insertion, 164, inactivated SfiB114 without affecting ftsA+. It was thought possible that Tn1000 insertion 164 was sufficiently near the terminus of the ftsA gene to allow a slightly truncated protein to complement the ftsA mutation in TKF12, yet prevent expression of a 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 ftsA and the proximal region of ftsZ. This recombinant plasmid, named

Figure 6.5

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

B - BamH1

E - EcoR1


B
Amp
E
pLG550
E
Kan
pLG339
E
B
Tet
E
B
ftsA
secA
E
ftsZ envA
E
E
BxE
BxE
ligate
Kan
pLG553
B
ftsA
E

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, CSH26 ΔF6) 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 flourography 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

were reported to have sizes of 50 kD (FtsA), 45 kD (FtsZ), 31 kD (EnvA) and 92 kD (SecA). Thus it was expected to be relatively easy to correlate the effects of pLG552::Tn1000 on the production of gene products from pLG552.

7.3 Analysis of polypeptides programmed by plasmids carrying sfiB114 and sfiB114::Tn1000

The maxi-cell strain CSH26 ΔF6 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 disappearence 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

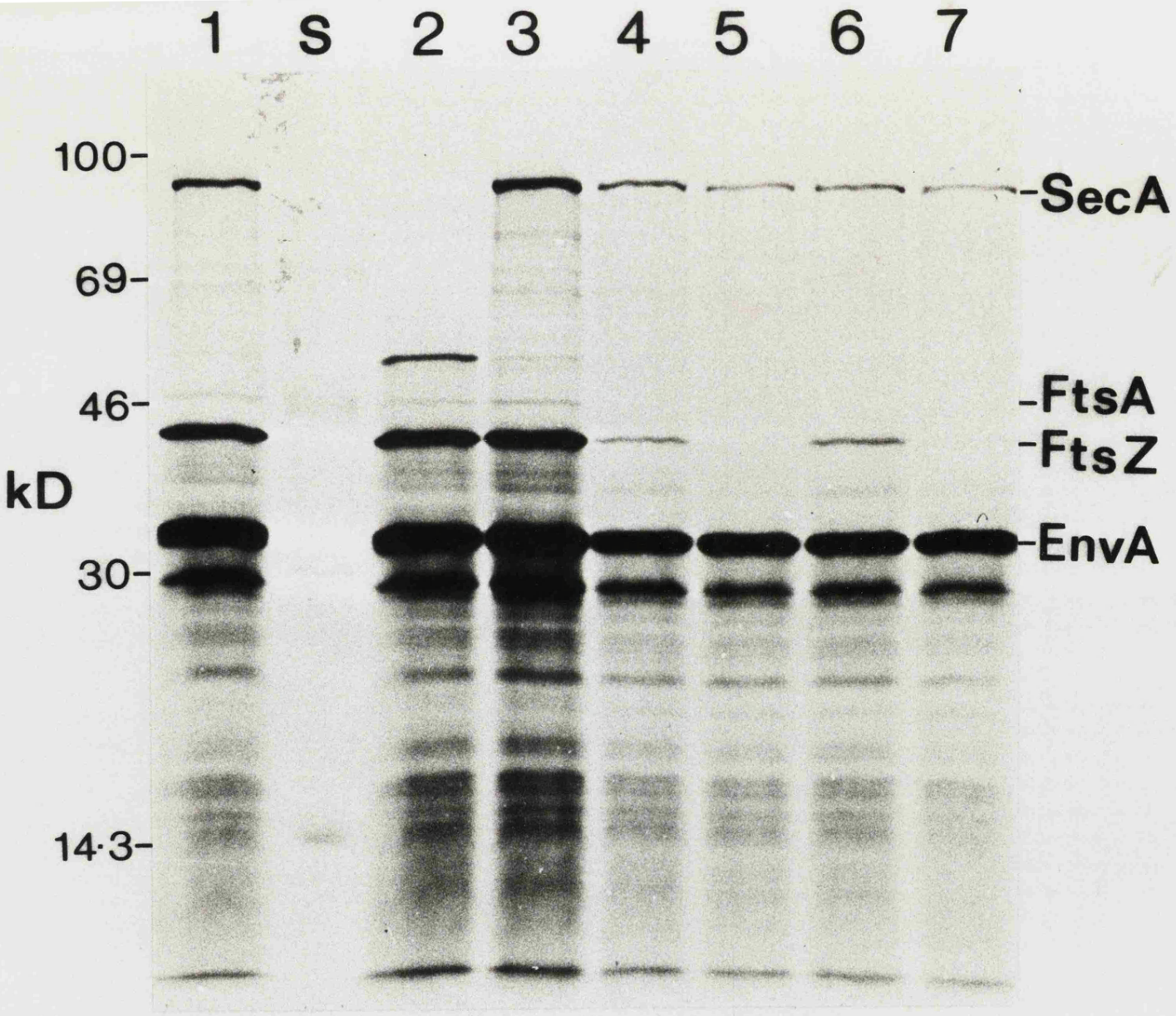
3 - pLG552::Tn1000-2

4 - pLG552::Tn1000-62

5 - pLG552::Tn1000-164

7 - pLG552::Tn1000-165

8 - pLG552::Tn1000-174


1
S
2
3
4
5
6
7
100-
69-
46-
30-
14·3-
kD
SecA
FtsA
FtsZ
EnvA

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 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 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 ftsZ (chapter 6), which parallels their more distal position upstream of ftsZ, is a direct consequence of increased 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 ftsA (chapter 6). However pLG552SfiB114::Tn1000 plasmids 174, 62 and 165, which fail to complement 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

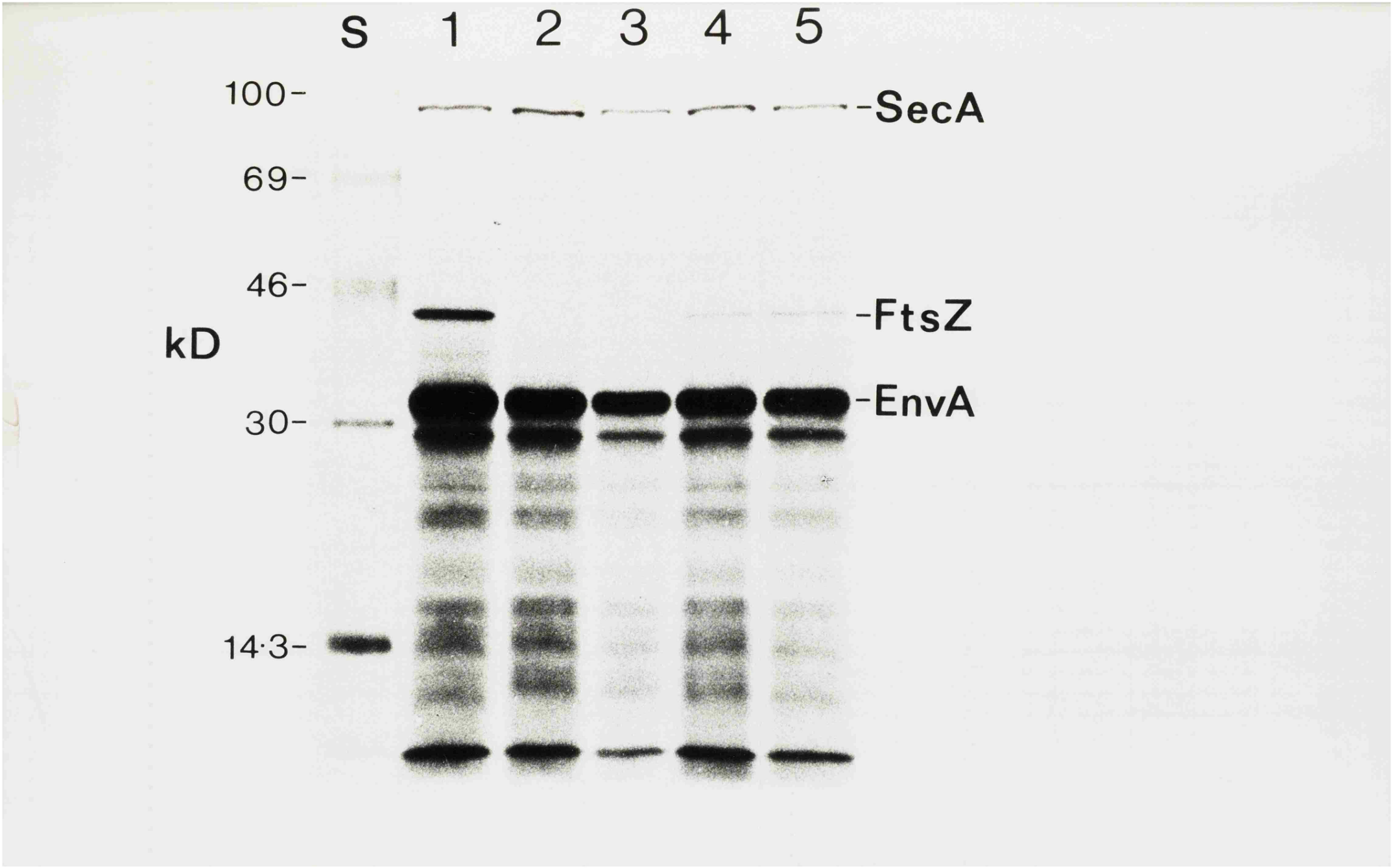

S
1
2
3
4
5
100-
69-
46-
kD
30-
14·3-
-SecA
-FtsZ
-EnvA

Figure 7.3

A longer exposure of the autoradiograph shown in Fig. 7.2. Tracks represent.

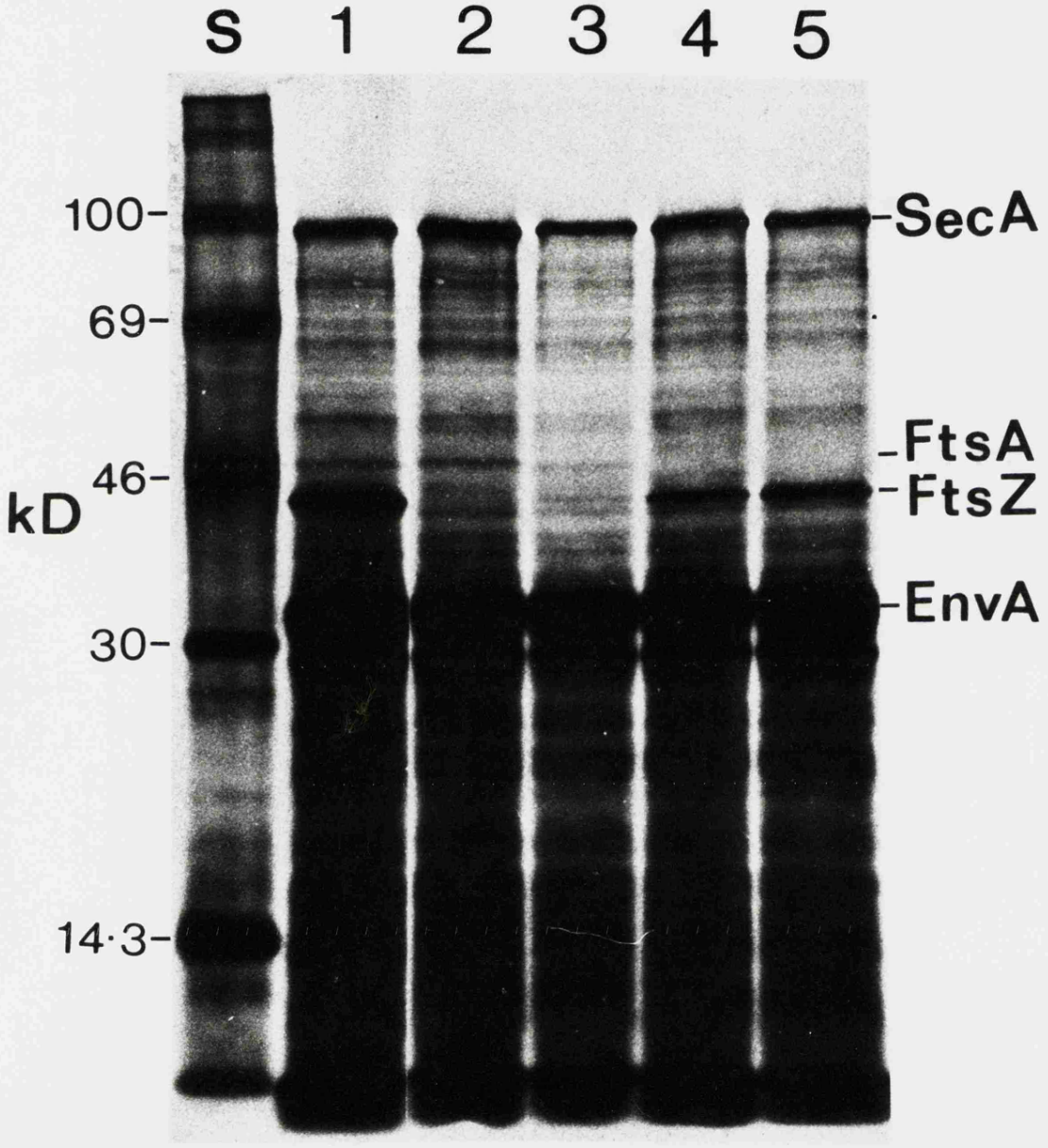
1 - pLG552

2 - pLG552::Tn1000-164

3 - pLG552::Tn1000-174

4 - pLG552::Tn1000-62

5 - pLG552::Tn1000-165


S
1
2
3
4
5
100-
69-
46-
30-
14·3-
kD
SecA
FtsA
FtsZ
EnvA

complementation data presented in Chapter 6. In particular, the results confirmed that Tn1000 insertions into the distal area of the coding region of ftsA and the proximal region of ftsZ could inactivate the SfiB114 phenotype and eliminate or significantly reduce the synthesis of FtsZ protein.

7.4 Tests for a ftsZ promoter on the 3.5 kb HindIII fragment

The results from the previous section left unexplained how insertions into ftsA could prevent or reduce expression of ftsZ. One possibility is that ftsZ is expressed primarily from either the ftsA promoter or from other promoters within the ftsA coding sequence. Luktenhaus and Wu (1980) had observed that expression of ftsZ from the 3.5 kb HindIII fragment carrying ftsZ and envZ (see Fig 6.3) was weak but they were unable to conclude whether or not 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 in-vitro transcription translation system.

The Zubay 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 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 E.coli recB strain (i.e. defective in the major E.coli exonuclease, exonuclease V) its use avoids degradation of linear DNA restriction

fragments used as templates (Jackson et al., 1983).

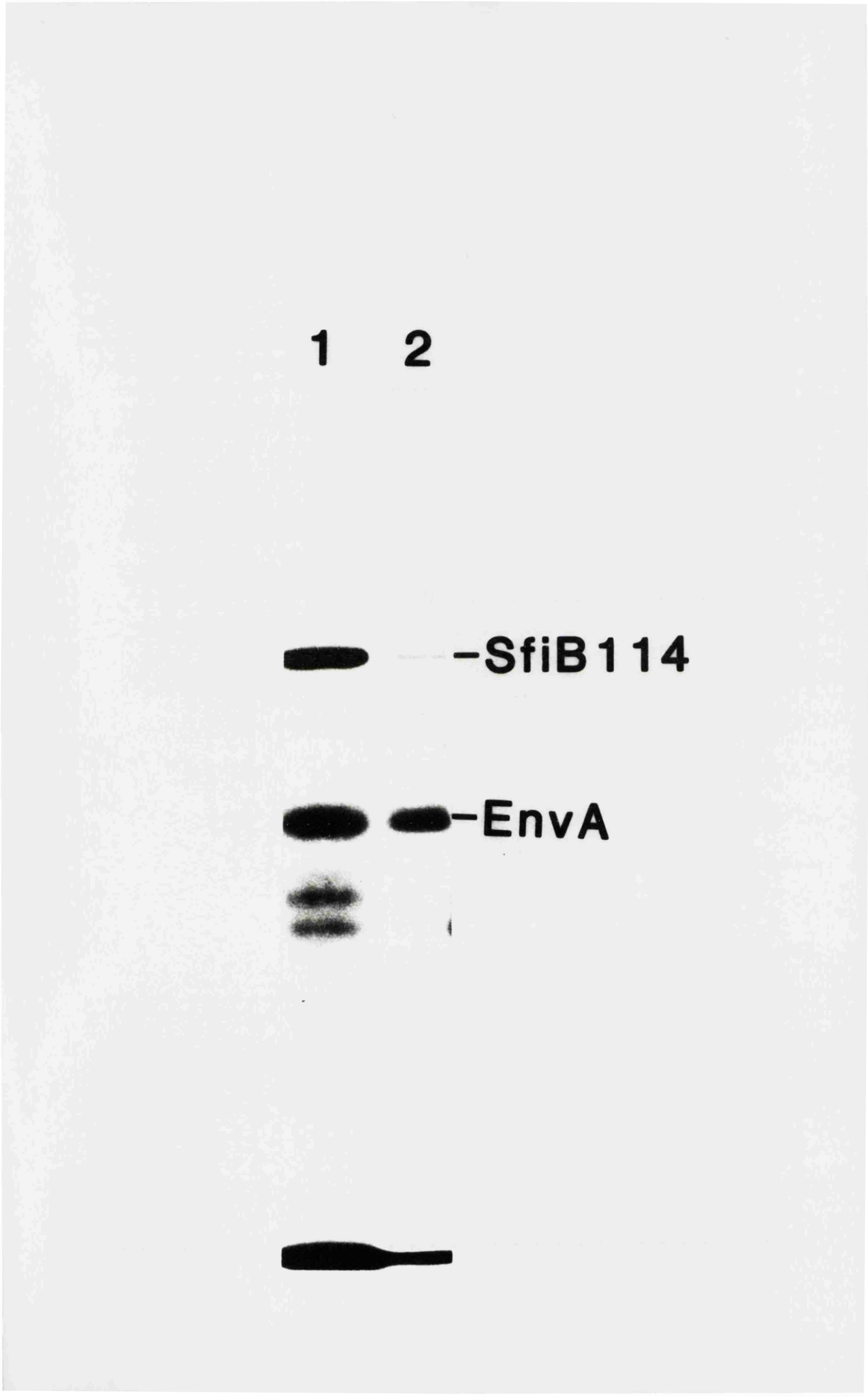
The 3.5 kb HindIII restriction fragment carrying ftsZ and envA, obtained from plasmid pLG552 DNA and therefore carrying sfiB114, was purified from an 0.8% agarose gel using the DE81 procedure (see methods). After concentration by ethanol precipitation and resuspension in TE buffer, this DNA fragment was used to programme the Zubay system using a recB S30 extract. The resulting ^{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 ftsZ is present on the 3.5 kb fragment.

7.5 Discussion

Results presented here have indicated that the sfiB114 mutation is an allele of the previously characterised essential cell division gene ftsZ and that an element within the ftsA coding sequence is required for efficient expression of ftsZ. While this work was in progress Luktenhaus (1983) published a paper in which two different sfiB alleles sulB25 (Johnson, 1977) and sulB9 (Gayder et al., 1976) were shown to be allelic to ftsZ. Luktenhaus (1983) also found that the gene products of these alleles, SulB25 and SulB9, migrated more slowly than the wild type FtsZ (SfiB$^+$) protein in SDS-PAGE. This result indicated that the location of the sfiB25 and sfiB9 mutations was within the ftsZ coding sequence producing polypeptides with an altered size or mobility in SDS-PAGE. These results confirmed the conclusion that sfiB and 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 in-vitro transcription-translation system and Track 2 represents expression of pLG552 in maxi cells.


1 2
-SfiB114
-EnvA

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

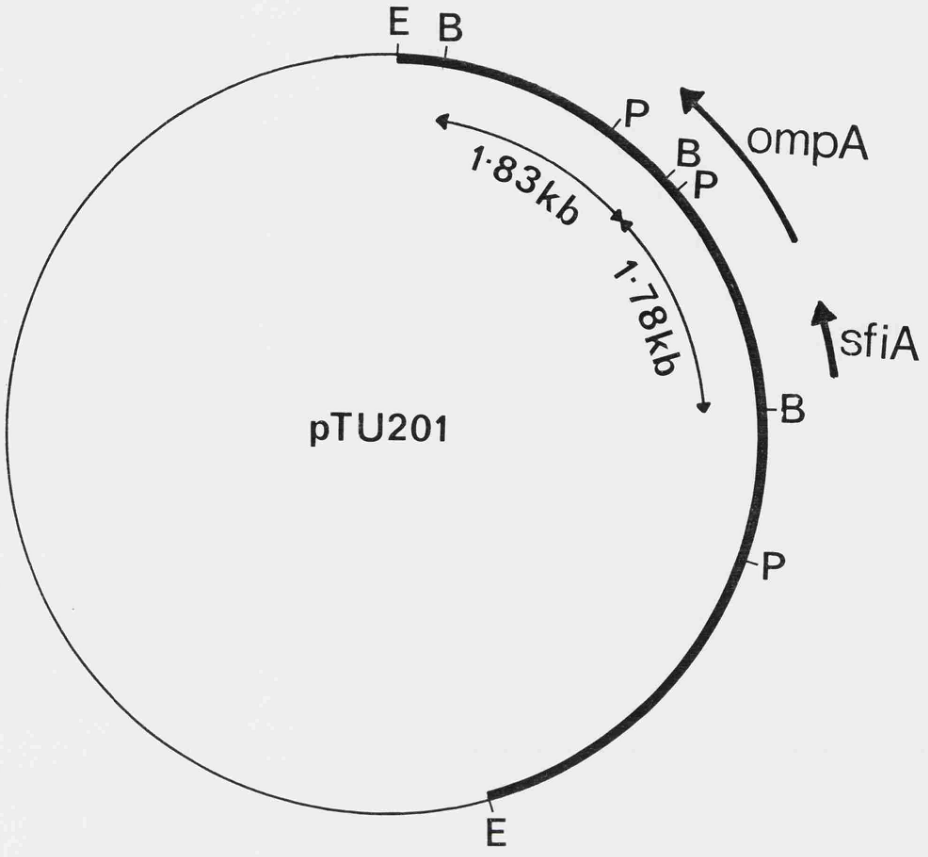
A map of the plasmid pTU201 showing the position of the ompA and sfiA genes. The light line represents the vector pBR325. No restriction pattern for the pBR325 segment is shown because the orientation of the chromosomal insert is unknown.

E - EcoR1

B - BamH1

P - PvuII

Note that the PvuII site shown in the 1.78 kb BamH1 fragment consists of two PvuII sites very close together.


E
B
P
B
P
ompA
1·83kb
1·78kb
sfiA
pTU201
B
P
E

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 acheived 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^{o}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, tsl strain as a host for maxi-cell experiments. Such a strain was obtained, GC2493. However, as described in the previous section the, tsl allele does not fully repress the SOS functions (including DNA repair) even during growth at 30°C. Consequently GC4293 (tsl, 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 concentation 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

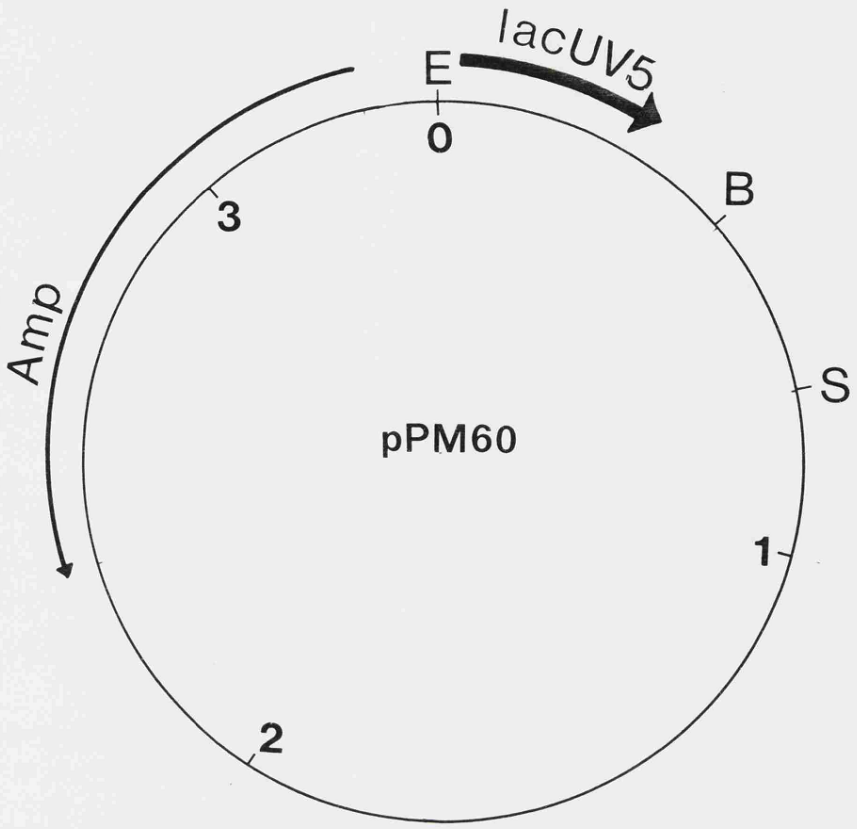
Figure 8.2

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 - EcoR1

B - BamH1

S - Sal1


lacUV5
E
0
B
S
1
2
3
Amp
pPM60

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*$_q$ 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*$_q$.

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 convienient 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 *lac*UV5 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 *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 F'*lacI*$_q$ to ampicillin resistance on nutrient agar plates (Fig 8.3). Two classes of transformant were obtained after growth at 37^{o}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 *sfiA* fragment inserted into the BamH1 site. The IPTG sensitive transformants of this second class were expected to harbour plasmids carrying the *sfiA* gene downstream of the *lac*UV5 promoter, transcription from the contiguous *lac*UV5 promoter being in the same direction as the *sfiA* gene. In contrast the IPTG resistant transformants would carry recombinant plasmids having the *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

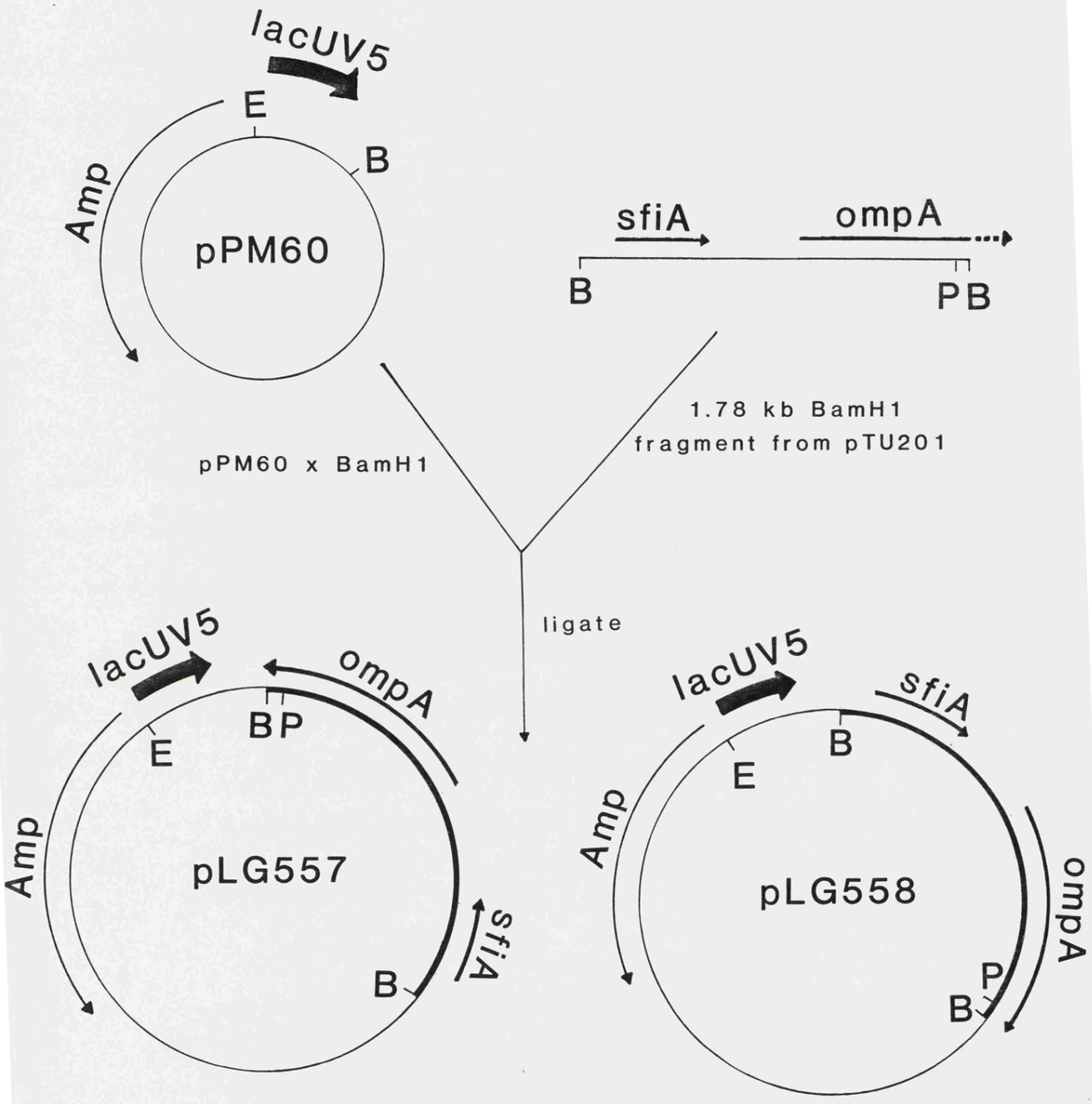
Figure 8.3

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


lacUV5
E
B
Amp
pPM60
sfiA
ompA
B
PB
1.78 kb BamH1
fragment from pTU201
pPM60 x BamH1
ligate
lacUV5
ompA
BP
E
Amp
pLG557
sfiA
B
lacUV5
sfiA
B
E
Amp
pLG558
ompA
P
B

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 posessed 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 CSH26 ΔF6F'$lacI_q$ carrying pPM60, pLG557 or pLG558 were grown in minimal media + casamino acids at 37°C to A_{450} = 0.1 and IPTG was added to a final concentration of 40 μg/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 CSH26ΔF6F'$lacI_q$ pLG558 causes a rapid cessation of cell division, an effect not seen with CSH26Δ F6F'$lacI_q$ carrying pLG557 or pPM60. In addition, even in the absence of IPTG the mass per cell of CSH26 ΔF6F'$lacI_q$ is approximately 1.8 X higher in the presence of pLG557 and pLG558 compared to pPM60. This increase in cell size in strains carrying pLG557 or pLG558 was thought to be due to

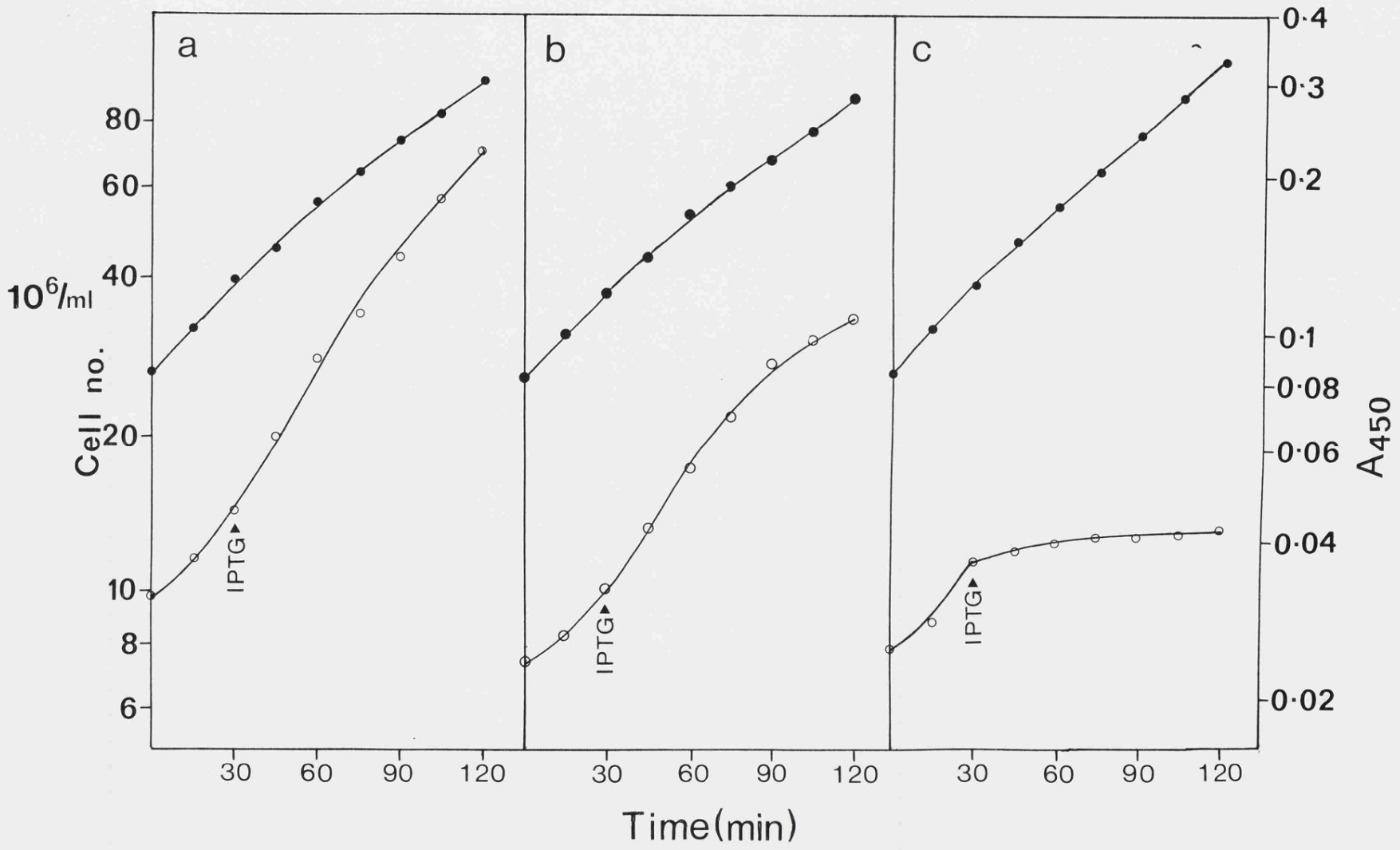
Figure 8.4

The effect on the cell number and cell mass of adding the *lac* inducer IPTG to cultures of CSH26ΔF6F'*lacI*^{q} growing in minimal medium + casamino-acids carrying the plasmids :

a) pPM60

b) pLG557

c) pLG558


a
b
c
80
60
40
20
10
8
6
10^6/ml
Cell no.
IPTG
0·4
0·3
0·2
0·1
0·08
0·06
0·04
0·02
A_{450}
30
60
90
120
Time(min)

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 demonstating 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'$lacI_q$ 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, CSH26ΔF6F'$lacI_q$ 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)

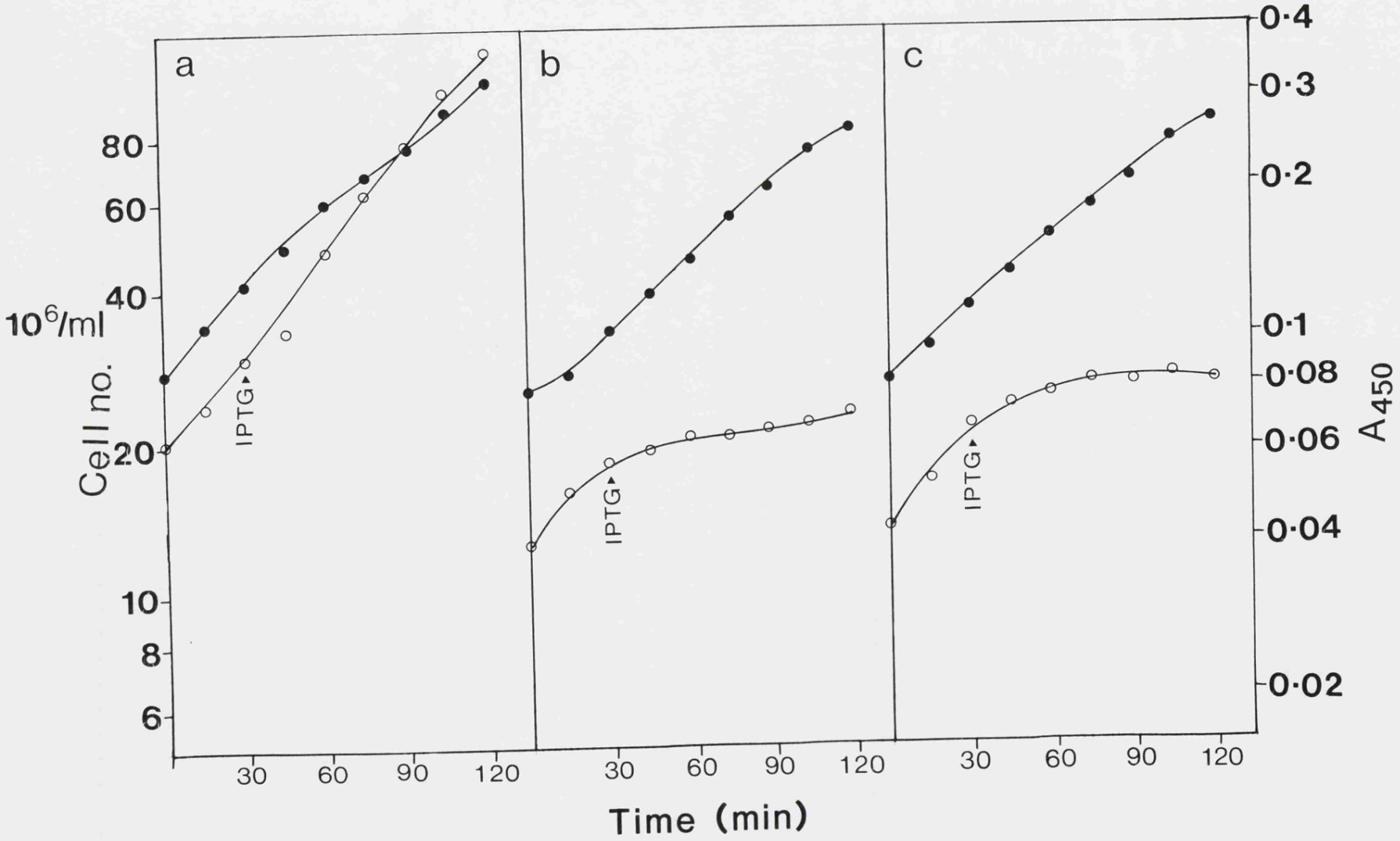
Figure 8.5

The effect on cell number and cell mass of adding IPTG to cultures growing in minimal media containing casamino-acids.

a) GC4276 (*sfiB114*) F'*lacI*^{q} pLG558

b) CSH26 ΔF6 F'*lacI*^{q}, pLG558, pLG552

c) CSH26 ΔF6 F'*lacI*^{q}, pLG558, pLG554


a
b
c
80
60
40
20
10
8
6
10^6/ml
Cell no.
IPTG
IPTG
IPTG
30
60
90
120
30
60
90
120
30
60
90
120
Time (min)
0·4
0·3
0·2
0·1
0·08
0·06
0·04
0·02
A_{450}

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 BamH1 fragment carrying *sfiA* downstream of a *lac*UV5 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) descibed 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 consistant 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 sfiA and sfiB as reported by Huiman et al. (1984). However, quite surprisingly host cells with multiple copies of sfiB114 on a recombinant plasmid (but with $sfiB^+$ present on the chromosome) were almost completely sensitive to the effects of pLG558 in the presence of IPTG although in the absence of the lac inducer filamentation was suppressed. This result is in contrast to the fact that pLG552 was shown to suppress 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 tsl mutant due to the high copy number of pLG558. The fact remains a puzzling one that the 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 $FtsZ^+$ function will be discussed later.

CHAPTER 9

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 *lac*UV5 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 *lac*UV5 promoter using the *lac* inducer IPTG. Maxi-cells were therefore prepared from CSH26 ΔF6 F'*lacI*$_q$ 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, CSH26ΔF6 F'*lacI*$_q$ was transformed with pLG552, pLG554, pLG558 + pLG552 and pLG558 + pLG554. Maxi-cells were prepared for both single and double transformants and ^{35}S-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$^+$ (FtsZ) seems to be expressed at a higher level (approximately 1.8 fold) than SfiB114, using EnvA and SecA as internal standards. Secondly the presence of pLG558 seems to actually increase the production of both SfiB$^+$ and SfiB114 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 (sfiB114). Similar results were obtained in several different experiments indicating a specific effect on the abundance of SfiB$^+$, SfiB114 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$^+$ and SfiB114 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

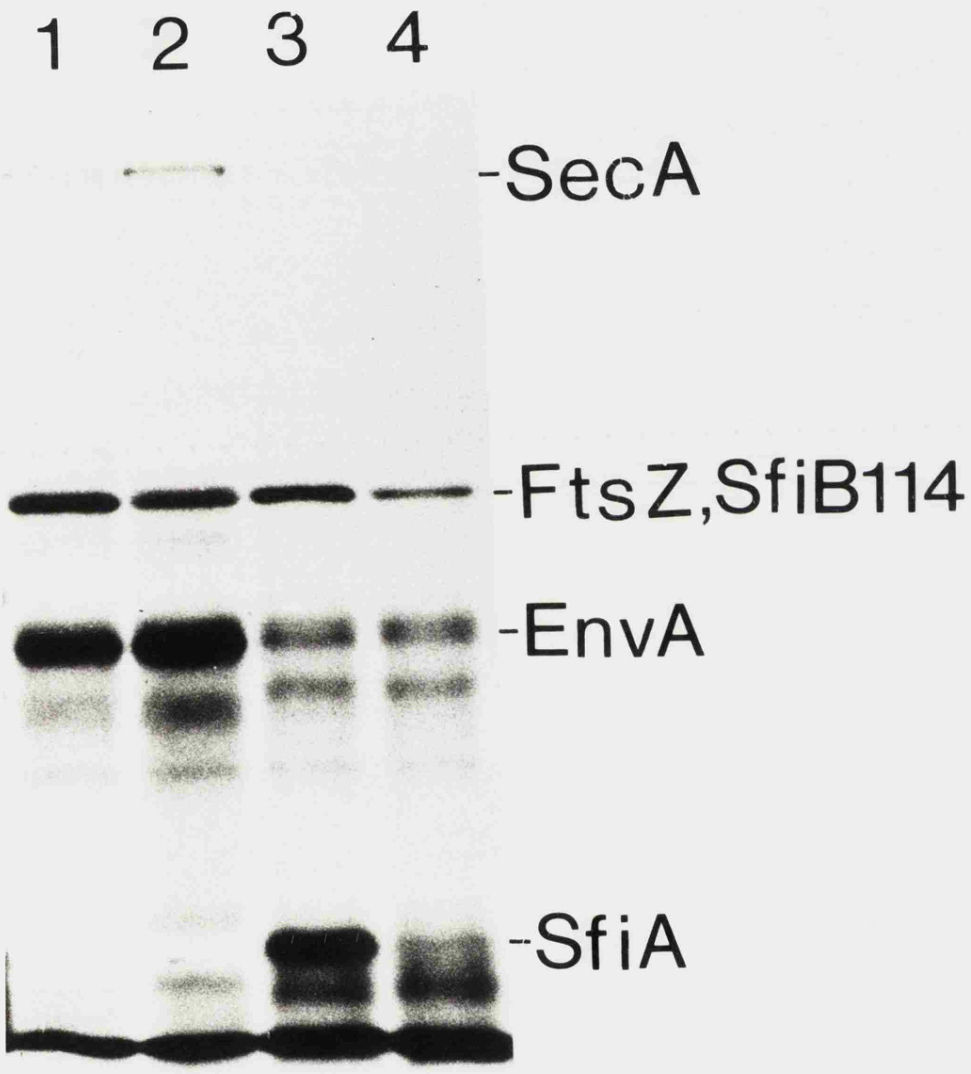
An autoradiograph of ^{35}S-methionine labelled maxi-cells analysed using an 11% polyacrylamide gel. Tracks represent maxi-cells carrying :

1 - pLG554

2 - pLG552

3 - pLG554 + pLG558

4 - pLG552 + pLG558


1 2 3 4
-SecA
-FtsZ,SfiB114
-EnvA
-SfiA

9.2 (pLG552) and Fig. 9.3 (pLG554). These results show that both the SfiB$^+$ and SfiB114 proteins are stable thoughout 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 densitometricaly 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.

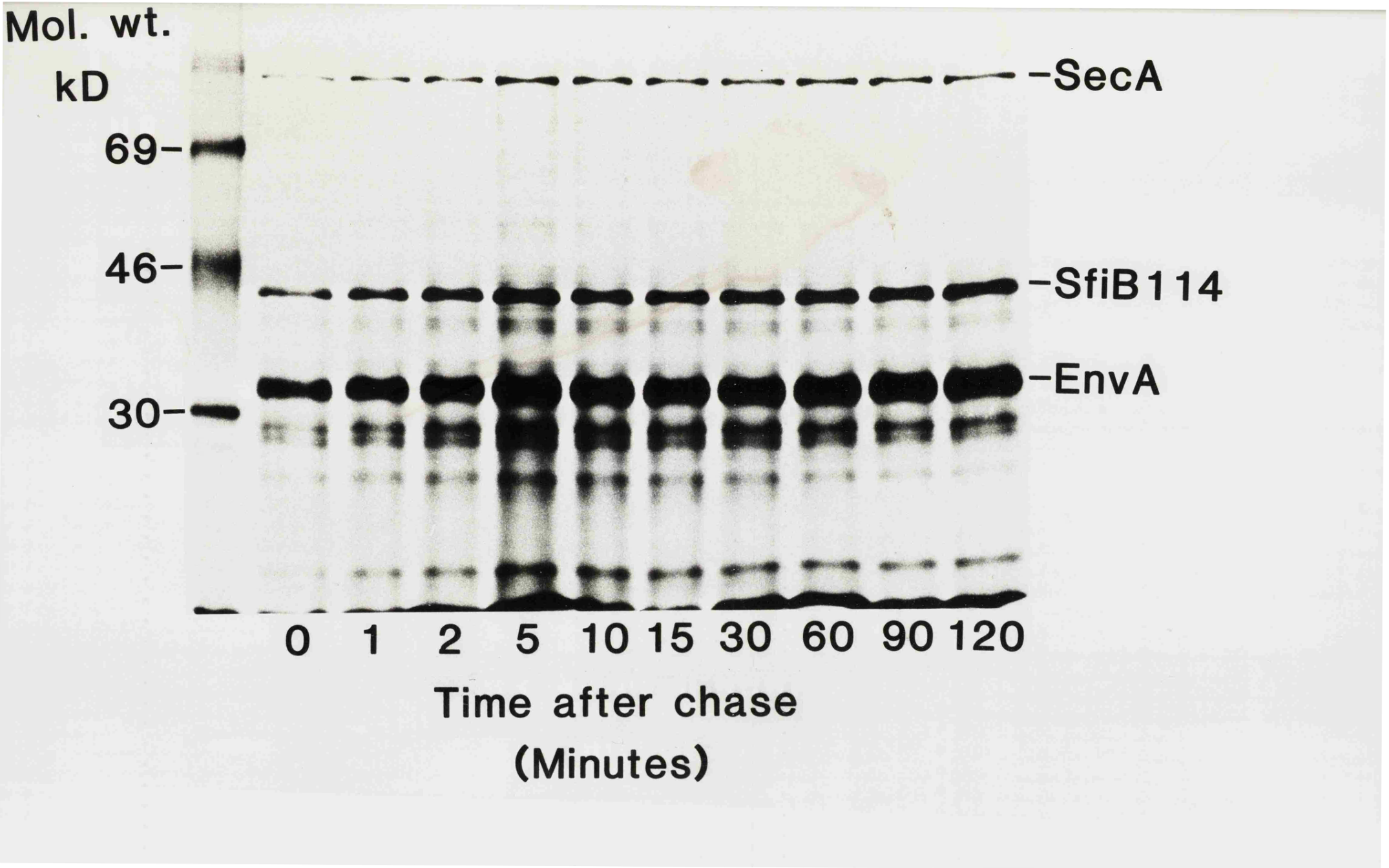

Mol. wt.
kD
69-
46-
30-
-SecA
-SfiB114
-EnvA
0 1 2 5 10 15 30 60 90 120
Time after chase
(Minutes)

Figure 9.3

An autoradiogram of maxi-cells containing pLG554, pulse labelled with ^{35}S-methionine and analysed using an 11% polyacrylamide gel.

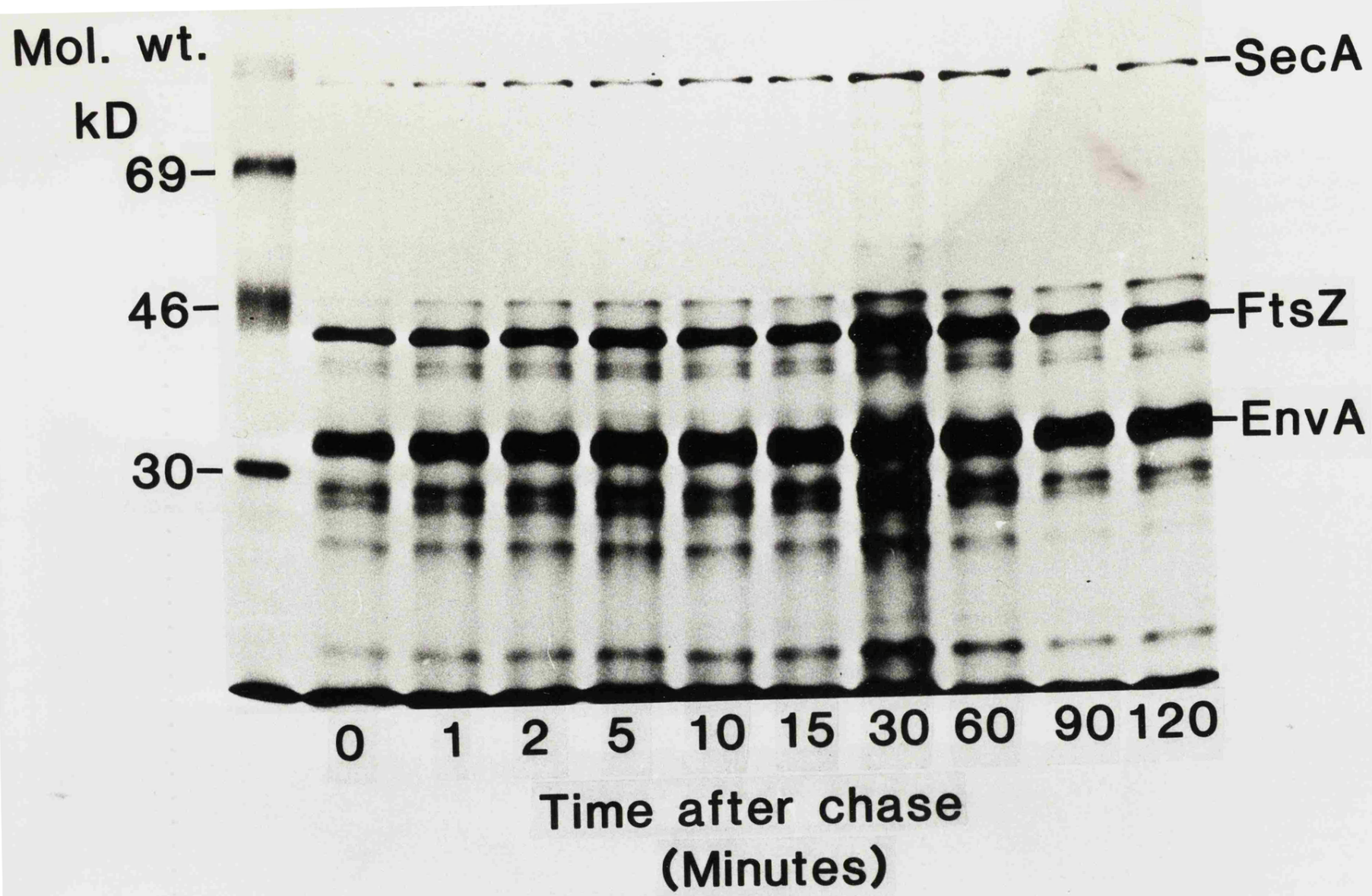

Mol. wt.
kD
69-
46-
30-
SecA
FtsZ
EnvA
0
1
2
5
10
15
30
60
90
120
Time after chase
(Minutes)

Figure 9.4

An autoradiogram of maxi-cells containing pLG558, pulse labelled with ^{35}S-methionine and analysed using an 15% polyacrylamide gel.

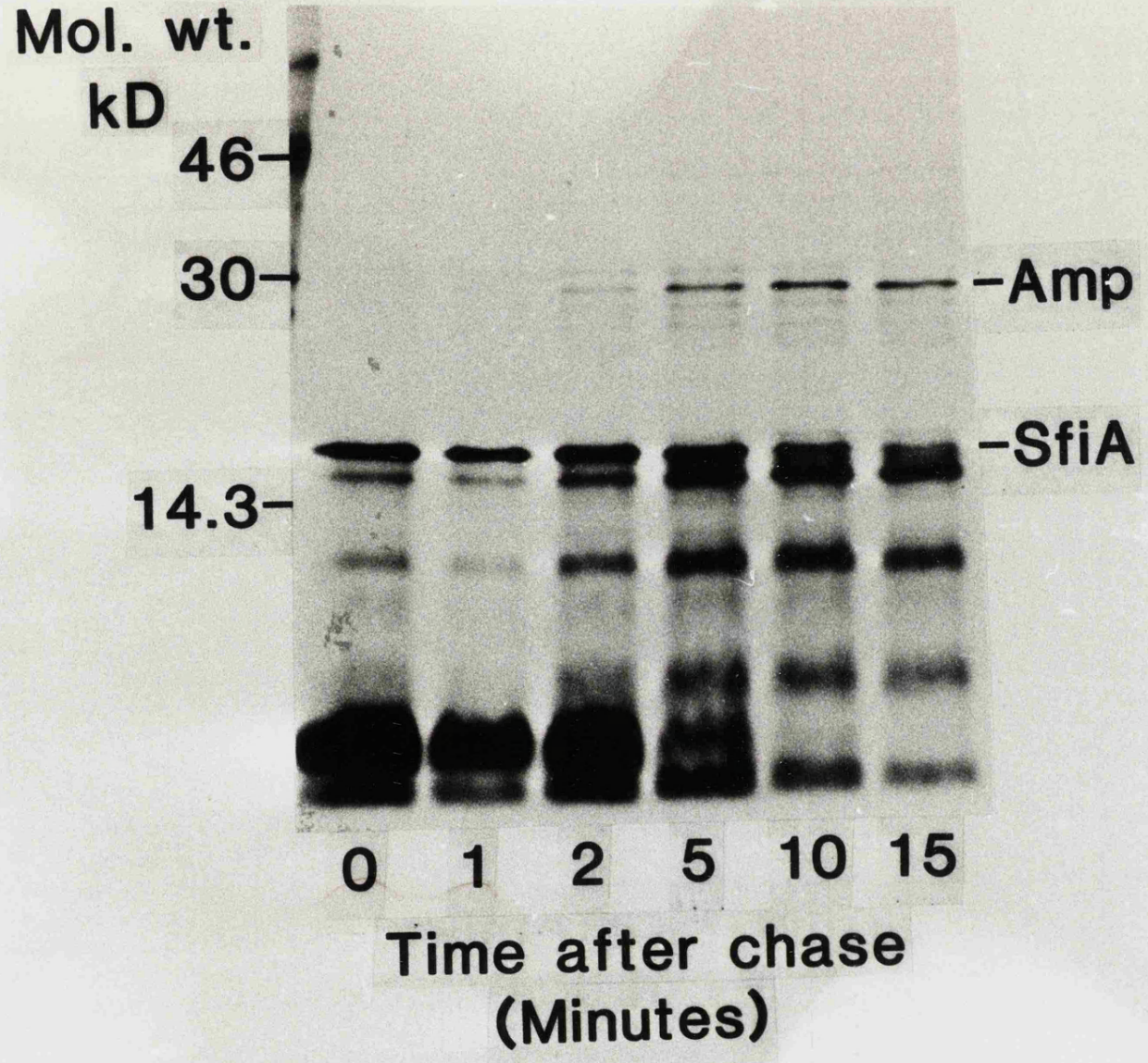

Mol. wt.
kD
46
30
14.3
Amp
SfiA
0
1
2
5
10
15
Time after chase
(Minutes)

Figure 9.5

An autoradiogram of maxi-cells containing pLG552 + pLG558, pulse labelled with ^{35}S-methionine and analysed using an 15% polyacrylamide gel.

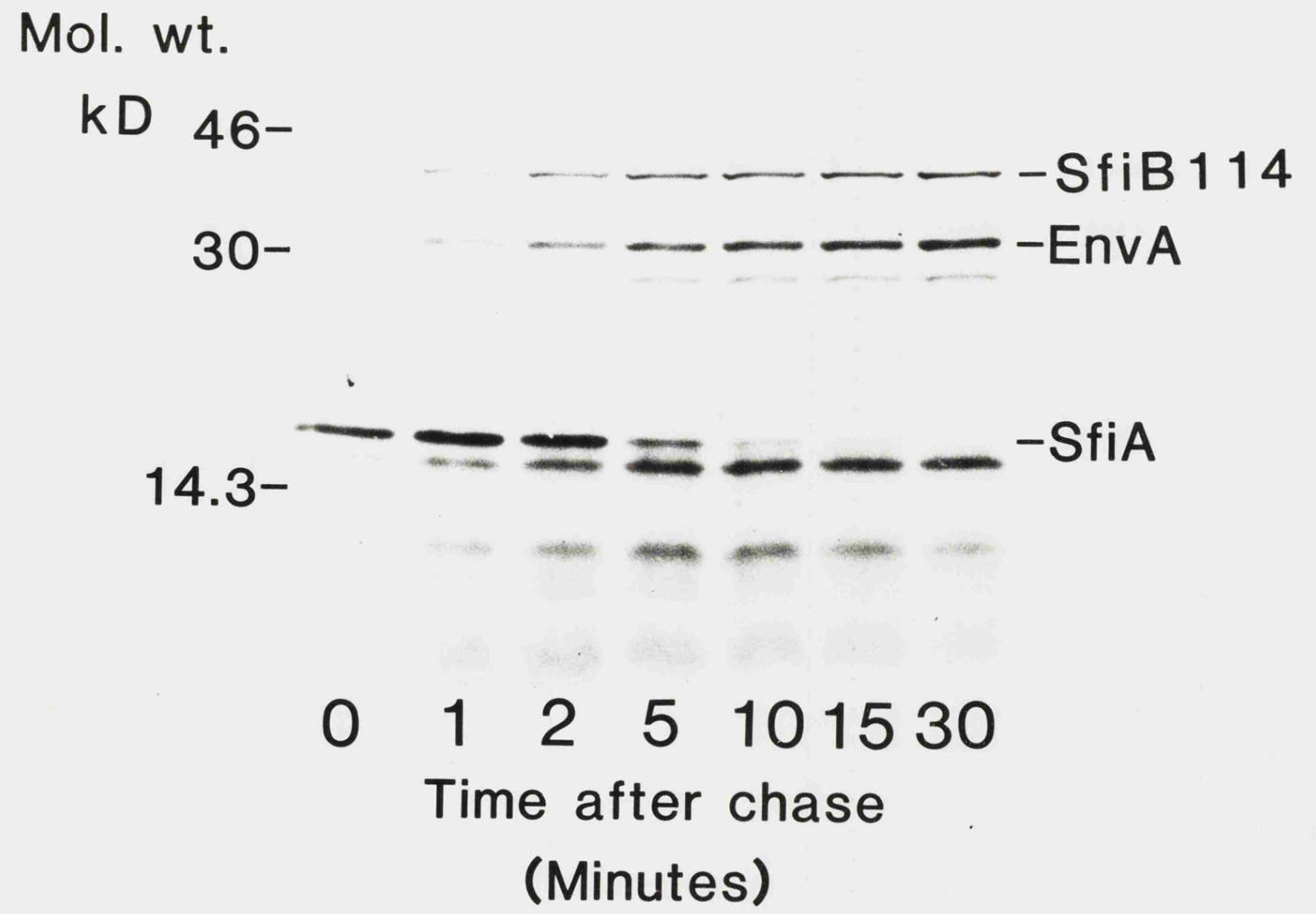

Mol. wt.
kD
46-
30-
14.3-
-SfiB114
-EnvA
-SfiA
0 1 2 5 10 15 30
Time after chase
(Minutes)

Figure 9.6

An autoradiogram of maxi-cells containing pLG554 + pLG558, pulse labelled with ^{35}S-methionine and analysed using an 15% polyacrylamide gel.

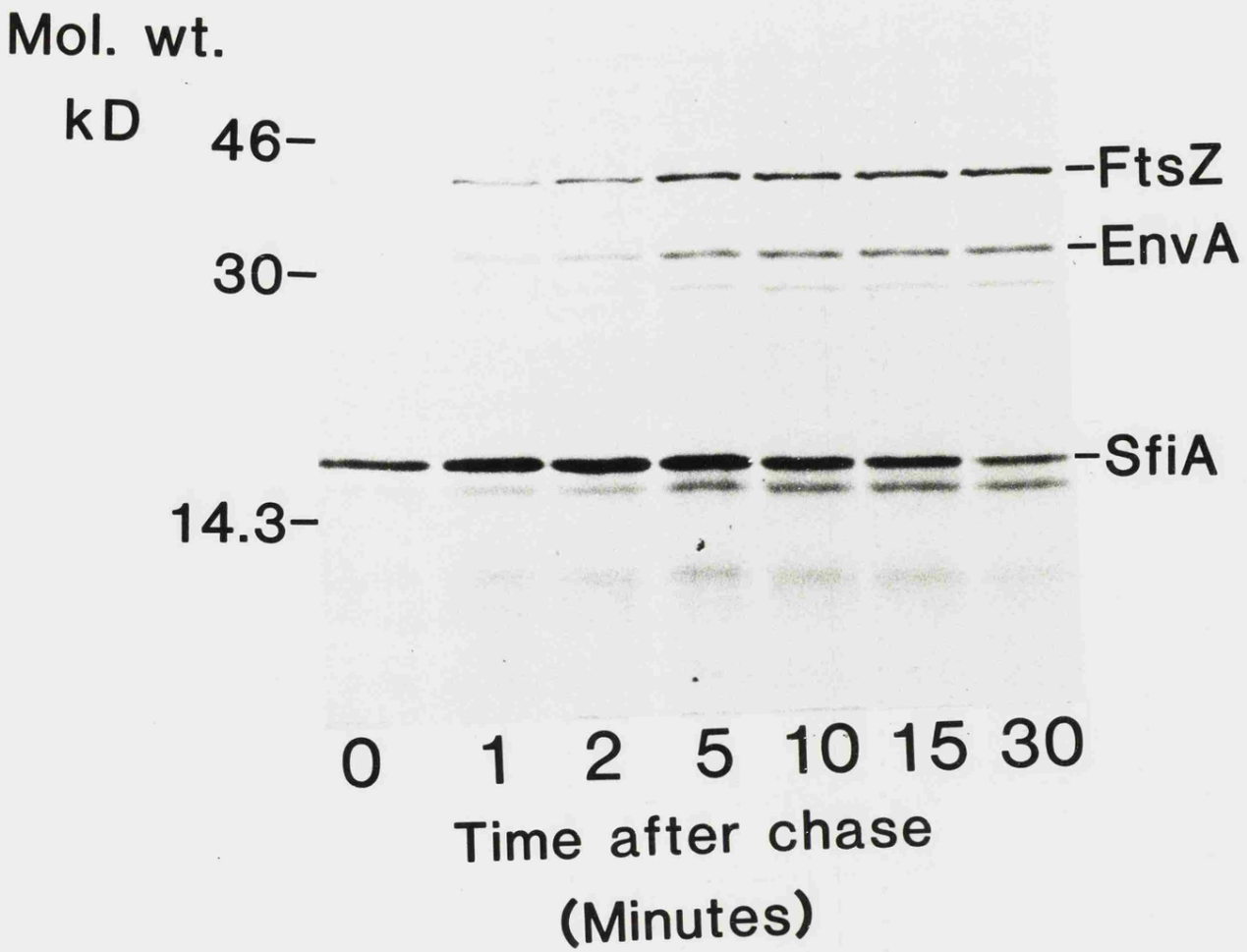
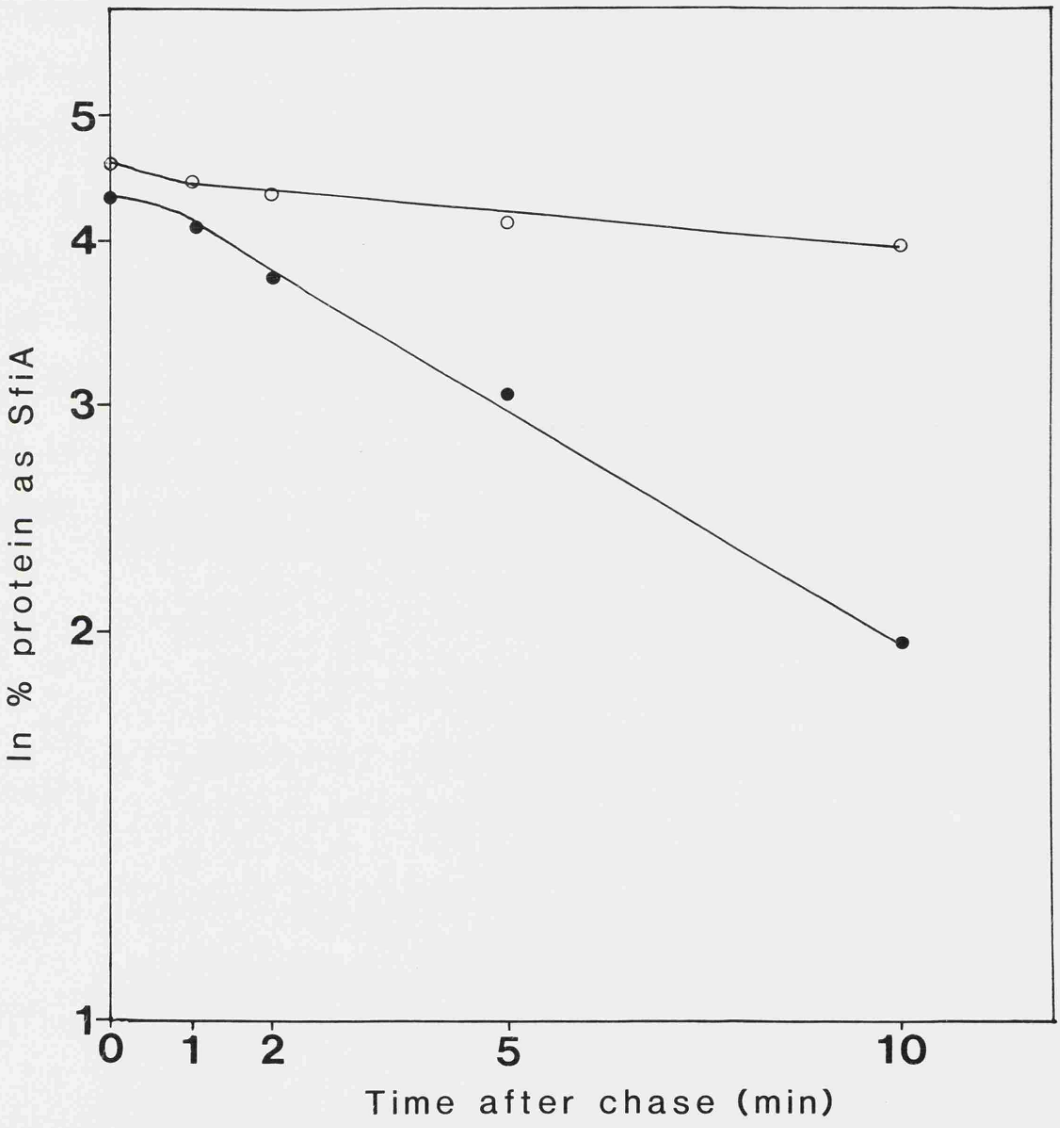

Mol. wt.
kD
46-
30-
14.3-
FtsZ
EnvA
SfiA
0 1 2 5 10 15 30
Time after chase
(Minutes)

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 2/ gradient. Similar calculations were done on Fig. 9.4 (pLG558 alone) (data not shown).


5
4
3
2
1
ln % protein as SfiA
0
1
2
5
10
Time after chase (min)

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^{o} 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^{o}) (a gift from J.Pratt and M.Jackson) and ^{35}S-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.

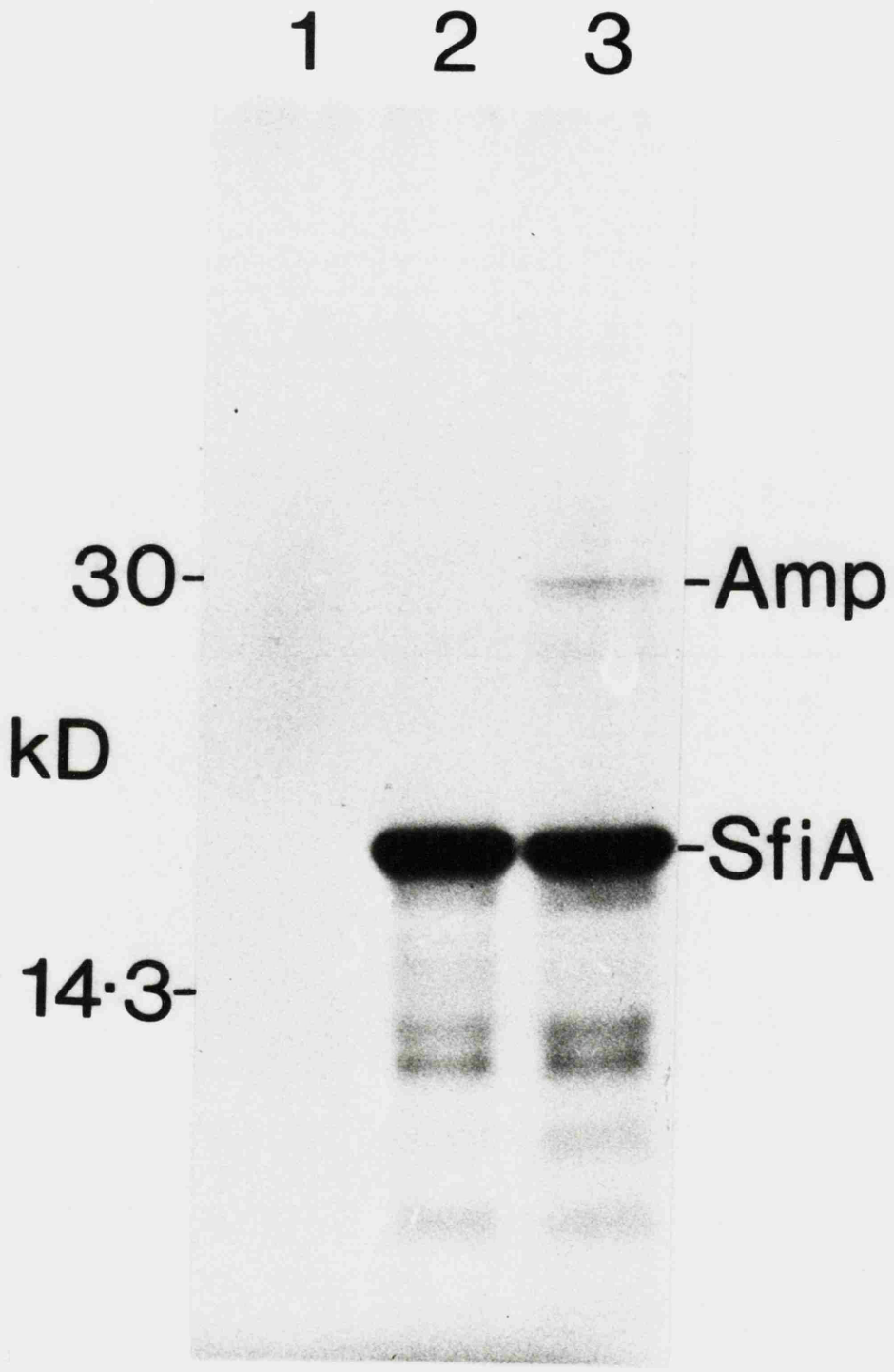
Figure 9.8

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

1 - No added DNA

2 - Purified 1.78 kb BamH1 fragment from pLG558

3 - Intact plasmid pLG558


1 2 3
30-
-Amp
kD
-SfiA
14·3-

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).

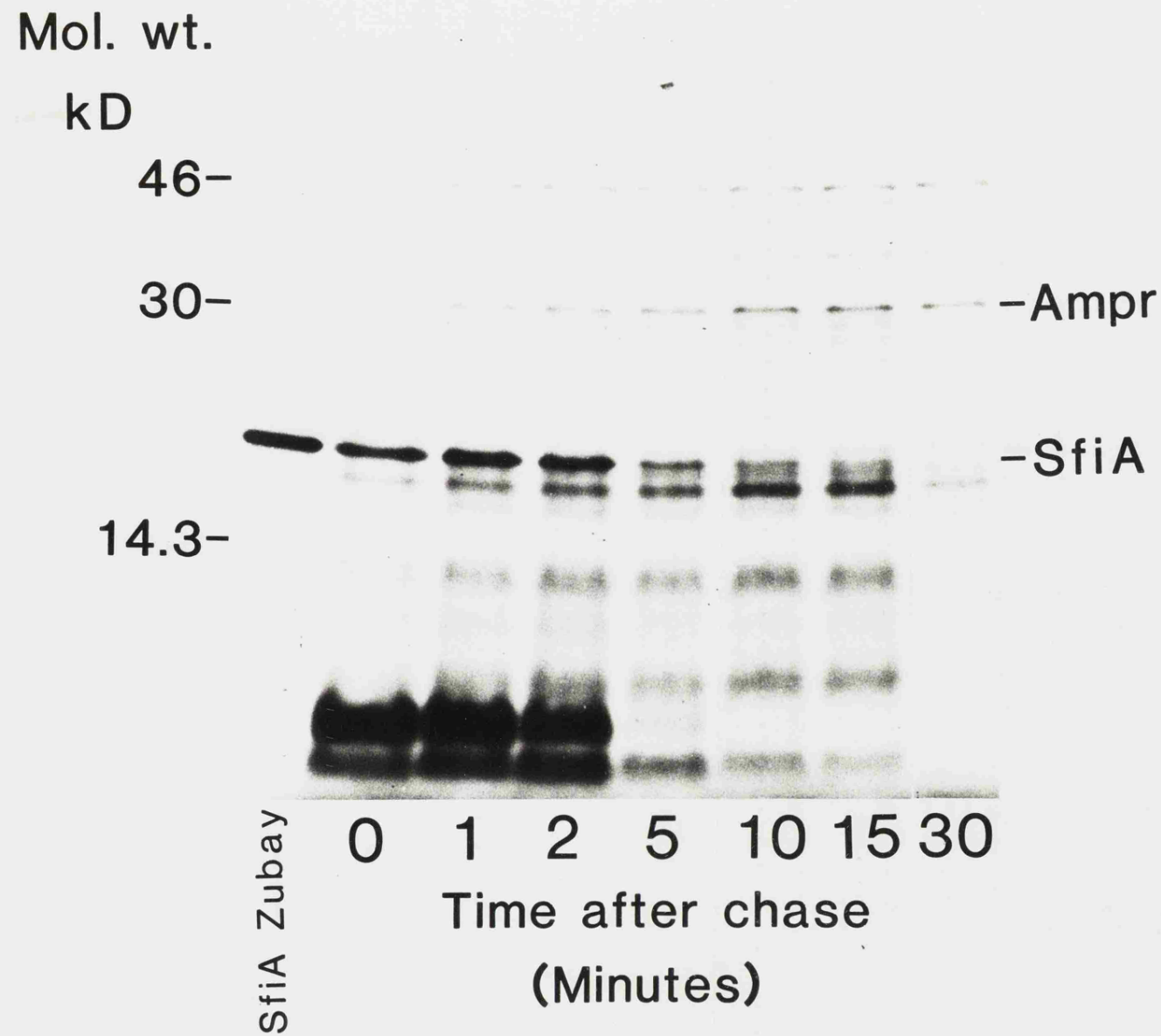
9.7 Discussion

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 BamH1 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.


Mol. wt.
kD
46-
30-
14.3-
-Ampr
-SfiA
0 1 2 5 10 15 30
Time after chase
(Minutes)
SfiA Zubay

allele of 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 ftsZ(sfiB). This result provides evidence for an interaction between SfiA and $SfiB^+$ 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, $SfiB^+$ 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 degredation of SfiA. However, it is difficult to explain the suppression of SOS mediated division inhibition by sfiB mutations 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 $SfiB^+$ and SfiB114 in the presence of SfiA and indeed the difference in the rates of synthesis of $SfiB^+$ and SfiB114 is intriguing. Although the difference in the rates of synthesis between $SfiB^+$ and SfiB114 may imply that the location of the sfiB114 mutation is within the ftsZ promoter region rather than the coding sequence this is not necessarily the case. Luktenhaus (1983) examined the gene products of two sulB alleles, sulB9 and sulB25, and found that both proteins were altered in size compared to the wild type FtsZ ($SfiB^+$) protein showing that both mutations are located within the coding region.

W.Donachie has claimed (personal communication) that ftsZ is repressed by its own gene product. The sfiB114 mutation , if present within the ftsZ coding sequence, might therefore alter the FtsZ protein reducing its efficiency to repress its own synthesis resulting in a higher level of sfiB114 expression compared to $sfiB^+$. Similarly the observed increase in the levels of synthesis of $SfiB^+$ and SfiB114 in maxi-cells in the presence of SfiA may result from a reduced affinity of SfiB, in association with SfiA, for its own promoter region. This effect would seem to add more indirect evidence for the postulated 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 envA, secA and $sfiB^+$/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 SfiA. Such a primary breakdown product was not observed by Mizusawa and Gottesman (1983) in -infection experiments used to measure the half-life of the SfiA protein. This was possibly due to the relatively higher background seen using the λ-infection gene expression system (compared to maxi-cells) obscuring such a protein band. Alternatively, this breakdown product may be more rapidly degraded in λ-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 sub-cellular 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 CHS26ΔF6 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 membane 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

Figure 10.1

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+}

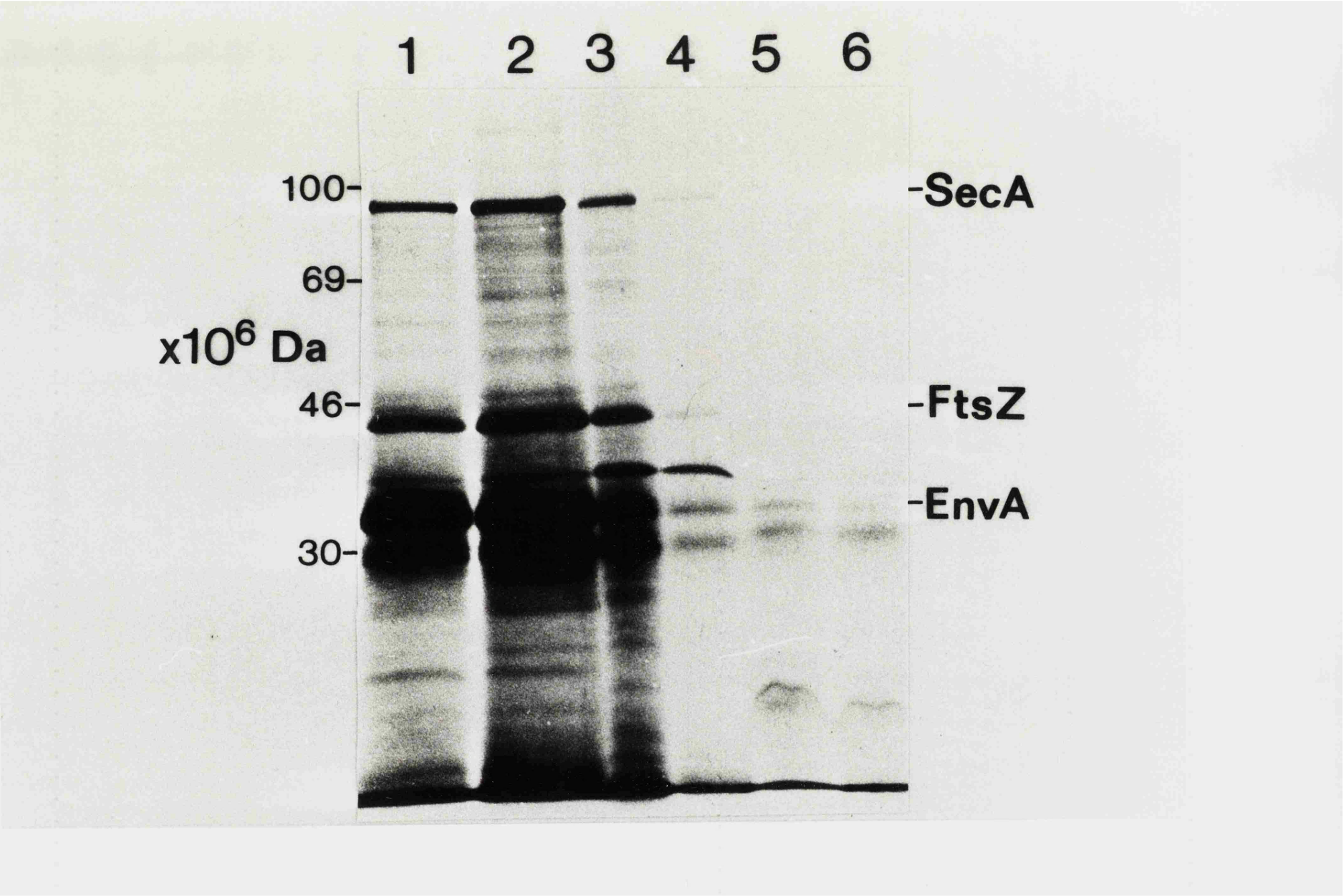
2 - Cytoplasm - Mg^{2+}

3 - Inner membranes + Mg^{2+}

4 - Inner membranes - Mg^{2+}

5 - Outer membranes + Mg^{2+}

6 - Outer membranes - Mg^{2+}


1
2
3
4
5
6
100-
69-
x10^6 Da
46-
30-
-SecA
-FtsZ
-EnvA

fractionated pLG558 containing maxi-cells. However, maxi-cells containing both pLG558 and pLG554 were sucessfully 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 Mg^{2+} and so the affinity of the SfiA protein for the inner membrane was severely reduced. Secondly, the SfiA protein has been reported to form agregates 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 agregates 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+}

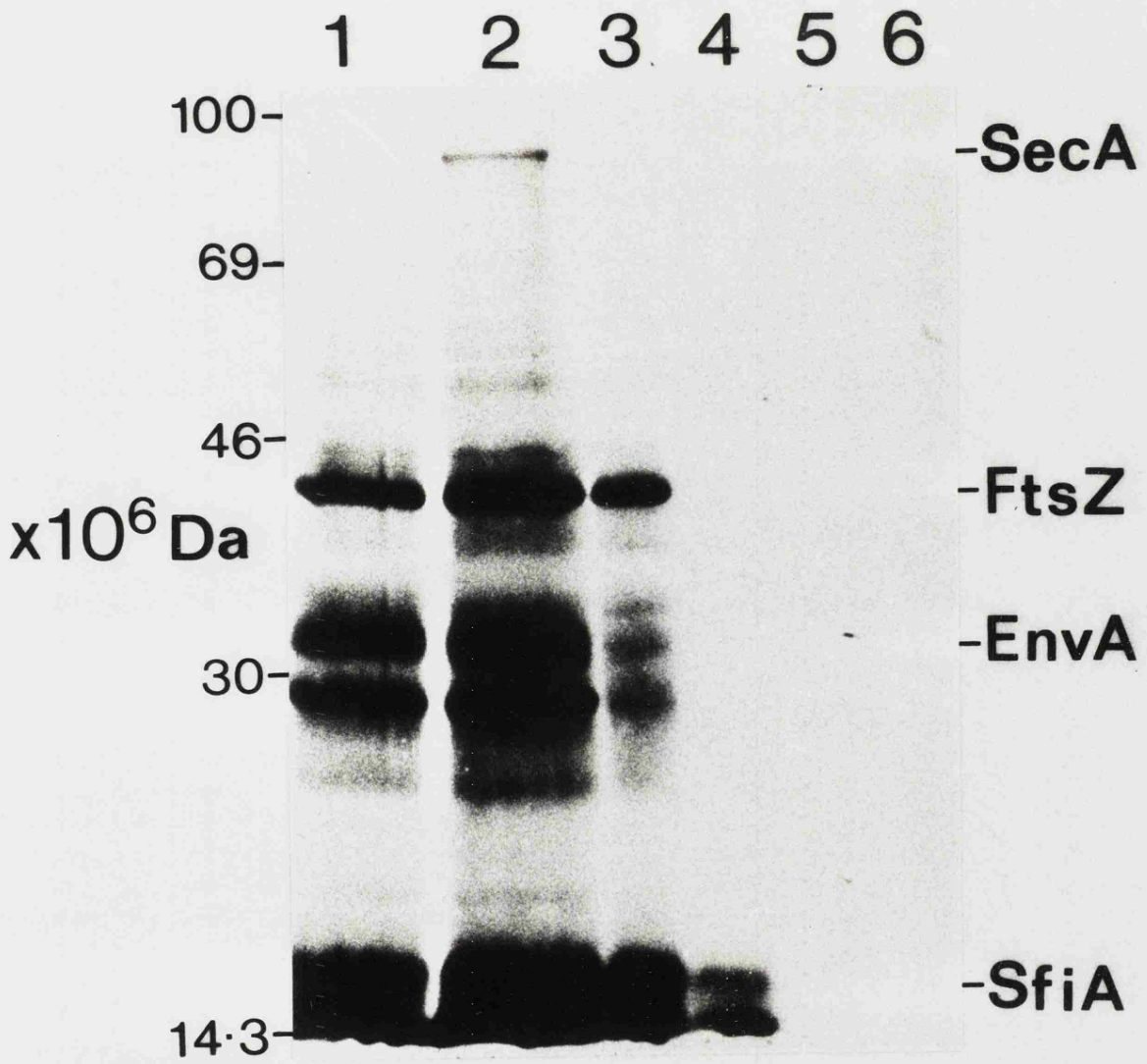
2 - Cytoplasm - Mg^{2+}

3 - Inner membranes + Mg^{2+}

4 - Inner membranes - Mg^{2+}

5 - Outer membranes + Mg^{2+}

6 - Outer membranes - Mg^{2+}


1 2 3 4 5 6
100-
69-
46-
30-
14·3-
x10⁶ Da
-SecA
-FtsZ
-EnvA
-SfiA

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.

CHAPTER 11

Discussion

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 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 ftsZ$^+$ copy number or a chromosomal 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 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 FtsZZ may cause competition between FtsZ and inactive 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 sfiB as well as the intriguing diploid experiments presented in Chapter 8. For example, in Chapter 8 it was shown that although a chromosomal sfiB114 mutation could suppress the IPTG sensitive filamentation caused by pLG558, sfiB114 present on plasmid pLG552 in a sfiB$^+$ host strain could not. In this case either the inclusion of FtsZ$^+$ into a FtsZ$^+$/SfiB114 mixed multimer or competition between FtsZ$^+$ and SfiB114 for septation sites could create a situation where SfiA (via FtsZ$^+$) could inhibit cell division through FtsZ$^+$ but not when the SfiA insensitive protein 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 ftsZ (in a strain containing a temperature sensitive suppressor) as has been accomplished with ftsA (Luktenhaus and Donachie, 1979).

Further indirect evidence for a central role of ftsZ in cell division control has come from the fact that despite intensive investigation, only one allele of ftsZ has been isolated (ftsZ84) compared to many for the adjascent ftsA and 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 lon mutations (e.g. filamentation following UV-irradiation, poor lysogenisation and mucoidy). Moreover, this mutation was suppressed by multiple copies of ftsZ and was mapped to the 2 minute region of the E.coli chromosome. Belhumeur and Drapeau (1984) interpreted this as showing the isolation of an allele of ftsZ ("ftsZ71") which yielded a protein more sensitive to the action of SfiA. However, ftsZ71 has not been shown to specifically map at the locus associated with 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 machinary 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, PZ_1 immediately preceeding the ftsZ coding region and PZ_2 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 PZ_1 and PZ_2 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 PZ_2 is affected by mutations in ftsA, ftsI or ftsZ but not by the unlinked ftsE gene. Donachie (1984) has also measured the transcription from PZ_2 at different growth rates and found that the rate of

Table 11.1: The effect of chromosomal mutations on expression from PZ_2. 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).

Mutation	Ratio (42°C/30°C)
ftsE35(ts)	1.0
ftsA13(ts)	1.0
ftsA16(am)*	5.0
ftsQ1(ts)	3.0
ftsZ84(ts)	1.7
ftsIkb(ts)	2.8
ftsIbs(ts)	3.1
envA	0.7
envK27	1.1

* 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 PZ_2 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.

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Inactivation of essential division genes, *ftsA*, *ftsZ*, suppresses mutations at *sfiB*, a locus mediating division inhibition during the SOS response in *E. coli*

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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 *sfiB*114 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 *sfiB*114 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 *sfiB*114 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 *Bam*HI-*Eco*RI DNA fragment was subcloned which carried *ftsA* and coded for a 47-K polypeptide but did not carry *sfiB*114 and did not complement *ftsZ*. We conclude that *sfiB*114 is located within *ftsZ* coding for a 42-K polypeptide. Nevertheless, insertions into *ftsA* coding the 47-K polypeptide suppress the *sfiB*114 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 *ftsA*, indicating the presence of a regulatory region essential for maximal expression of *ftsZ*.

Key words: SOS/u.v./division inhibition/*sfiB*/*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 RADI 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, *(tsl)* 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, *(tif)*, 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 filament formation in both *tsl* and *tif* 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 *sfiB*114 is located within *ftsZ* and that Tn1000 insertions in *ftsA* suppress the effects of *sfiB*114 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 *sfi*B

Donor	Recipient	Co-transduction frequency
*leu*B$^+$, *pbp*3$^-$	*leu*B$^-$, *pbp*3$^+$	88%
*leu*B$^+$, *fts*A$^+$	*leu*B$^-$, *fts*A$^-$	59%
*leu*A$^+$, *sfi*B$^+$	*leu*A$^-$, sfiB$^-$114	41%
*leu*A$^+$, *env*A$^-$	*leu*A$^-$, *env*A$^+$	41%
*leu*B$^+$, *azi*$^+$	*leu*B$^-$, *azi*$^-$	25%

The *leu*$^+$ marker was selected in each cross and the recipients scored for the unselected marker.

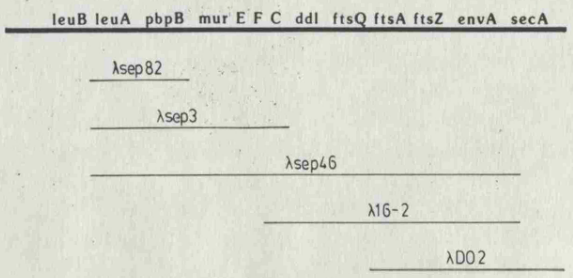

Fig. 1. A schematic diagram showing the organisation of known cell wall and division genes in the two minute region of the *E. coli* chromosome and the portions covered by the λ-transducing phages described in the text.

out. However, using *leu*$^+$ as a primary selection it was possible by several two factor crosses to localise *sfi*B$^+$ between *fts*A and *azi* (Table I). In a three point cross involving *leu*A, *sfi*B and *env*A, *sfi*B could not be separated from *env*A (see Figure 1). For the reasons outlined above, it was not possible to carry out three factor crosses involving *pbp*B or *fts*A, *sfi*B and *leu*A. Alternative methods involving λ transducing phages were then employed in an attempt to position *sfi*B more precisely.

Mapping by λ transducing phages

The two minute region is well covered by specialised λ transducing phages (Fletcher *et al.*, 1978; Lutkenhaus *et al.*, 1980) and some of these were tested for the presence of *sfi*B$^+$ in lysogens of a host strain carrying *sfi*B114. This *sfi*B allele was previously reported to be recessive in partial diploids (Huisman *et al.*, 1980c). Phages of interest were used to lysogenise strain GC2490 (*tsl, sfi*B114) by selection for *leu*$^+$, and the lysogens tested for the restoration of temperature-sensitive filamentation. Three of these phages, λsep82, λsep3 and λsep46 include *E. coli* DNA and *leu*A to *pbp*B, *mur*C and *env*A respectively (see Figure 1).

All three phages failed to complement *sfi*B114. However, *sfi*B$^+$ was recovered by marker rescue from λsep46-infected cells after selection for *leu*A$^+$, (see Materials and methods) indicating that at least part of the *sfi*B$^+$ gene is present in this phage. Lysogens derived from λ16-2 (carrying *mur*C to *env*A) also failed to show complementation of *sfi*B and although this phage, by comparison with λsep46 should contain *sfi*B$^+$, marker rescue was not observed. This may be simply explained by the very low lysogenisation frequency of λsep46 and consequently the greater probability of detecting marker rescue.

Since λDO2, a phage carrying *fts*A, *env*A and *sec*A (see Figure 1) also failed to complement *sfi*B114 (R.D'Ari, personal communication), despite the inferred presence of *sfi*B$^+$, we concluded that *sfi*B114 must be a dominant mutation. Consequently we decided to clone directly the *sfi*B114 region from total *E. coli* chromosomal DNA.

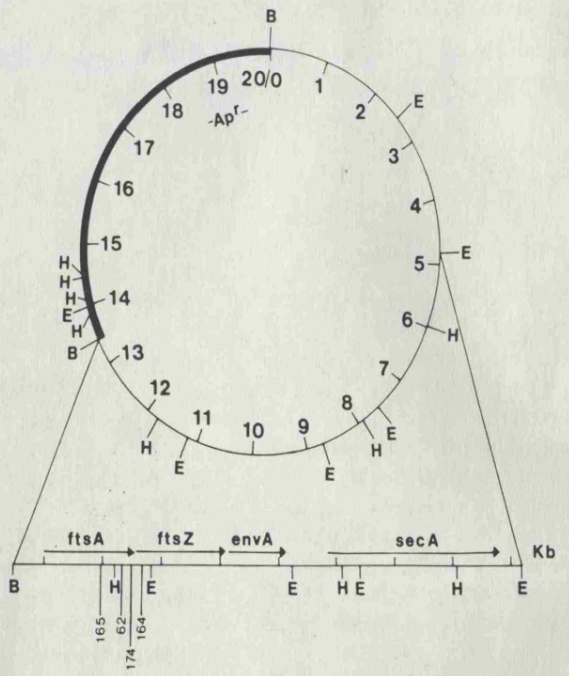

Fig. 2. Restriction map of pLG550. The bold portion of the map indicates the vector pOU71 region. The positions and directions of transcription of the known genes are presented in the expanded section showing the organisation of the *E. coli* DNA insert. The direction of transcription of *env*A is deduced from our unpublished data (I.B.Holland and V.Darby). Numbered arrows show the positions of the *sfi*B114::Tn1000 insertions discussed in the text. Abbreviations used are B, *Bam*HI, E, *Eco*RI and H, *Hind*III.

Cloning sfiB114

Chromosomal DNA from an *sfi*B$^+$ strain (MC4100) and an *sfi*B114 strain (GC2490) was prepared, cut with *Bam*HI and ligated into the *Bam*HI 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°C or by using a λ$^+$ lysogen as a host at 42°C.

Re-ligated banks of *Bam*HI fragments in pOU71 were used initially to transform TKF12 λ$^+$ (*fts*A) to temperature resistance, thus selecting for recombinants carrying *fts*A$^+$ fragments. These were obtained from both *sfi*B$^+$ (pLG551) and *sfi*B114 (pLG550) gene banks and were found to carry inserts of ~13.5 kb extending from *fts*A to ~5 kb beyond *sec*A 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 *fts*A mutation and partial complementation of the *fts*Z mutation (see Discussion).

To test the presence of *sfi*B alleles, a *tsl, sfi*B$^+$, λ$^+$ strain was transformed with both pLG550 and pLG551 and transformants were shifted to 42°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).

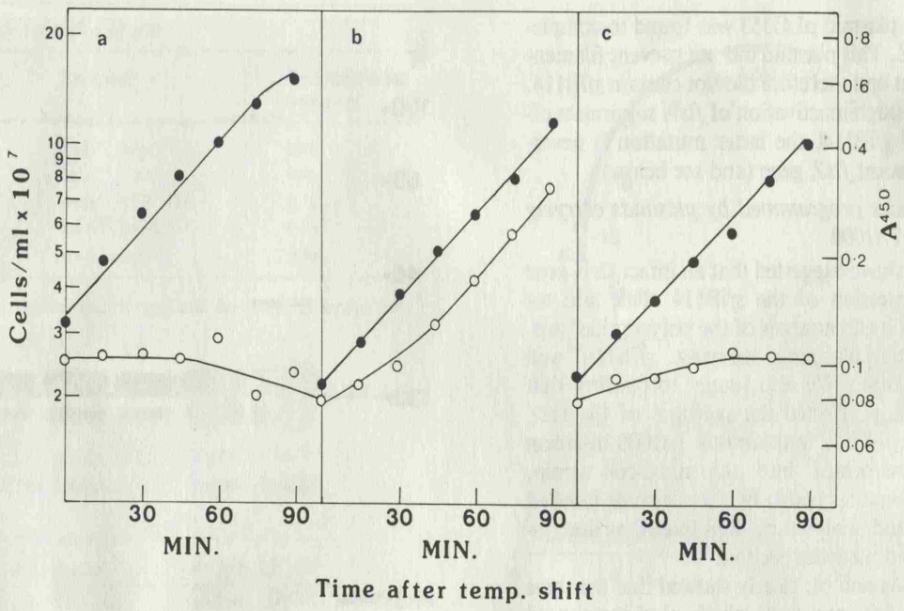

Fig. 3. Inhibition of cell division at 42°C in various *tsl* strains. Exponentially growing cultures of GC2490/1 λ⁺ *(tsl)* cells bearing plasmids **(a)** pOU71, **(b)** pLG550 and **(c)** pLG551 were shifted to 42°C at time 0, cell mass (A_{450} ●——●) and cell number (○——○) were monitored at intervals.

Table II. MMS-sensitivity of *sfi*B⁺ and *sfi*B114 strains

Strain	Surviving fraction on MMS plates
PAM162/1 (*lon*⁻, *sfi*B26)	5.8×10^{-1}
PAM660 (*lon*⁻, *sfi*B⁺)	3×10^{-5}
PAM660 (pOU71)	5×10^{-5}
PAM660 (pLG550)	2.6×10^{-2}
PAM660 (pLG551)	$<1.7 \times 10^{-6}$

Strains carrying *sfi*B⁺ or *sfi*B114 recombinant plasmids and a *lon* chromosomal mutation were plated on NA plates including 250 μl/LMMS 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 *sfi*B114 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 *sfi*B114 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 *sfi*B114 more precisely, we sought to use Tn1000 insertional mutagenesis. For greater convenience in the isolation of these insertions the *Bam*HI fragment carrying *sfi*B114 was isolated from pLG550 *in vitro* and religated into the vector pLG339 to form pLG552 (see Materials and methods). Vector pLG339 (Stoker *et al.*, 1982) is present in ~8 copies at all temperatures, and carries the more convenient Kan^R marker for selection purposes in conjugal crosses. pLG552 displayed very similar complementation properties for *fts*A and *fts*Z to those observed with pLG550. We have observed that all strains carrying *sfi*B⁺ or *sfi*B⁻ plasmids grow more slowly than their parental hosts. Despite the enhanced copy number of pLG339, however, we have not observed any additional deleterious effects on growth. Plasmid pLG552 was then transformed into the F⁺-donor strain, RB308. This strain was mated with GC2490/1 carrying chromosomal markers, *tsl*⁻, *sfi*B⁺ and Tn1000 insertions selected by plating out for Kan^R recipients (see Materials and methods). Using this selection protocol we anticipated that GC2490/1 heterozygotes *sfi*B114/*sfi*B⁺, should not filament at 42°C due to the dominant *sfi*B114 allele, whilst Tn1000 insertions into the *sfi*B114 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 *sfi*B114, three inactivated the *fts*A gene in functional tests and were located on the physical map at a position exactly corresponding to that assigned to *fts*A by Lutkenhaus and Wu (1980). A further insertion (164) did not inactivate *fts*A (Figure 2). Unfortunately, the precise limits of *fts*Z on the physical map were not previously established (Lutkenhaus and Wu, 1980). However, this insertion did abolish *fts*Z in complementation tests, indicating that this insertion does lie within *fts*Z. Other insertions into pLG552 did not suppress *sfi*B114 and mapped well outside the *fts*A, 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 *fts*A and possibly *fts*Z suppressed the *sfi*B114 phenotype. To resolve these possibilities, an attempt was made to clone from a plasmid carrying *sfi*B114, the chromosomal region coding for *fts*A independently of *fts*Z. Inspection of Figures 1 and 2 (see also Lutkenhaus and Wu, 1980) indicates that *fts*A resides on an *Eco*RI-*Bam*HI fragment which also carries only the proximal portion of *fts*Z. Accordingly, a 2.1-kb *Eco*RI-*Bam*HI 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 resulting plasmid pLG553 was found to complement *fts*A but not *fts*Z. This plasmid did not prevent filamentation in a *tsl*$^-$ mutant and therefore did not contain *sfi*B114. We conclude that although inactivation of *fts*A suppresses efficiently the effect of *sfi*B114, the latter mutation is nevertheless within the adjacent *fts*Z gene (and see below).

Analysis of polypeptides programmed by plasmids carrying sfiB114 and sfiB114::Tn1000

The results described above suggested that an intact *fts*A gene was required for expression of the *sfi*B114 allele and we sought to confirm this by an analysis of the polypeptides programmed by various plasmids carrying *sfi*B114 and *sfi*B114::Tn1000 insertions. We also sought to confirm that insertion 164 specifically affected the synthesis of the FtsZ protein. The plasmid pLG552 and various Tn1000 insertion derivatives were transformed into the maxi-cell strain, CSH26ΔF6 and polypeptides coded by the plasmids labelled with [^{35}S]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 *fts*A which also inactivated *sfi*B114 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 *fts*Z 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, pLG552 also coded for a 92-K polypeptide, apparently identical to the SecA polypeptide (Oliver and Beckwith, 1982) since inactivation by Tn1000 of this gene leads to truncation of this polypeptide (data not shown).

These results provided clear evidence that an intact *fts*A was required for maximal expression of *fts*Z and explained the suppression of *sfi*B114 by insertions into *fts*A. The *fts*A 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 pLG552. More significantly, Tn1000 insertions (62, 165, 174) which inactivated the *fts*A function also resulted in the disappearance of this protein as well as loss or reduced expression of *fts*Z. 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 *fts*A function and failed to affect the synthesis of the 47-K polypeptide confirming that this is the *fts*A product. Insertion 164 on the other hand completely blocked the synthesis of the 42-K, FtsZ protein and inactivated the *fts*Z function and this confirmed our conclusion that this insertion lies within the *fts*Z gene.

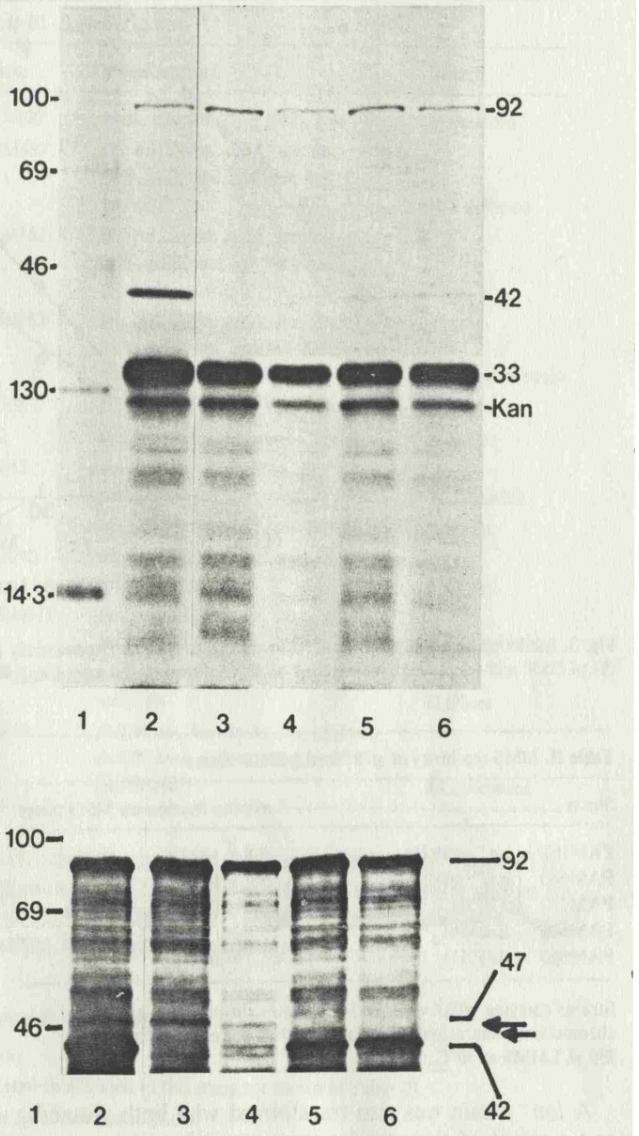

Fig. 4. (a) Synthesis of polypeptides coded for by pLG552 and pLG552 carrying Tn1000 insertions in *sfi*B114 in maxi-cells. Maxi-cells were labelled with [^{35}S]methionine as described in Materials and methods and the products analysed on an 11% polyacrylamide gel, followed by fluorography. Tracks represent: 1, ^{14}C standards; 2, pLG552; 3–6, pLG552::Tn1000 insertions 164, 174, 62 and 165 respectively. Polypeptides corresponding to SecA (92 K) FtsA, (47 K) FtsZ (42 K) and EnvA (33 K) are indicated. **(b)** A section of the gel shown in **(a)** after a longer exposure. Arrows indicate the presumed truncated forms of FtsA due to insertions 174 **(track 4)** and 62 **(track 5)**. Figure also indicates that the more distal the insertion into *fts*A the greater the reduction in FtsZ synthesis, compare the 42-K band in **tracks 4, 5** and **6**.

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 *fts*A and *fts*Z (see also Lutkenhaus *et al.*, 1980 for definition of the *fts*A and *fts*Z loci) are required for division in the last few minutes of the cell cycle. The current findings that a dominant mutation at the *sfi*B locus was suppressed in partial diploids by inactivation of either *fts*A or *fts*Z was therefore quite consistent with the hypothesis that either of the products of these genes is the target for the SOS (*sfi*A) inhibitor.

The *fts*A gene product was previously demonstrated to be an ~50-K polypeptide coded by a 2.1-kb *Bam*HI-*Eco*RI 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 FtsA consistently ran at a position corresponding to 47 K. The *Bam*HI-*Eco*RI fragment did not, however, carry the *sfi*B114 allele and failed completely to complement *fts*Z. Lutkenhaus and Wu (1980) also presented evidence that the region clockwise to *fts*A on the *E. coli* map coded for the *fts*Z locus. We

have shown in this study that this region codes for a 42-K polypeptide. Analysis of proteins synthesised by plasmids carrying *sfi*B114::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 *fts*A. 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 *sfi*B114 if it is located in *fts*Z. Lutkenhaus and Wu (1980) have also observed that a region to the left of *fts*Z was important for maximal expression of this gene consistent with our findings.

Concerning the mechanism of reduced synthesis of *fts*Z by insertions into *fts*A, 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 *fts*Z is transcribed in the same clockwise direction as *fts*A but from an independent promoter. Moreover, the 3.5-kb *Hind*III fragment (see Figure 3), which covers only the distal region of *fts*A and the whole of *fts*Z, has been isolated and was found to programme the synthesis of *fts*Z *in vitro* (our unpublished data) with high efficiency. On the other hand, the data of Lutkenhaus and Wu (1980) appeared to show that the *fts*Z promoter alone was insufficient *in vivo* to facilitate normal expression of *fts*Z. Consequently we cannot rule out the possibility that *in vivo* expression of *fts*Z is augmented by transcription from the *fts*A promoter. In consequence the suppression of *fts*Z and *sfi*B114 observed in this study by insertions into *fts*A could simply be due to transcriptional polarity. However, it is difficult to conceive of any mechanism involving transcription emanating from the *fts*A 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 increasingly distal insertions of Tn1000 into *fts*A separate the *fts*Z promoter from an auxillary control region located in the *distal* region of *fts*A, necessary for high level expression of *fts*Z. Since these effects on *fts*Z expression were observed in host cells carrying a wild-type *fts*A$^+$ allele on the chromosome, any positive regulating effects of the FtsA protein itself on the *fts*Z gene can be ruled out.

In marked contrast to insertions 62, 165 and 174, insertion 164, whilst inactivating *sfi*B114, had no effect on *fts*A function and the 47-K protein was still synthesised. Moreover, we were able to show that the *fts*Z function was also inactivated and the synthesis of a 42-K polypeptide was blocked by this insertion. This indicated that insertion 164 was within *fts*Z. This together with the demonstration that the 2.1-kb *Bam*HI-*Eco*RI fragment carries *fts*A but not *sfi*B114 leads us to conclude that *sfi*B114 lies within *fts*Z. Moreover, whilst this manuscript was in preparation, Lutkenhaus (1983) reported that another *sfi*B (*sul*B25) mutation was, in fact, an *fts*Z allele.

Finally, some comment is required on the previously reported recessivity of *sfi*B114 and other *sfi*B alleles. In the case of *sfi*B114, the presence of a recessive allele in partial diploids appeared to be demonstrated unequivocally by subsequent P1 transduction analysis (Huisman *et al.*, 1980c).

Table III. Bacterial strains

Strains	Strain markers	Source
MC4100	*araD*, Δ*lac*IPOZYA (169), *rps*L, *thi*	P.Bassford
PAM660	*thi, thr, leu*B6, *pro*A, *his, lon, lac*Y, *gal*K, *ara, xyl, mtl, rps*L, *tsx, sup*E	B.R.Johnson
PAM162	*thi, thr, leu*B6, *pro*A, *his, lon, lac*Y, *gal*K, *ara, xyl, mtl, rps*L, *tsx, sup*E, *sfi*B26	
PAM162/1	*thi, thr, leu*B6, *pro*A, *his, lon, lac*Y, *gal*K, *ara, xyl, mtl, rps*L *tsx, sup*E, *azi, sfi*B26	Laboratory strain
RB308	F$^+$, *deo*C, *thy*A, *lac*Y, *rec*A	R.Buxton
D22	*trp, pro, his, rps*L, *env*A	A.Salem
TKF12	*thr, leu, thi, pyr*F, *thy*A, *ilv, fts*A12, *his, arg, lac, ton*A	W.Donachie
SP63	*trp*,(am), *tyr*(am), *ilv, pbp*B63	B.Spratt
GC2490	*thr, pro, his, lac, gal, leu*A, *tsl rps*L, *sfi*B114	R.D'Ari
GC2490/1	*thr, pro, his, lac, gal, leu*A, *tsl, leu*$^+$, *sfi*B$^+$	R.D'Ari
CSH26ΔF6	*ara*, Δ*(lac, pro)*, Δ*(rec*A, *srl)*F6 *rps*L, *thi*	D.Oliver
JFL100	*fts*Z84, *ilv, his, thy*A, *deo, ara*(Am), *lac*125(Am), *gal*U42 (Am), *tyr*T, (*sup*Fts*A*81)	J.Lutkenhaus

Nevertheless, both in this study and that recently reported by Lutkenhaus (1983), *sfi*B (*sul*B) alleles were clearly shown to be dominant. Dominant alleles of *sfi*B 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 sulphonate (MMS) was used to distinguish sensitive strains *(lon)* from wild-type or *lon* strains also carrying *sfi*B. MMS (250 μl/l) was incorporated into oxoid nutrient agar (NA) and strains patch-tested. Strains mutant at *env*A 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 (A_{450} = 0.1) Luria broth 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 *fts*Z mutant, where survival at 42°C was $<10^{-7}$, 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 ZB1 with a 30 μm orifice.

Phage transduction

P1 transducing lysates were prepared as described by Buxton and Holland (1973) and transductions carried out as described by Willetts *et al.* (1969). Where necessary, recipients were grown in nutrient broth for 2 h to allow expression of specific markers before plating onto selective media. λ 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

(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

RB308 (F^+) carrying pLG552 and the recipient strain GC2490/1 (*tsl, sfi*$^+$) were grown in nutrient broth to A_{450}, 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 transconjugants, containing pLG552 (Kan^R) mobilised for transfer by insertion of Tn1000, were screened for the inactivation of *sfi*B114 present in pLG552. This was achieved by patching out individual colonies at 42°C; *tsl*$^-$ *sfi*$^+$/*sfi*B114 heterozygotes grew at 42°C whereas, *tsl*$^-$ *sfi*$^+$/*sfi*B114::Tn1000 heterozygotes failed to grow.

Tn1000 insertions were mapped from analysis of the *Bam*HI, *Eco*RI and *Hind*III 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 *Bam*HI fragments since Tn1000 contains an asymmetric *Bam*HI site.

Synthesis of plasmid coded polypeptides in maxi-cells

Plasmids of interest were transferred into the maxi-cell strain, CSH26ΔF6 and labelled with [^{35}S]methionine as described by Stoker *et al.* (1983). The labelled cells were lysed in SDS and analysed by SDS-PAGE followed by autoradiography (Boyd and Holland, 1979).

Construction of pLG552 and pLG553

pLG550 was restricted with *Bam*HI and fragments ligated into the *Bam*HI site of previously restricted pLG339. Recombinants having the chromosomal fragment from pLG550 cloned into pLG339 were isolated by transforming TKF12 (*fts*A$^-$) with the ligation mixture and screening for kanamycin-resistant, temperature-resistant recipients. Cloning into the *Bam*HI site of pLG339 was confirmed by the inactivation of the plasmid encoded tetracycline resistance gene. Plasmid pLG553 was constructed similarly from a *Bam*HI-*Eco*RI double restriction digest of pLG550 prior to ligation into pLG339 cut with *Eco*RI and *Bam*HI.

Curing

PAM660/1 was cured of pLG550 by growing overnight in nutrient broth in the absence of ampicillin and then plated on NA plates; pOU71 and its derivatives are poorly maintained without selection and so >90% of the resulting colonies were amp^S and lacked the plasmid.

Marker rescue

Lysates of the defective λ transducing phages λsep82, λsep3 and λsep46 were used to transduce GC2490$^+$ to *leu*$^+$ transductants were tested for the presence of the λ transducing phages by their ability subsequently to yield a *leu*$^+$ transducing lysate after u.v. irradiation. Those failing to produce such a lysate were presumed to have acquired *leu*$^+$ by marker rescue. In the case of λsep46, where lysogens are recovered only rarely, many of these recombinants were shown to have acquired *sfi*B$^+$, presumably by marker rescue. The presence of the *sfi*B$^+$ allele was detected by screening individual clones of the *tsl, sfi*B114 host for filamentation at 42°C by phase contrast microscopy.

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ABSTRACT

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

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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 P1 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.