

An Investigation of Cell Cycle Genes
in Escherichia coli

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A thesis presented for the degree of
Doctor of Philosophy

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October 1981



ABSTRACT

A new gene has been identified and designated ftsQ. This gene maps in the cluster of cell division genes at 2 minutes on the E. coli K12 genetic map, adjacent and anticlockwise to ftsA. It appears that, ^{as suggested for} ftsA, there ^{may be} a periodic requirement for the ftsQ gene product in the cell cycle. The cloning of ftsQ and ftsA on a 2.2kb Eco RI restriction fragment, and the construction of deletion derivations, maps ftsQ within a 1.0kb Eco RI - Pvu II fragment.

The ftsZ promoter also lies within the 2.2kb Eco RI fragment, and this has been identified by transcriptional fusion to the galK gene. There are other active promoters on this fragment, but these have not yet been precisely located.

Determination of the DNA sequence of the 2.2kb Eco RI ^{fragment} is not yet complete, but three 'islands' of sequence data have been obtained around the Bam HI, Bgl II and Hind III sites. The sequence around the Bam HI site may contain the initiation codon and ribosome binding site for the ftsQ gene, but only part of the RNA polymerase binding sequence. ftsQ gene product has not yet been identified.

Acknowledgements

I would like to thank Dr. Donachie for his supervision and Dr. Hayward for his advice.

The initial "TOE" isolation was performed by Dr. Ken Begg. The physiological analyses were performed jointly by Dr. Ken Begg and myself. I would like to especially thank Ken for all his help and specialist physiological knowledge without which the TOE selection would never have been.

I thank also Oli Andersson and Heather Houston for their help and ceaseless patience with the sequencing, Susan Wilkie and Vicky Derbyshire for their help in the lab., Moira for the typing, and Jo Rennie for the photography. Lastly I would like to thank Dr. George Salmond for his help in the lab, and his friendship and excellent "J.P.S. Heavy" beyond it.

Abbreviations

Ap	ampicillin
bp	base pair
Cm	chloramphenicol
dNTP	deoxyribonucleotide
ddNTP	dideoxyribonucleotide
ug	microgram
g	gram
Kb	kilobase
Kd	kilodalton
um	micrometres
min	minute
mRNA	messenger RNA
ml	millilitre
mM	millimolar
MW	molecular weight
nm	nanometres
OD	optical density
PBP	penicillin binding protein
RF	replicative form
Tc	tetracycline
TE	10mM Tris Hcl pH 7.4, 1mM EDTA
ts	temperature sensitive
XG	5-bromo-4-chloro-3-indolyl- β -D-galactoside
PEG 6000	Polyethylene glycol 6000

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CHAPTER 1Introduction

Bacterial cell division has been the subject of intensive study for many years. These studies have revealed some important aspects of cell division although little is known about this process at the molecular level. The recent technological advances in molecular biology and genetic engineering provide the opportunity to improve our knowledge of all cellular processes at the molecular level. E. coli has been characterised very well physiologically, biochemically and genetically, making it an ideal organism for studying fundamental processes such as cell division. In this chapter I will discuss some important aspects of cell division that are relevant to this project.

1.1 DNA replication and the timing of cell division

E. coli contains a circular genome of double stranded DNA, about 1200um in length (Cairns, 1963). DNA replication is initiated at a point 88 minutes on the genetic map (Bachmann and Brooks Low, 1980) and a pair of replication forks move away from each other until they meet at a terminus at 32 minutes (Masters and Broda, 1971; Bird, Louarn, Martuscelli and Caro, 1972; Fayet and Louarn, 1978; Louarn, Patte and Louarn, 1979). This takes about 40 minutes and is called the C period (Cooper and Helmstetter, 1968). This period is constant at different growth rates, except when DNA precursors or energy requirements are in limited supply, as in very slow growth rates (Cooper and Helmstetter, 1968). There is a second constant, the D period which is the time between completion of a round of replication and cell separation. This is about

20 minutes (Cooper and Helmstetter, 1968). Initiation of DNA replication therefore occurs 60 minutes (C + D) before cell division, and cells growing with a doubling time of less than 60 minutes initiate rounds of replication in the previous cycle (Donachie, 1968). Donachie (1968) showed that the initiation of DNA replication correlated strongly with the mass M_i (initiation mass) which was constant and independent of C, D and T (growth rate).

In their 'Unit Cell Concept', Donachie and Begg (1970) observed that cells of E. coli growing at a doubling rate of 60-70 minutes divide when they are 3.4 μ m long to produce two daughter cells, each 1.7 μ m long. They suggested that 1.7 μ m is the minimum length of E. coli cells, and that the mass of such a unit cell must be equal to the initiation mass M_i . However, cell volume and cell length vary with growth rate according to the equations:

$$\bar{V} = \bar{V}_u \cdot 2^R \quad \text{and} \quad \bar{L} = \bar{L}_u \cdot 2^{(R/3)}$$

where \bar{V} and \bar{L} are the mean cell volumes and lengths respectively and R is the growth rate (Donachie, 1981), so that cells growing with a doubling time of less than 60 minutes will behave as a group of independent unit cells, and divide 60 minutes after each doubling in number of unit cell equivalents. Donachie, Begg and Vicente (1976) observed that when cells reach a certain critical length, the rate of elongation doubles (provided the growth rate is unaltered) and 20 minutes later the cell divides. This critical length was found to be twice that of the minimum cell length (i.e. that of a unit cell), and it was suggested that this may be a trigger for cell division (Donachie, Begg and Vicente, 1976).

There are two types of model to explain the coupling of cell division to DNA replication. Witkin (1967) suggested the existence of

* \bar{V}_u and \bar{L}_u are the \bar{V} and \bar{L} of the unit cell.

a division inhibitor which prevents untimely division and is induced when DNA replication is interrupted. Recent evidence to support this model will be discussed later. Other models suggest that DNA replication or termination of DNA replication triggers the synthesis of a product which is essential for division to occur (Clark, 1968). Jones and Donachie (1973) observed there to be a brief requirement for RNA and protein synthesis between termination of chromosome rounds and cell division, and termed this 'termination protein synthesis'. It is not known whether a few specific proteins are required to be synthesised, or whether there is a general requirement for protein synthesis, but this does support the hypothesis of Clark (1968) for a division trigger. Inouye (1971) showed however, that DNA replication is not an absolute requirement for cell division, and the introduction of a recA⁻ mutation uncouples DNA replication and division so that even in the absence of DNA replication, cells divided to form DNA-less but normal sized cells.

Pierucci and Helmstetter (1969) found that a 40 minute period of protein synthesis is required before division, even when DNA_{replication} has been previously completed. This is a fixed period of protein synthesis rather than a particular amount of protein synthesis. There are therefore three clock-like processes that lead up to cell division, the C period, the D period and a 40 minute period of protein synthesis. These processes are summarised in Fig. 1.1.

1.2 Genetics of cell division

Table 1.1 shows a list of genes that are involved more or less specifically in cell division. Genes involved in initiation and elongation of DNA replication also affect cell division because of the

Fig. 1.1. Model of the cell cycle in *E. coli*

Cell mass doubles every T minutes, and this initiates two processes, DNA replication (t_{DNA}) and synthesis of division proteins (t_{DIV}). This protein synthesis is completed after 40 minutes and initiates a sequence of events (IA) that do not involve RNA, or DNA or protein synthesis. Termination of chromosome replication (T_{DNA}) induces synthesis of termination protein (TP) which interacts with the division proteins at stage P_{DIV} , a septum is formed and at T_{DIV} the cells divide. (Adapted from Jones and Donachie, 1973).

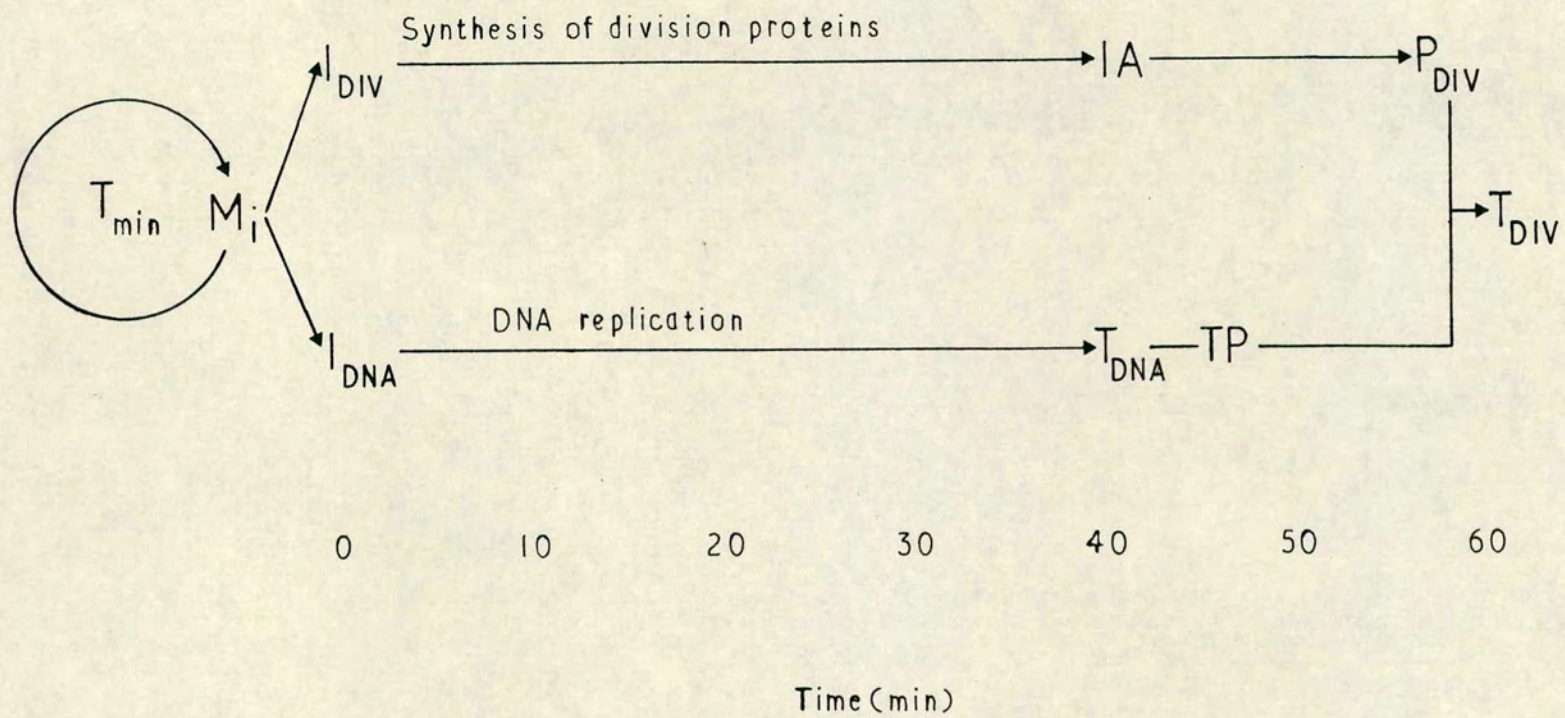


Fig 1.1

coupling of these processes but will not be discussed further, as their primary role is not in cell division. As cell division is essential for cell survival (at least on agar plates) most of the mutants in genes in Table 1.1 are conditional lethals (usually temperature sensitive). Several of the genes (lon, recA, lexA, su1A and su1B) are involved in the SOS system and their relationship to division will be discussed in the next section.

The isolation of cell division mutants has had several uses:

- a) a gene is identified that plays a role in division,
- b) physiological analyses, using cell number as an assay for division in a mutant strain, may indicate what this role in division is, and when in the cell cycle the gene product is required,
- c) biochemical assays of mutant strains at permissive and non-permissive temperature may lead to correlation between the temperature sensitive lesion and loss of a known enzyme activity,
- d) amber mutants may be used to identify gene products,
- e) mutants may be used for cloning regions of the bacterial chromosome.

The cell division genes in Table 1.1 have been mapped to many loci on the genetic map, and it is probable that some cell division genes have not yet been identified. Several workers have isolated mutants that map in genes around the 2 minute region of the genetic map, clockwise to leu (Hirota, Ryter and Jacob, 1968; Van de Putte, van Dillewijn and Rorsch, 1964; Reeve and Clark, 1972; Normark, 1970; Allen et al., 1974; Donachie et al., 1979; Begg, Hatfull and Donachie 1981), and through the use of λ transducing phage, the organisation of these genes is better understood than any other cell division genes. Several

Table 1.1 Genetic loci of genes involved in cell division in E. coli

<u>Genetic locus</u>	<u>Map Position (mins)</u>	<u>Phenotypic Trait of Mutants</u>	<u>Reference</u>
envA	2	Chain formation	Normark (1970)
ftsA	2	Multinucleate filaments at restrictive temperature	Ricard and Hirota (1973) Lutkenhaus and Donachie (1979)
ftsB	32-34	Multinucleate cells and chromosomeless cells at restrictive temperature	Ricard and Hirota (1970)
ftsC	4-9	Multinucleate cells and chromosomeless cells at restrictive temperature	
ftsD	86	Multinucleate filaments at restrictive temperature	Richard and Hirota (1973)
ftsE	73	Multinucleate filaments at restrictive temperature	Richard and Hirota (1973)
ftsF	82	Multinucleate filaments at restrictive temperature	Richard and Hirota (1973)
ftsG	29-30	Multinucleate filaments at restrictive temperature	Richard and Hirota (1973)
ftsH (ASH124)	89	Multinucleate filaments at restrictive temperature	Holland and Darby (1976)
ftsH (y-16)	69	Multinucleate filaments at restrictive temperature	Santos and de Almeida (1975)
ftsQ	2	Multinucleate filaments at restrictive temperature	Begg, Hatfull and Donachie (1980)
ftsZ	2	Multinucleate filaments at restrictive temperature	Lutkenhaus, Wolf-Watz and Donachie (1980)
fil ts	88	Multinucleate filaments at restrictive temperature	Stone (1973)

Table 1.1 continued

<u>Genetic locus</u>	<u>Map Position (mins)</u>	<u>Phenotypic Trait of Mutants</u>	<u>References</u>
min A	10	min A, min B double mutants misplace	Adler, Fisher, Cohen and Hardigree (1967)
min B	29-30	septa forming small chromosomeless cells	Teather, Collins and Donachie (1974)
lon (capR)	10	Radiation sensitive	Donch, Green and Greenberg (1968) Hua and Markovitz (1972).
sep (fts I, pbp B)	2	Multinucleate filaments at restrictive temperature	Fletcher et al 1978 Spratt (1977)
rec A	58	Continued cell division in absence of DNA replication	Castellazzi, George and Buttin (1972a,b)
lex A	90	Continued cell division in absence of DNA replication	Mount <u>et al</u> (1972)
su1 A (sfi A)	22	Suppressor of lon	George, Castellazzi and Buttin (1975).
su1 B (sfi B)	2	Suppressor of lon	George, Castellazzi and Buttin (1975)
ts20	1	Multinucleate filaments at restrictive temperature	Nagai and Tamura (1972)
ts52	35	Multinucleate filaments at restrictive temperature	Zusman et al (1972)

genes involved in murein biosynthesis also map in this region of the chromosome, and the enzyme activities of some of these genes are known. mur E is thought to code for mesodiaminopimelic acid^{adding} enzyme, mur F for D-alanyl-D-alanine adding enzyme, mur C for L-alanine adding enzyme and ddl for D-alanine : D-alanine ligase (Lugtenberg, and van Schijndel-van Dam 1972, 1973, Miyakawa, Matsuzara, Matsubishi and Sugino, 1972). PAT84, originally mapped as an ftsA mutant (Ricard and Hirota, 1973) but redefined as a separate locus ftsZ (Lutkenhaus, Wolf-Watz and Donachie, 1980) appears to have a reduced level of D,D-carboxypeptidase at the restrictive temperature (Mirelman, Yashouv-Gan and Schwarz, 1976, 1977). envA mutants have a reduced level of N-acetylmuramyl-L-alanine amidase activity, and show increased permeability to several antibacterial agents (Wolf-Watz and Normark, 1976). The biochemical activity of the ftsA gene is not known. The structural organisation of these genes at 2 minutes will be discussed in detail in section 1.5.

minA minB mutants are not defective in septum formation but are defective in the positioning of the septa, so that these strains produce small DNA-less cells called minicells (Adler, Fisher, Cohen and Hardigree, 1967). The division potential (number of septa per unit cell equivalents) is unaltered, so that the size distribution becomes very heterogeneous (Teather, Collins and Donachie, 1974). These mutants are most useful for their practical applications of examining protein synthesis directed by phage or plasmids (Adler, Fisher, Cohen and Hardigree, 1967).

Apart from some interesting observations about the map position of division genes (i.e. that several map in a cluster at 2 minutes), the physiological and biochemical studies have yielded very little information

about the role of the genes in division. Vicente, Otsuji and Donachie (unpublished data) hoped to overcome these problems by isolating amber mutants in essential genes (the OV series). By using a temperature sensitive suppressor, conditional lethal amber mutants were isolated, which are temperature sensitive for the synthesis of the essential gene product, rather than temperature sensitive for the activity of the product as in missense mutants. Physiological analyses with such mutants can be used to determine when in the cell cycle the gene product is required to be synthesised, or whether the product can be re-used in successive cycles. Amber mutants can also be used very effectively in the identification of gene products and this is particularly useful when there is little biochemical information available (Lutkenhaus and Donachie, 1979).

In practice, this system of isolating amber mutants has been limited by the suppression levels of the temperature sensitive suppressor that was used (Sup F_{A81}^{ts}), which allows only about 13% suppression at the permissive temperature (Smith, Barnett, Brenner and Russell, 1970). Nonetheless mutants have been isolated and used to identify a new gene mur G (Salmond, Lutkenhaus and Donachie, 1980), and to identify the ftsA gene product (Lutkenhaus and Donachie 1979). Two amber mutants were isolated in ftsA (OV8 and OV16) and used to show that ftsA may play a special role in cell division (Donachie et al., 1979). The mutant OV16 was synchronised by sucrose gradient centrifugation and samples shifted from the permissive temperature to the restrictive temperature and vice versa at different times during the cell cycle. The results indicated that the ftsA gene product was required to be synthesised periodically during a 10-15 minute period prior to division (Donachie et al., 1979).

It is not known whether the ftsA gene is actually periodically synthesised or if cells are only periodically competent to respond to its synthesis. Lutkenhaus, Moore, Masters and Donachie (1979) determined that the gross pattern of protein synthesis during the cell cycle does not change, even when cells reach certain critical dimensions, and therefore ftsA would be an atypical gene as regards its timing of synthesis, if it were periodically synthesised.

Although these physiological and biochemical studies have been slow to yield information about the mechanism of division, progress has been made in two particular fields of study, the SOS system and its relationship to cell division, and the fine structure of the genes located at 2 minutes on the genetic map.

1.3 The SOS system and the division response

When cells of E. coli are UV-irradiated or their DNA replication interrupted in some way (introduction of DNA synthesis inhibitors, addition of mutagens, for example) a series of events takes place in the treated cell which have collectively been called the SOS response (Radman 1975). The obvious initial response is the induction of synthesis of a 40 Kilodalton protein, the product of the recA gene (Inouye and Pardee, 1970; Gudas and Pardee, 1976; Sedgwick, 1975) which mediates the induction of DNA repair mechanisms and various prophages (Roberts, et al., 1978), and leads to an inhibition of division. Induction of these functions does not occur in cells that are mutated in either the recA gene or lexA gene (Witkin, 1976). recA protein has been shown to be active as a protease, cleaving both the λ repressor (Roberts et al., 1978) and the lexA gene product (Little et al., 1980), and mutations in

the recA gene abolish the protease activity. lexA which codes for a 24 kilodalton protein is autoregulated (Brent and Ptashne, 1980) and represses its own synthesis, and that of the recA protein (McPartland et al., 1980). Therefore recA protein itself must be among the induced functions, as is observed (Gudas and Mount, 1977). However, the introduction into the cell of a high copy number plasmid carrying the recA gene does not lead to induction of the SOS system (McEntee, 1977), even though the concentration of recA protein is observed to increase, so there must be some other activation of the recA product for SOS induction. It is thought that effectors produced by DNA damage (such as oligonucleotides) are responsible for this activation. Mutants have been isolated in recA (tif) (Morand, Goze and Devoret, 1977), and lexA (ts1) (Mount, Walker and Kosel, 1975) which induce the SOS functions at the restrictive temperature without any DNA damage being required.

If cells are given a low dose of UV-irradiation (10Jm^{-2}), the SOS system is induced, and division is inhibited.. This is however a transient effect, and after about 90 minutes, division resumes and the filaments divide to produce normal cells. This resumption of division is dependent upon the lon (cap R) gene product (Adler and Hardigree, 1965), which has been identified as a 94 kilodalton protein (Schoemaker and Markovitz, 1981). The lon product acts as a repressor of the gal operon and derepression in lon mutants leads to over production of colonic acid and mucoidy (Mackie and Wilson, 1972). Shineberg and Zipser (1973) showed that lon gene product also has a protease activity and degrades nonsense fragments of β -galactosidase. A further effect of lon mutants is the decreased lysogenisation of some bacteriophages (Walker et al., 1973). Suppressor mutants of lon (sul) map at one of two loci, sul A, or sul B

Fig. 1.2. The SOS system and the division response

In an uninterupted cell, lex A synthes^{es} its gene product (□) which acts as a repressor for its own synthesis, and the synthesis of rec A protein and sul A protein. Division continues normally.

However, when the cell is UV-irradiated, or DNA replication inter-upted in some way, effector molecules (▲) are released and activate the rec A protein (○) to its protease form (●). Amongst the SOS responses, the rec A protein cleaves the lex A product (⊖), which leads to depression of its own synthesis, rec A protein and also sul A protein. sul A is therefore induced and this leads to an inhibition of division.

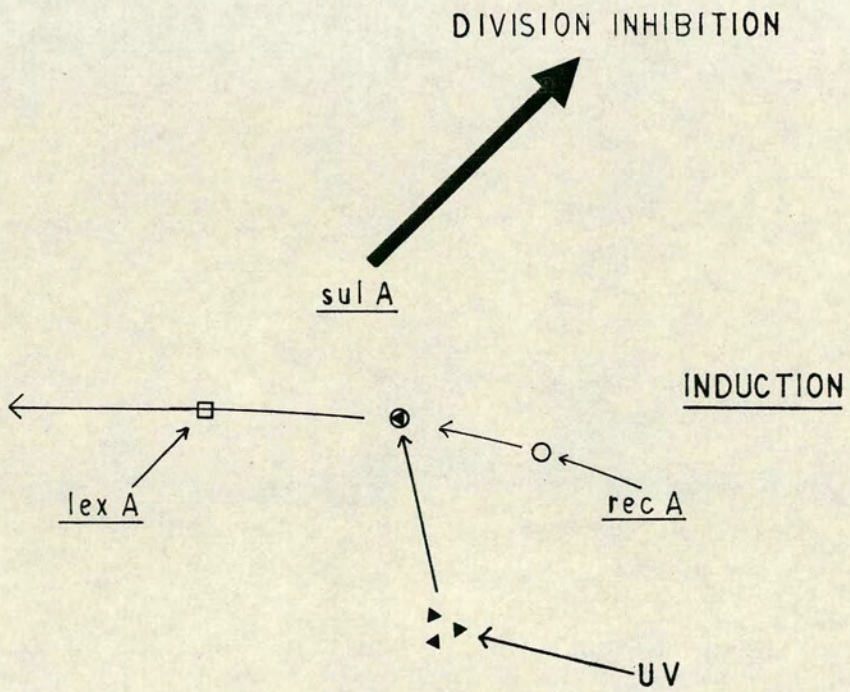
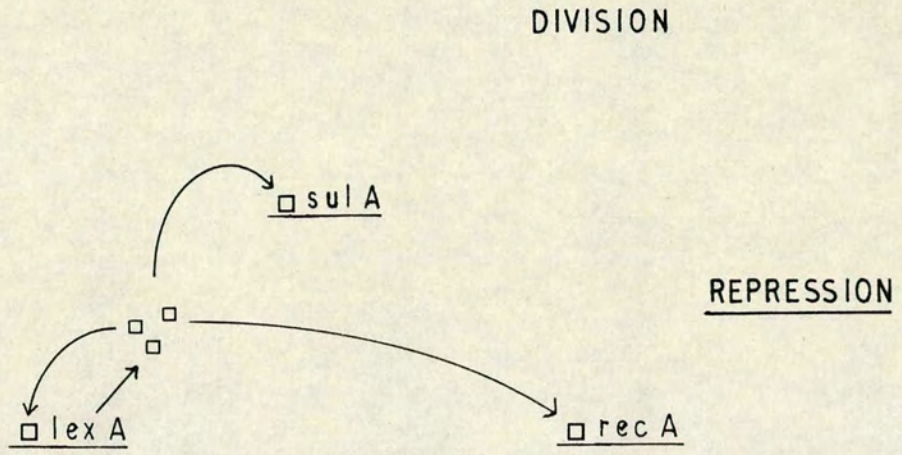


Fig 1.2

(Johnson, 1977) and these are identical to the map positions of sfi A and sfi B respectively, the suppressors of tif and ts1. These suppressor mutants therefore offer a link between the SOS system, and its division response.

Witkin (1967) and George et al., (1975) postulated previously the presence of a Repair Associated Division Inhibitor (RADI), and Huisman, D'Ari and George (1980) suggested that RADI may be the product of one of the suppressor loci. Huisman and D'Ari (1981) tested this hypothesis by genetically fusing the control region of su1 A to the lac Z gene in vivo using the MudAplac phage constructed by Casadaban and Cohen. The introduction of either the ts1 or tif mutation into the fused strain resulted in induction of the su1 A gene at the restrictive temperature as assayed by the production of β -galactosidase. The ts1 (lex A) effect is not mediated through the rec A gene, as ts1 rec A⁻ double mutants still induce β -galactosidase at the restrictive temperature. Huisman and D'Ari (1981) suggested that on UV-irradiation the following series of events occurs: Alteration to the DNA replication complex causes the generation of effector molecules which activate the rec A protein to its protease form which cleaves the lex A repressor. This leads to derepression of the su1 A gene which was repressed by lex A, and thus results in inhibition of division. They also speculate that lon may act in its protease form to cleave the su1 A product and allow division to resume, once DNA repair is complete (Fig. 1.2).

The mechanism by which su1 A inhibits division is not known although it has been proposed that it may act via the su1 B gene (Darby ^{Univ. Leicester} Ph.D. thesis, 1981). The relevance of the SOS system to the present study is that su1 B maps at a position approximately 2 minutes on the genetic map

of E. coli in the cluster of cell division genes (Johnson, 1977). Darby (Ph.D. Thesis) demonstrated that a 2.5Kb Eco RI restriction fragment from this region offers cells protection to low doses of UV-irradiation, although the sul B gene has not been precisely located.

1.4 The fts A - env A gene cluster

As discussed in 1.2 there are several division specific genes that map at 2 minutes on the E. coli genetic map, clockwise of leu. They include env A, fts A and sep (pbp B). Several genes involved with the biosynthesis of peptidoglycan, mra A, mra B, mur C, mur E, mur F ddl and probably mur G also map in this region (Bachmann and Brooks Low 1980).

Fletcher et al., (1978) isolated a family of λ transducing phages that contained chromosomal DNA from the 2 minute region that complemented some of the temperature sensitive mutants. The phage were isolated by induction of a phage integrated in leu and testing for transduction of chromosomal markers, and are all defective (require a λ helper). The largest transducing phage isolated, complements env A (the furthest marker from leu that was tested) and contains a 26.4Kb fragment of chromosomal DNA, sufficient to code for 22 genes assuming an average protein molecular weight of 40 Kilodaltons (Fletcher et al., 1978). This phage complements seven of the mutants that were tested. The genes were ordered by complementation analysis with smaller transducing phage. The gene order is shown in Fig. 1.3. The mutant PAT84 had previously been classified as an fts A mutant (Hirota, Ricard and Shapiro, 1971; Ricard and Hirota, 1973) but was not complemented by any of the transducing phage. Fletcher et al., suggested that the PAT84 mutation is either in another gene clockwise to env A, or is a dominant mutation in the fts A gene.

Fig. 1.3. Gene order in the 2 minute region of the E. coli K12 chromosome, as determined by Fletcher et al., (1978).

(not to scale).

Fig. 1.4. Transducing phage λ 16-2.

Diagram showing the chromosomal insert in λ 16-2. Only the central part of the phage is shown. Single lines represent the λ phage arms, and the open box the chromosomal insert. Hind III (\blacktriangledown), Eco RI (\triangle) and Bam HI (\downarrow) restriction endonuclease sites in the chromosomal insert are shown, as well as the bacterial genes that are carried on this phage. Each segment below the phage represents 1 Kilobase. (Adapted from Lutkenhaus, Wolf-Watz and Donachie, 1980).

leuA sep murE murF murC ddl ftsA envA

Fig 1.3

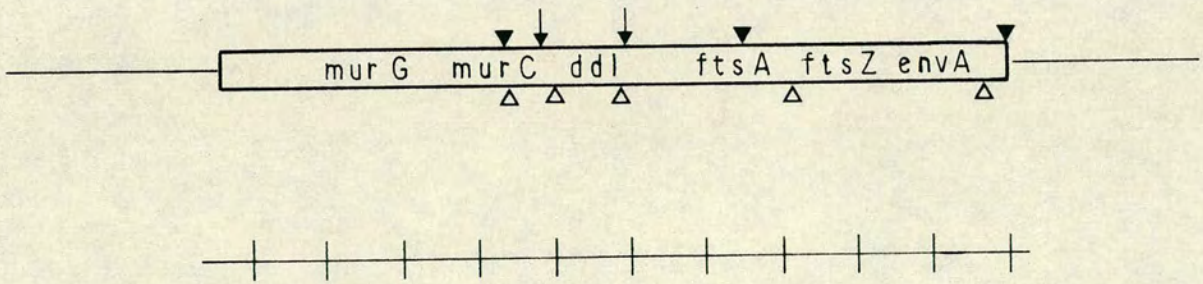


Fig 1.4

The coding capacity of the transducing phages suggested that there may be genes present in this region that had not been identified. A mutant (AX655) was complemented by these transducing phages and mapped in a gene between fts A and leu that had not been identified, and this gene was designated sep. Irwin et al., constructed a λ transducing phage carrying an 18.2Kb Eco RI restriction fragment that complemented the sep mutation, and, on infection of E. coli cells, over-produces penicillin binding protein 3 (PBP-3). Irwin et al., (1979) suggest that the sep gene is probably identical to pbp B (Spratt 1977) and fts I (Suzuki et al., 1979), based on the finding that a sep^{ts} mutant lacked PBP-3 activity at 30°C and 42°C, although this has not yet been genetically confirmed.

Lutkenhaus and Donachie (1978) isolated a proficient ^{plaque forming} transducing phage, λ 16-2 (Fig. 1.4) containing a 10.5Kb chromosomal DNA insert which complemented mutations in the genes env A, fts A, ddl and mur C. Analysis of this phage, and derivatives of it led to the determination of transcriptional units and gene products in this part of the cluster (Lutkenhaus and Donachie, 1979; Lutkenhaus, Wolf-Watz and Donachie, 1980; Lutkenhaus and Wu, 1980).

The fts A gene product was identified as a 50 Kilodalton protein (Lutkenhaus and Donachie, 1979) proved unequivocally by recombining the amber mutation from OV16 onto the λ 16-2 transducing phage, and examining the patterns of protein synthesis in a UV-irradiated cell system. The phage carrying the amber mutation failed to synthesis^e only one protein, of 50 Kilodaltons and this was restored on infection of a strain carrying an amber suppressor. This is therefore the fts A gene product. Five other proteins were also observed that were coded for by the chromosomal

insert in $\lambda 16-2$ (Lutkenhaus and Wu, 1980).

$\lambda 16-2$ also complements the PAT84 mutation, and complementation analysis with deletion derivatives of $\lambda 16-2$ mapped the PAT84 mutation not in fts A but in a new gene designated fts Z (Lutkenhaus, Wolf-Watz and Donachie, 1980), between fts A and env A.

The products of known genes complemented by $\lambda 16-2$ were identified by making a comparison between the patterns of protein synthesis and complementation of mutants in deletion derivatives of $\lambda 16-2$ (Lutkenhaus and Wu, 1980). This data is shown in Fig. 1.5. The gene products were identified as env A (31Kd), fts Z (45Kd), fts A (50Kd), ddl (30Kd) and mur C (65Kd). An additional protein was synthesised from $\lambda 16-2$ and $\lambda \Delta R1$ of 48Kd (Lutkenhaus and Wu, 1980). These phage complement a mutant from the OV series (OV58) which defines a new gene designated mur G (Salmond, Lutkenhaus and Donachie, 1980). The 48 Kilodalton protein is probably the product of this gene. The OV58 mutant swells and lyses at the restrictive temperature so mur G is probably involved in peptidoglycan biosynthesis, as are its neighbouring genes mur E, mur F, mur C and ddl (Salmond, Lutkenhaus and Donachie, 1980).

The 30 Kilodalton protein was assigned to the ddl gene as it was the only known gene on the 3.2Kb Hind III fragment that codes for this protein (Lutkenhaus and Wu, 1980). Evidence in this project indicates that another gene is present on this fragment so this identification must come into question.

The direction of transcription and the location of promoters in the $\lambda 16-2$ transducing phage were determined by analysing protein synthesis from $\lambda 16-2$ derivatives, and also by examining the effects of P_L

Fig. 1.5. Deletion derivatives of $\lambda 16-2$ and their patterns of protein synthesis

Open boxes represent the bacterial insert in each transducing phage. The boxes are not closed when the extent of the deletion is not known. Restriction endonuclease sites Hind III (\blacktriangledown), and EcoRI (\triangle) are shown in $\lambda 16-2$, but only in the deletion derivatives when they help to define the extent of the deletion.

+ signs above each phage indicates the ability for that phage to synthesise the protein shown at the top (adapted from Lutkenhaus and Wu, 1980).

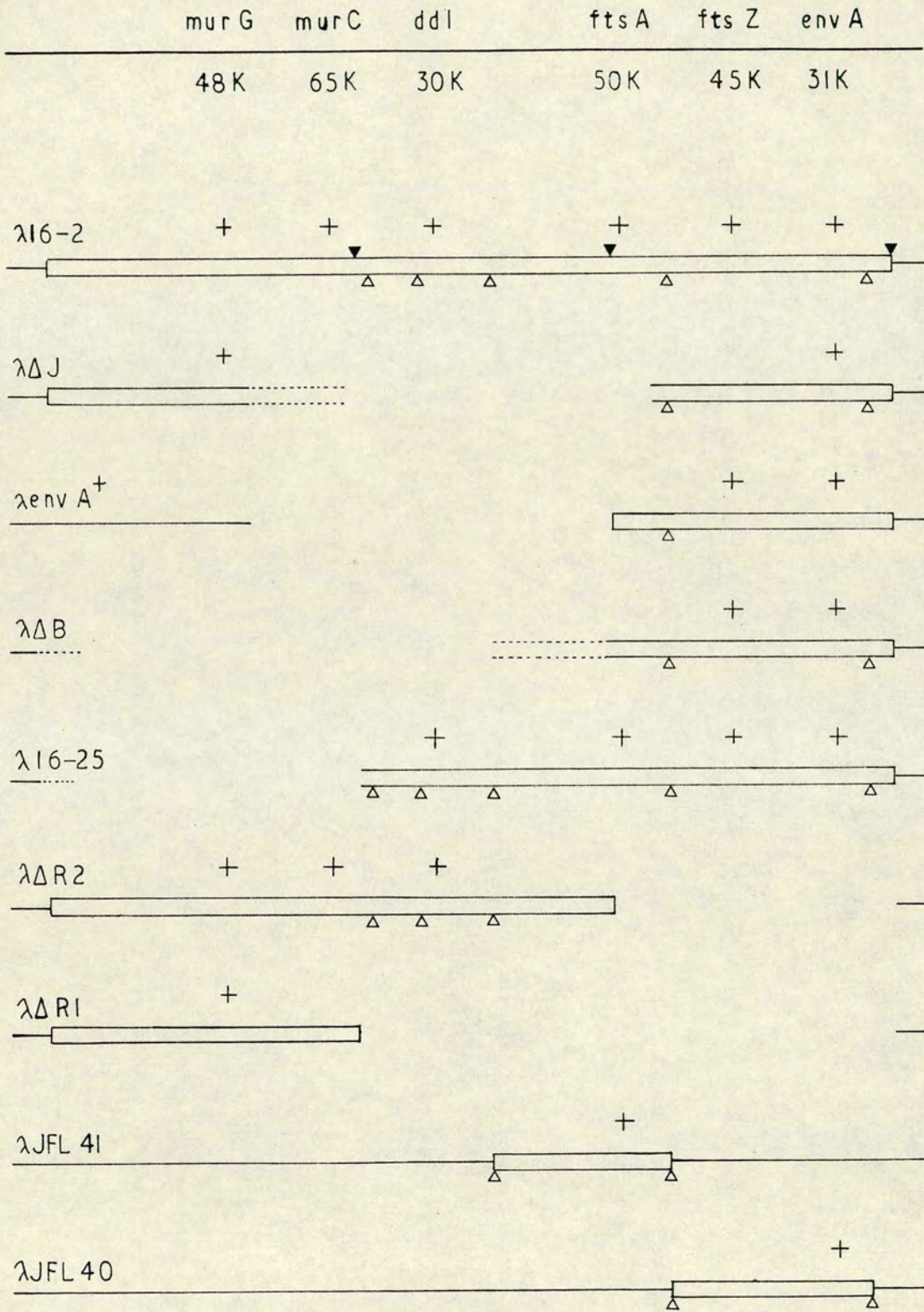


Fig 1.5

Fig. 1.6. Genetic organisation of genes carried on λ 16-2.

The proteins synthesised by each gene are represented as open boxes, and the direction of transcription shown above these boxes. mur G, mur C and ddl may all be in one transcriptional unit, or mur G may be in a separate unit to mur C and ddl. Promoters are represented by a letter P. The exact position of the genes is uncertain, but is close to that in the diagram. Restriction endonuclease sites Hind III (\blacktriangledown) and Eco RI (\triangle) are also shown. (Adapted from Lutkenhaus and Wu (1980)).

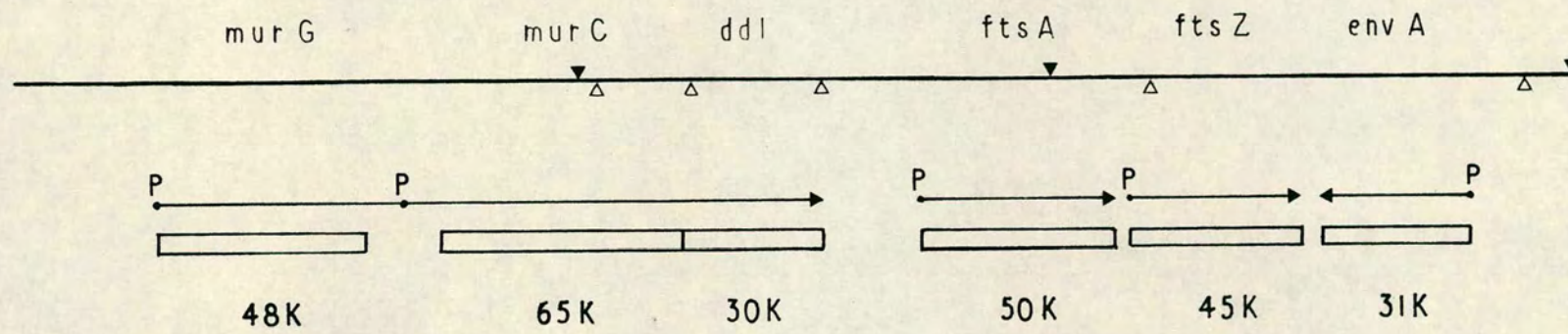


Fig 1.6

on the expression in N_{am} hybrid immunity derivatives. The transcription units and their direction of transcription are shown in Fig. 1.6.

The Hind III site that maps within the fts A gene lies at least 135 nucleotides from the C-terminal end of the fts A gene, as estimated by the size of hybrid proteins synthesised across the Hind III site (Lutkenhaus and Wu, 1980). It was also observed that fts Z could be expressed without the DNA anticlockwise to the Hind III site in fts A but only weakly, and synthesis of the 45Kd fts Z gene product was stimulated if this DNA was present. This pattern of synthesis correlated well with the complementation patterns of the relevant transducing phages. This raises the question as to whether there is an additional promoter or control site within the fts A gene that affects expression of the fts Z gene (Lutkenhaus and Wu, 1980).

1.5 Aims of this project

A novel isolation procedure was devised for isolating cell division mutants. A new gene was identified which mapped adjacent to fts A, in the gene cluster at 2 minutes on ^{the} E. coli. ^{genetic map.} An analysis of fts A and fts Q was facilitated by the cloning of the genes on a 2.2Kb Eco R1 restriction fragment. Several restriction sites were mapped and the position of the genes defined by complementation analyses with deletion derivatives of the 2.2Kb Eco R1 fragment. These derivatives were also used to attempt to identify the fts Q gene product, and the transcription organisation of the fts Q, A, Z genes. A start was made to the DNA sequencing of the 2.2Kb fragment, and some circumstantial evidence obtained that the fts Q promoter region may be cleaved by Eco R1. It was hoped that these studies would reveal the mechanism of the controls of the fts genes and their role in cell division.

CHAPTER 2Materials and Methods

See Table 2.1 for a list of bacterial and phage strains used in this project.

2.1 Media and Buffers

L-broth	Difco Bacto Tryptone	10g
	Difco Bacto Yeast Extract	5g
	Sodium Chloride	10g
	Water to 1 Litre	
	pH adjusted to 7.2	
L-agar:	Difco Bacto Tryptone	10g
	Difco Bacto Yeast Extract	5g
	Sodium Chloride	10g
	Difco Agar	15g
	Water to 1 Litre	
	pH adjusted to pH 7.2	
Oxoid Nutrient Broth:	Oxoid Nutrient Broth, No. 2	25g
	Water to 1 Litre	
Oxoid Nutrient Agar:	Oxoid Nutrient Broth, No. 2	25g
	Davis, N.A. Agar	12.25g
	Water to 1 Litre	
MacConkey Agar:	Bacto-Peptone	17g
	Difco Protease-Peptone	3g
	Bacto Bile Salts No. 3	1.5g
	Sodium Chloride	5g
	Neutral Red	0.03g
	Bacto Agar	15g
	Bacto Crystal Violet	0.001g
	Water to 1 Litre	

Sugars were added at a final concentration of 1%.

BBL Top Agar:	Baltimore Biological Laboratories	
	Trypticase	10g
	Sodium Chloride	5g
	Difco Agar	5.5g
	Water to 1 Litre	

Minimal Medium:

M9 (x4):	$\text{Na}_2 \text{HPO}_4$	28g
	$\text{KH}_2 \text{PO}_4$	12g
	NaCl	2g
	$\text{NH}_4 \text{Cl}$	4g
	Water to 1 Litre	
	pH adjusted to 7.0	

Sugars were added to 1x M9 at a final concentration of 0.2%.

Amino acids were added to 1x M9 at a final concentration of 2ug/ml.

For minimal agar, Davis N.Z. Agar was added to a final concentration of 12.52g/Litre.

Bacterial Buffer:	$\text{KH}_2 \text{PO}_4$	3g
	$\text{Na}_2 \text{HPO}_4$	7g
	NaCl	4g
	$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	0.2g
	Water to 1 Litre	

Phage Buffer:	$\text{KH}_2 \text{PO}_4$	3g
	$\text{Na H}_2 \text{PO}_4$	7g
	NaCl	5g
	MgSO_4	0.25g
	Ca Cl_2	0.015g
	Gelatin	0.001%
	Water to 1 Litre	

Antibiotics were added to the above media at a final concentration of:

Tetracycline (Tc)	20ug/ml
Ampicillin (Ap)	50ug/ml
Chloramphenicol (Cm)	100ug/ml

Table 2.1 Bacterial strains

<u>Strain</u>	<u>Relevant features</u>	<u>Source of reference</u>
W3110	Prototroph	Laboratory stock
AB2497	thy A arg E leu his pro thr thi galK	Laboratory stock
JC10-240	recA srl380::tn10 thr300 ilv 318 rel 1 thr 1 spc 300 Hfrpo 45	Csonka and Clark (1980)
AA125	(lacZ) ^Δ (trp ED) ^Δ (gal-uvrB) ^Δ his tsx ton A lacI ³ sup ⁰	Laboratory stock
TKF12	ftsAts thr thi leu pyrF thyA ilvA his arg lac tonA tsx	Wijsman and Koopman (1976)
NEM259	met SuII SuIII r ^{-m+}	Laboratory stock
C600K-	galK thr leu thi lac tonA supE	R. Hayward
N100	galK recA pro his	R. Hayward
DS410	minA minB prototroph	N. Willetts
JM101	(lac pro) ^Δ thi strA end A sbc B15 SupE F'tra D36 proAB lacI Z ^ϕ M15	K. Murray
TOE1	fts Q ^{ts} thyA argE leu his pro thr thi	This work
TOE13	ftsAts thyA argE leu his pro thr thi	This work
Phage	<u>Phage strains - Bacterial genes on phage</u>	
λNEM616	lacZ	Wilson and Murray (1979)
λNEM607		Murray, Brammar and Murray (1977)
λ16-2	murG murC ddI fts Q fts A fts Z env A	Lutkenhaus and Donachie (1979)
λ16-4	murG murC ddI ftsQ ftsAts ftsZ env A	Lutkenhaus and Donachie (1979)
λ16-2 E	ddI ftsQ ftsA ftsZ envA	J. Lutkenhaus
λddI+	murG murC ddI	J. Lutkenhaus

2.2 Growth of Bacterial Cultures

Stationary phase cultures were prepared by inoculating a single colony from an agar plate into fresh medium and incubating overnight at the appropriate temperature with vigorous shaking (New Brunswick Gyrotory Shaker). Exponentially growing cultures were prepared by diluting a fresh stationary phase culture 1 in 50 into fresh medium and incubating as above for 2-3 hours.

2.3 Monitoring Cell Growth

Cell mass was followed by measuring the optical density of a culture at 540nm (unless otherwise stated) using a Perkin-Elmer Coleman Model 55 Spectrophotometer.

Cell size and number were measured in a Coulter Counter Model ZB interfaced with a Coulter Channelizer (Coulter Electronics Ltd., Harpenden, England). 0.2mls of bacterial samples were mixed thoroughly with 0.2mls of filtered 0.5% formaldehyde solution. For counting, a 50ul sample was diluted into 8mls of filtered azide-saline solution (NaCl 36g, NaN_3 2g, water to 4 litres). Bacterial cultures to be used for Coulter analysing were always grown in filtered media.

2.4 Synchronisation of Bacterial Cultures using Sucrose Gradients

Sucrose gradients were prepared by layering 3mls each of 12%, 10%, 8%, 6% and 4% Sucrose in filtered broth successively into a test tube. Cells from 200mls of an exponentially growing culture (OD_{540} 0.5-0.6) were harvested by centrifugation (10 mins, 5000g, 20°C), and resuspended in 1ml of fresh broth. 0.5mls was layered onto each of two

gradients and centrifuged in an MSE Mistral centrifuge with a swing-out rotor, at 2000rpm. The required centrifugation time varies for different bacterial strains but is usually between 10 minutes and 13 minutes. The small new-born cells were removed from the top of the gradient using a syringe and needle, in 0.1-0.2mls and inoculated into 30-40mls of fresh broth (Gudas and Pardee, 1974).

2.5 Transduction of Chromosomal Markers using P1

P1 phage lysates were prepared by plating a mixture of 1×10^8 cells, 1×10^8 P1 phage and 3mls Top Agar (50°C) onto an L-broth agar plate containing 10^{-3} M Ca^{++} , and incubating for 6-7 hours at the appropriate temperature. 2mls of phage buffer were added to the plate, the top agar removed, and clarified by centrifugation in a bench centrifuge.

10mls of recipient cells were grown to stationary phase in L-broth supplemented with 10^{-3} M Ca^{++} , harvested by centrifugation in a bench centrifuge and resuspended in 1ml of broth. 0.1ml of recipient cells were mixed with 0.1ml of a P1 lysate (contained approximately 5×10^7 phage particles) and incubated at 37°C for 10 minutes. 0.5mls of phage buffer added, and dilutions spread onto selective plates, (Masters, 1970).

2.6 Transduction of Chromosomal Markers using λ transducing phage

For spot test complementation of a temperature sensitive bacterial strain, 0.2mls of an overnight culture of the strain were mixed with 3mls of Top Agar and poured onto a broth agar plate. When the agar

had set, a loopful of phage suspension containing approximately 10^8 phage was placed onto the agar, and allowed to dry in. The plate was incubated at the non-permissive temperature for 15 hours, and examined for temperature resistance in the phage spot (Lutkenhaus and Donachie, 1979).

For transduction of the env A⁺ phenotype, the plates were incubated at 30°C for 16 hours, and then a loopful of cells from the turbid patch streaked out onto broth agar plates containing 5ug/ml Rifampicin (Normark, 1970).

2.7 Construction of λ lysogens

0.2mls of an overnight bacterial culture and 3mls of Top Agar were mixed, poured onto a broth agar plate and allowed to set. The phage lysate was spotted onto the bacterial lawn and when dry incubated at the appropriate temperature overnight. Cells were picked from the turbid spot and streaked out to isolated colonies on a fresh agar plate. Single colonies were tested in a cross-streak for sensitivity to infection by λ virulent and a λ homoimmune phage. Lysogens were sensitive to infection by λ virulent, but insensitive to superinfection by a λ homoimmune phage.

2.8 Hfr Crosses

The donor Hfr culture was prepared by inoculating fresh broth 1 in 50 from an overnight stationary phase culture and incubating at the appropriate temperature without shaking, until OD_{540} 0.3-0.4. An equal volume of this culture was mixed with an exponentially growing culture of the F⁻ recipient, and incubated without shaking. Samples were removed

at the appropriate times, and the conjugation terminated by vortexing. Dilutions were spread out immediately onto selective plates.

2.9 Preparation of λ phage and λ phage DNA

2.9.1 Induction of a λ lysogen by UV-irradiation

Lysogenic bacteria were grown in L-broth to OD_{540} 0.5, harvested by centrifugation at 8,000g, 10 minutes, at 20°C and resuspended in 10^{-2} M $MgSO_4$. The cells were UV-irradiated in a glass petri dish with a UV dose of 400 ergs/mm², and then diluted four-fold into fresh pre-warmed L-broth. The flask was covered with foil and shaken vigorously for 2½-3 hours at 37°C. 1ml of chloroform was added per 500mls of culture and the lysate clarified by centrifugation for 10 minutes at 8,000g, 4°C.

2.9.2 Liquid Infection with λ

The host strain was grown in L-broth supplemented with 10^{-2} M $MgSO_4$ to an OD_{540} 0.5. phage were added at an m.o.i. slightly in excess of 1, and the culture shaken vigorously for 3-4 hours at 37°C, or until the OD_{540} reached a minimum. Chloroform was added, and the lysate clarified as above.

2.9.3 Plate lysate preparation of λ phage

0.2mls of an exponentially growing culture were mixed with 0.1ml of a lysate containing 10^7 phage particles and incubated at room temperature for 10 mins. 3mls of Top Agar were added and the mixture poured onto a fresh L-broth Agar plate. After 7 hours incubation at 37°C

2mls of phage buffer were added and the top layer removed, and clarified in a bench centrifuge.

2.9.4 Titration of λ phage

The titre of phage lysates was estimated by diluting to 10^{-6} and 10^{-8} and mixing 0.1ml of each dilution with 0.2mls of exponentially growing cells. After absorption, 3mls of Top Agar were added, and the mixtures poured onto broth agar plates and incubated overnight. Phage titres were $1-5 \times 10^9$ pfu/ml.

2.9.5 Concentration of λ phage with PEG 6000

40g/L of NaCl and 10% (w/v) PEG 6000 were dissolved in a clarified phage lysate, and the phage precipitated at 4°C overnight. The precipitated phage were harvested by centrifugation (5000g 10 minutes, 4°C) and resuspended in phage buffer. DNAase and RNAase were added at 10ug/ml and incubated at room temperature for 30 minutes (Yamamoto, et al., 1970).

2.9.6 Concentration of λ phage on CsCl Step Gradients

CsCl solutions were prepared in phage buffer to densities of 1.3, 1.5 and 1.7. 3mls of each were layered into the bottom of a nitrocellulose tube with a pasteur pipette, the lightest solutions first, and the heavier solutions through these. The lysate was layered onto the CsCl and the gradients spun at 22 Krpm 4°C for 3 hours in an SW25.1 rotor. Phage bands were collected through the side of the tube with a syringe and a 19G needle.

2.9.7 Preparation of λ phage DNA

CsCl was removed from phage preparations by dialysis against phage buffer for 1-2 hours at 4°C. Dialysis tubing was always pre-boiled in 5mM EDTA for 15 mins and washed thoroughly in distilled water before using. Phage were extracted three times with an equal volume of pre-equilibrated phenol. The aqueous layer was dialysed against TE for 16-20 hours with four changes of TE to remove all traces of phenol. DNA concentration was estimated by measuring OD₂₆₀, taking OD₂₆₀ of 1.0 to be approximately 50ug/ml DNA.

2.10 Preparation of plasmid DNA by Isopycnic Centrifugation

100mls of cells harbouring a high copy number plasmid were grown to stationary phase in selective media. The cells were harvested by centrifugation at 8000g, 10 minutes at 4°C, and resuspended in 1.5mls of sucrose solution (Table 2.2). 0.5mls of 0.5M EDTA and 0.25mls of lysozyme (10mg/ml) were added, and the suspension kept on ice for 10 mins with occasional swirling. 4mls of lysis solution (Table 2.2) were added and the solution kept on ice for a further 10 mins with occasional swirling. The lysate was clarified by spinning at 15Krpm 4°C for 30 minutes in an 8 x 50 rotor. The supernatant was decanted and the volume made up to 9mls with TE. 9g CsCl and 0.9mls Ethidium Bromide (5mg/ml) were added to the cleared lysate, and the plasmid DNA banded by centrifugation for 60 hours, 15°C at 38 Krpm in a Ti 50 rotor. The band of closed circular plasmid DNA (the lower of the two bands) was visualised under long wavelength UV light and removed through the side of the tube with a syringe and 19G needle. Ethidium bromide was extracted from

the plasmid DNA with 5 changes of Amyl Alcohol (pre-equilibrated with water). The CsCl was removed by dialysis against TE for 1-2 hours at 4°C, and the DNA precipitated with 2 volumes of absolute ethanol and 1/10 volume 3M Potassium Acetate at -70°C for 30 minutes. The precipitated DNA was pelleted in a microcentrifuge at 4°C, dried in a vacuum desiccator and resuspended in 200ul of TE (see Clewell and Helinski, 1970).

2.11 Quick Method for preparation of plasmid DNA (Birnboim and Doly, 1979)

This method was used for rapidly screening strains that contain plasmids. Of the several methods available for screening plasmids, this proved the most useful and up to twelve plasmids can be prepared in a few hours, that are suitable for digestion with restriction enzymes.

Cells from an overnight culture were harvested by centrifugation for 1 minute in a microcentrifuge, and resuspended in 100ul lysis solution (Table 2.3). After 30 minutes on ice, 200ul of alkaline SDS solution (Table 2.3) were added, and after a further 5 mins. on ice, 150ul of high salt solution (Table 2.3) were added. The chromosomal DNA and cellular proteins were precipitated on ice for 30 mins, and removed by centrifugation for 5 mins in a microcentrifuge at room temperature. 400ul of the supernatant were mixed with 1ml of cold ethanol (-20°C) and the plasmid DNA precipitated at -70°C for 30 mins. After harvesting in a microcentrifuge, the nucleic acids were resuspended in 100ul of low salt solution (Table 2.3) and reprecipitated with 200ul of cold ethanol. The nucleic acids were again harvested by centrifugation, dried in a vacuum desiccator, and resuspended in 50ul TE. Of this preparation, 15ul were used for restriction digestion and agarose gel electrophoresis, or for transformation.

Table 2.2Solutions for Preparation of Cleared Lysates:

Sucrose Solution:	50mM Tris H Cl 40 mM EDTA 25% Sucrose pH adjusted to pH 8.0
Lysis Solution:	2 mls 10% Triton x -100 25 mls 0.5M EDTA pH 8.0 10 mls 1M Tris H Cl pH 8.0 Water to 200 mls.

Table 2.3Solutions for Birnboim Plasmid Preparations:

Lysis Solution:	25mM Tris H Cl pH 8.0 10mM EDTA pH 8.0 50mM Glucose 2mg/ml Lysozyme
Alkaline SDS Solution:	1% SDS 0.2M NaOH
High Salt Solution:	3M Sodium Acetate pH 4.8
Low Salt Solution:	0.1M Sodium Acetate pH 6.0

2.12 Digestion of DNA with Restriction Endonucleases

DNA was digested in a reaction mixture containing 1 μ g DNA, 1/10 volume of the appropriate 10x reaction buffer, 1 Unit of enzyme and water up to 20 μ l in a snap-cap Eppendorf tube, and incubated for 1 hour at 37 $^{\circ}$ C. The reaction conditions for each enzyme are shown in Table

2.4 If digesting DNA with more than one enzyme, and the reaction conditions were different for each enzyme, the DNA was precipitated, dried and resuspended between the reactions. Reactions were terminated by heating to 70 $^{\circ}$ C for 10 minutes, and then kept on ice.

2.13 Purification of DNA restriction fragments by sucrose gradient centrifugation

A two chamber linear gradient maker was used to pour 17ml 5-20% sucrose gradients. Sucrose solutions were made in TE and boiled for 15 minutes before using. 50-100 μ g of DNA was digested with the restriction enzyme and loaded onto the top of a gradient. The gradients were spun at 25 Krpm, 4 $^{\circ}$ C for 20 hours in an AH 627 swing-out rotor. The gradient was fractionated into 0.2mls samples and 10 μ l of each sample run on an agarose gel to determine which fractions contained the required restriction fragment. These fractions were pooled and the sucrose removed by dialysis against TE overnight. The DNA was ethanol precipitated, washed with 1ml of cold ethanol, dried and resuspended in 200 μ l TE.

2.14 Agarose Gel Electrophoresis (McDonnell et al., 1977)

Gel solutions were prepared by dissolving 1.6g agarose (Sigma, electrophoresis grade) in 200mls of Tris-Acetate buffer (40mM Tris, 20mM

Sodium Acetate, 100mM EDTA, pH 8.2), heating up to boiling point, and allowing to cool to hand-heat. Ethidium bromide was added to 0.5ug/ml and the solution poured into a perspex plate with 3mm surrounding spacers. Wells were formed with a perspex comb, and the gel allowed to set for 30 minutes.

4ul of loading buffer (50% Glycerol, 0.1% Bromophenol Blue in Tris-acetate buffer) were added to each DNA sample and loaded into the wells using a 50ul Hamilton syringe. Gels were run either between wicks of 3MM filter paper (Whatman) or in a tank submerged beneath buffer, towards the positive electrode, until the Bromophenol Blue dye had travelled about three quarters the length of the gel. Gels were photographed over long wavelength UV light, and photographed with Ilford FP4 film, using a 15 second exposure through a red filter.

2.15 Ligation of DNA fragments

DNA fragments with staggered ends were ligated in a reaction mixture containing 1-2ug DNA, 1/10 volume of 10 x ligase cocktail (66mM Tris pH 7.5, 1mM EDTA, 10mM Mg Cl₂, 1mM, ATP, 10mM DTT) and 1 unit of T4 ligase (New England Biolabs), and water to the appropriate volume. If the DNA molecules were to be encouraged to circularise, then the volume of the reaction mixture was 100ul. Otherwise volumes of 30-50ul were used. Ligation mixtures were incubated at 15^oC for 3-4 hours.

For ligation of DNA fragments with blunt ends, 30 units of ligase were used and the mixtures incubated overnight at 15^oC.

Table 2.4

Reaction conditions for Restriction Endonucleases

Enzyme	NaCl (mM)	K Cl (mM)	Tris HCl		MgCl ₂ (mM)	Source
			mM	pH		
Bam HI	150		6	7.9	6	Boehringer
Bgl II		6	10	7.5	6	BRL
Eco RI	50		100	7.5	5	Boehringer
Hae II			6	7.4	6	New England Biolabs
Hinc II	60		10	7.9	6.6	BRL
Hind III	60		10	7.4	7	Boehringer
Kpn I	6		6	7.4	6	BRL
MspI		6	10	7.4	10	BRL
Pst I	50		6	7.4	6	BRL
Pvu II	6		6	7.4	6	New England Biolabs
SaI I	150		6	7.9	6	New England Biolabs
Sau 3A	50		6	7.5	5	BRL
Sma I		20	6	8.0	6	Boehringer
Taq I	6		6	7.4	6	New England Biolabs
Xho I	150		6	7.4	6	New England Biolabs

All reactions also included 100ug/ml BSA and 6mM β -mercaptoethanol

2.16 Transformation and Transfection

To prepare competent cells, exponentially growing cells OD_{540} 0.5-0.6 were harvested in a bench centrifuge and resuspended in half the original volume of ice-cold 50mM $CaCl_2$. The cells were immediately centrifuged again and resuspended in 1/15 the original volume of 50mM $CaCl_2$, and kept on ice for at least 30 minutes.

For transformation, 0.2ml of competent cells were added to plasmid DNA, kept on ice for 30 minutes and then heat - pulsed for 5 minutes at $42^{\circ}C$. 1ml of fresh broth was added and the cell suspensions incubated at $37^{\circ}C$ (or $30^{\circ}C$ if the strain was temperature sensitive) for 1 hour to allow for expression of plasmid antibiotic resistance genes. 0.1ml of 10^{-1} and 10^{-2} dilutions in broth were spread on selective agar plates and incubated overnight. Transformation frequencies were usually about 10^6 transformants per μg DNA.

For transfection, 0.2mls of competent cells were added to phage DNA, kept on ice for 30 minutes and then heat - pulsed for 2 minutes at $42^{\circ}C$. The cells were returned to ice for a further 30 minutes. 3mls of top agar were added and the mixture poured onto an agar plate, and incubated overnight. Transfection frequencies were usually about 10^5 pfu/ μg DNA.

2.17 Preparation of minicells (Meagher et al., 1977).

Cells from 2 litres of a stationary phase culture of strain DS410 were harvested by spinning at 8,200g $4^{\circ}C$ for 10 minutes in a Sorvall rotor, and resuspended in 20mls of broth. Sucrose gradients were prepared by freezing ($-20^{\circ}C$) 35mls of 20% sucrose in M9 Glucose, in a 50ml tube and thawing slowly at $4^{\circ}C$. The concentrated cells were loaded onto four sucrose gradients and spun at 5000g, $4^{\circ}C$ for 20 minutes in a Sorvall

HB-4 rotor, and the minicell band (half-way up the gradient) removed with a syringe and 19G needle through the top of the gradient. The minicells were pelleted by spinning at 20,000g, 4°C, 10 minutes in an SS34 rotor, resuspended in 5mls of M9 Glucose, and layered onto two more sucrose gradients. Minicells were collected and pelleted as above and resuspended in 1ml M9 Glucose. This was layered onto one more sucrose gradient, and spun as above. The minicells were removed and harvested, and resuspended in 1ml of 30% Glycerol in M9 Glucose. Aliquots of minicells preparations were stored frozen at -70°C.

2.18 ³⁵S labelling of minicells

Minicells preparations were thawed and diluted with M9 Glucose to achieve a 1ml culture of OD₆₀₀ 0.2. The minicells were pelleted by spinning for 2 minutes in a microcentrifuge, and resuspended in 100ul of M9 Glucose. The preparations were pre-incubated at 37°C for 60 minutes to remove any bacterial mRNA. A solution was prepared containing 4/5 volumes of Difco methionine assay mix (25% in M9 Glucose) and 1/5 volumes ³⁵S-methionine, and an appropriate volume containing 10uCi of ³⁵S added to each minicell preparation. After labelling for 3 hours at 37°C, each sample was chased with 5ul of cold methionine (8mg/ml) for 3 minutes, and the minicells harvested by spinning for 2 minutes in a microcentrifuge. Each preparation was washed with 1ml of 0.05M Tris-HCl pH 6.8, repelleted, and resuspended in 25ul of loading buffer (0.6g SDS, 1ml β-mercaptoethanol, 4mls Glycerol, 12.5mls 0.5M Tris-HCl pH 5.8 and water up to 10mls). The samples were boiled for 3 minutes, 5ul of 0.1% Bromophenol Blue added and a 5ul sample removed and dried onto glass filters. Filters were

counted in 5mls of scintillation fluid (0.5% Butyl PBD in toluene) in a Packard Scintillation Counter, Model 3003.

2.19 SDS Polyacrylamide Gel Electrophoresis (Laemmli, 1970)

7-20% gradient gels were prepared for the separation of minicell proteins. 7% and 20% solutions of acrylamide were prepared with the solutions in Table 2.5 using the lower tris buffer. Before adding the TEMED, the solutions were degassed using a vacuum line. 16mls of each solution were placed in each side of a linear gradient maker and poured between glass plates (28cms x 16cms) separated by 1mm perspex spacers. The gel was overlaid with isopropanol, and allowed to set for 60 minutes. The stacking gel was prepared, degassed, and after removing the isopropanol from the gel, the TEMED added and the stacking gel poured onto the separating gel with a pasteur pipette. A thirteen tooth perspex comb was inserted and the gel left to set for 30-60 minutes. The comb was removed and the wells rinsed with running buffer. Samples were loaded into the wells, with a 50ul Hamilton syringe, so that the number of counts were approximately the same in each track (about 10^6 cpm/track) and run overnight at a constant current of 10mA. Marker proteins were run in tracks either side of the radioactive samples and included:

Bovine Serum Albumin	M.W. 67,000
Ovalbumin	M.W. 43,000
Carbonic Anhydrase	M.W. 30,000
Soyabean Trypsin Inhibitor	M.W. 20,000
α - lactoglobulin	M.W. 14,000

The acrylamide gel was removed from the glass plates, fixed in 45% Methanol, 9% acetic acid for 10 minutes, stained in 0.1% Coomassie blue, 45% Methanol, 9% acetic acid for 10 minutes, and destained for several hours with 4-5 changes of 7% acetic acid, 5% methanol. Fixing, staining and destaining were all at 37°C on a rotary shaker. Gels were dried down onto 3MM filter paper (Whatman) on a Bio-rad gel drier for 1 hour, and exposed to Kodak XH-1 film for 8-20 hours.

2.20 Assay for Galactokinase

Bacterial cultures were grown to OD₆₅₀ 0.3-0.5 in oxid nutrient broth and 1ml aliquots removed into a glass tube on ice. 40ul of lysis buffer (Table 2.6) and 1 drop of toluene added. The tubes were vortexed and incubated at 37°C for 15 minutes to evaporate the toluene.

¹⁴C - galactose (D - (1-¹⁴C) galactose, Amersham CFA 435) was diluted to a final specific activity of 4.5×10^6 dpm per umole, and filtered twice through DE81 paper in a swinnex filter. A reaction mixture was prepared containing 20ul mix 1, 50ul mix 2, and 10ul ¹⁴C - galactose for each assay, and 80ul distributed into the appropriate number of Eppendorf snap-cap tubes. 20ul of each toluenised culture was added to the tubes and the reactions incubated for 30 minutes at 32°C. The tubes were transferred to ice and two 20ul aliquots of each reaction mixture transferred to DE81 paper filters. The filters were washed in distilled water for four changes of water, blotted, dried in a 65°C oven, and counted in a Packard Scintillation Counter, Model 3003. The counts per minute for the two samples were averaged, and the average counts per minute of two blank filters (prepared by using a reaction mixture with 20ul broth

Table 2.5Solutions for SDS Polyacrylamide Gel Electrophoresis

Acrylamide Stock:	Acrylamide 30g N N' Methylene bisacrylamide 0.8g Water to 100mls Stored dark at 4 ^o C
Upper Tris (x 4):	Tris 6.06g SDS (10%) 4.0mls Water to 100mls pH adjusted to pH 6.9 with H Cl
Lower Tris (x 4):	Tris 18.17g SDS (10%) 4.0mls Water to 100mls pH adjusted to pH 8.8
Running Buffer:	Tris 3g Glycine 14.4g SDS (10%) 10mls Water to 1 Litre

For Separating Gel:/

For Separating Gel:

	<u>7%</u>	<u>20%</u>
Acrylamide Stock	4.7mls	13.2mls
10% Ammonium Persulphate:	25u1	25u1
Lower Tris Buffer (x4):	5mls	5mls
Water:	9.3mls	1.8mls
TEMED	15u1	15u1

For Stacking Gel:

Acrylamide Stock:	1.0ml
10% Ammonium Persulphate:	40u1
Upper Tris (x4)	2.5u1
Water:	6.5ml
TEMED:	15u1

instead of culture) subtracted from this figure. 20ul of two random samples was transferred to DE81 paper and dried without any washing. These were counted too and the average cpm calculated. The number of galactokinase units were calculated using the following equation:

$$\text{galactokinase units} = \frac{(\frac{\text{cpm-blank}}{2} \times 5) \times 5200}{\frac{(\text{Average of unwashed filters} \times 5 \times \text{time of incubation} \times \text{OD}_{650})}{4}}$$

(Adhya and Miller, 1979; Wilson and Hogness, 1966; McKenney et al., 1981).

Table 2.6Solutions for assay of galactokinase

Lysis buffer:	100mM	EDTA
	100mM	DTT
	50mM	Tris HCl pH 8.0
Mix 1:	5mM	DTT
	16mM	NaF
Mix 2:	8mM	Mg Cl ₂
	200mM	Tris HCl pH 7.9
	3.2 mM	ATP

CHAPTER 3Isolation of "TOE" mutants : identification of a new gene, fts Q3.1 Introduction

Cell division in *E. coli* requires the synthesis of the ftsA gene product in a 5-10 minute period during septum formation (Donachie et al. 1979). Mutants in the ftsA gene grow as filaments at the restrictive temperature which are characteristically invaginated at the division site. These observations suggest that the ftsA gene product acts late in septum formation, and is therefore unlikely to be the trigger that initiates the division process. Donachie et al. (1979) suggested from their data with synchronous cultures of OV16 that there is a periodic requirement for synthesis of the ftsA protein. It is not known if ftsA protein is normally synthesised only at the time of requirement in the cell cycle, or synthesised throughout the cycle but only the molecules made during the critical period are used for septation (Donachie et al. 1979). It was reasoned that there must be a class of genes that code for periodically required proteins. This class would include genes that code for proteins that the cell can only respond to periodically, and genes that are synthesised de novo periodically. ftsA is the only known gene of this class, although termination protein and the hypothetical "trigger" for cell division also fall in this class. A novel selection procedure was devised for isolating mutants in such a class of genes.

The "TOE" (Temperature Oscillation Enrichment) selection procedure involves temperature pulsing of an asynchronous culture at every mass doubling. The rationale is that mutants of the desired class would be trapped at the restrictive temperature during successive

cycles, and due to their periodic nature, will not be able to compensate for the lesion at the permissive temperature and filamentous growth will result. Mutants in genes that are not periodically required will recover at the permissive temperature and grow as normal cells. The filaments can be separated from the normal sized cells by filtration through a membrane and recovered at the permissive temperature. Temperature sensitive mutants can then be detected by replica plating of colonies at 30°C and 42°C.

3.2 Isolation of "TOE" mutants

The multiple auxotrophic strain AB2497 was grown to mid-exponential phase in oxid nutrient broth at 30°C, pelleted in a bench centrifuge and resuspended in $\frac{1}{2}$ volume of bacterial buffer. The cell suspension was irradiated in a glass dish with a UV dose of 1000 ergs/mm², diluted into fresh oxid nutrient broth and grown to stationary phase overnight at 30°C.

10mls of this culture were diluted into 200mls of oxid nutrient broth at 30°C, grown to OD₅₄₀ = 0.5 and diluted 1 in 2 into fresh prewarmed oxid nutrient broth at 42°C. 1.5 minutes was required for this culture to equilibrate to 42°C, and then it was heat-pulsed for a further 5 minutes. The culture was cooled to 30°C by placing on ice, and incubated at 30°C again until the OD₅₄₀ = 0.5. Heat pulsing and growth were repeated twice more and the whole culture filtered through a sterile 14u millipore membrane. The membrane was washed through with 200mls of bacterial buffer and placed in 10mls of oxid nutrient broth. Cells were allowed to grow up at 30°C to stationary phase overnight. The temperature oscillation enrichment was repeated with this culture for a second cycle and the cells from the filter again grown to stationary

Fig. 3.1. Phage mapping of TOE1 and TOE13 mutations

The upper part of the diagram shows the complementation pattern of λ transducing phages. A + sign represents the ability for that phage to complement a mutant in the above gene. The bacterial insert in each phage is represented as an open box.

$\lambda 16-4$ is a transducing phage similar to $\lambda 16-2$ except an fts A^{ts} allele has been recombined onto the phage. (Lutkenhaus and Donachie, 1979).

$\lambda dd1^+$ is identical to $\lambda \Delta R2$ show in Fig. 1.5.

The construction of $\lambda FH16$ and $\lambda GH200$ is described in chapter 4.

Restriction sites Hind III (\blacktriangledown) and Eco RI (\triangle) are shown.

The map location of fts Q is defined by the complementation pattern of TOE1 and TOE13 with these transducing phages, shown in the lower part of the diagram.

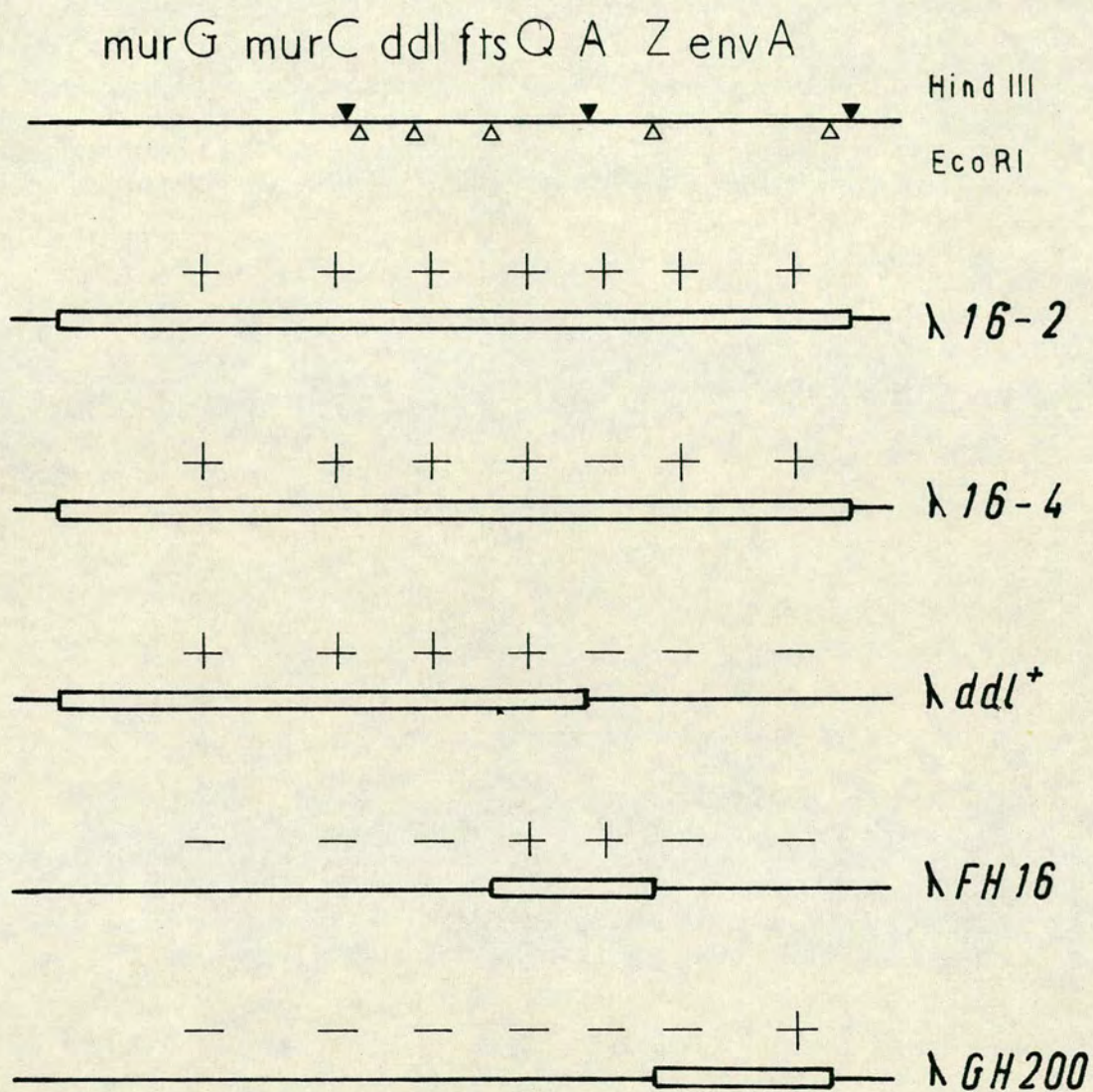


Fig 3.1

TOE I		TOE I3		
+	+	+	+	$\lambda 16-2$
+	-	-	-	$\lambda 16-4$
+	-	-	-	λddl
+	+	+	+	$\lambda FH16$
-	-	-	-	$\lambda GH200$

phase. Dilutions of this culture were spread onto oxoid nutrient plates and incubated overnight at 30°C so that each plate had about 100 small colonies. These master plates were replica plated onto two oxoid nutrient agar plates, which were incubated overnight at 30°C and 42°C. About 1% of clones were temperature sensitive and these were picked from the 30°C plate for further examination.

3.3 Characterisation of "TOE" mutants

From the initial selection two temperature sensitive *mutations*, designated TOE 1 and TOE 13, were co-transducible with leu (temperature resistance was 75% co-inherited when the mutants were transduced to leu⁺ with P1 grown on W3110) and were complemented in spot tests with λ 16-2. The results of a more detailed complementation analysis with *derivatives* of λ 16-2 are shown in Fig. 3.1. This complementation data suggests that the TOE 1 mutation maps in a previously unidentified gene that we have designated ftsQ (Begg, Hatfull and Donachie 1980). ftsQ is adjacent to ftsA and anticlockwise of it. Complementation data with TOE 13 (Fig. 3.1) maps the mutation in ftsA.

At the restrictive temperature TOE 13 grows as long filaments which are characteristically invaginated at the division site, as are other ftsA mutants (Donachie *et al.* 1979). TOE 1 differs from TOE 13 in that at the restrictive temperature it grows as long filaments which are straight sided and not invaginated (Fig. 3.2).

TOE 1 also differs from TOE 13 in that the ability to form colonies at the restrictive temperature can be reversed by addition of 1% NaCl to the media. The effect of intermediate NaCl concentrations

Fig. 3.2. Photograph of a filamentous cell of TOE1 grown at 42°C

This filamentous cell of strain TOE1 (fts Q^{ts}) has been growing for four generations at 42°C and has reached a length of approximately 64um.

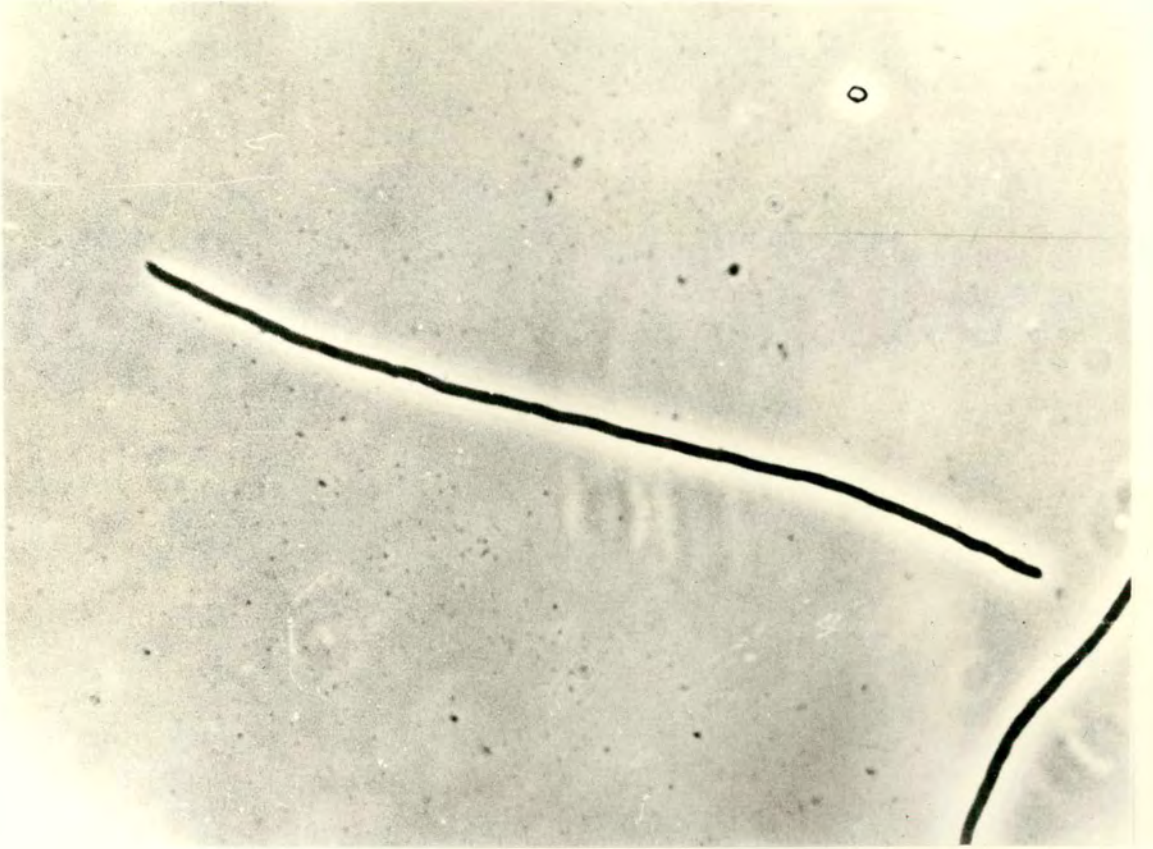


Fig 3.2

Table 3.1. Effect of NaCl concentration on colony forming ability of TOE1 and TOE13 at 42⁰C

	<u>% NaCl in agar</u>					
	<u>0.5</u>	<u>0.6</u>	<u>0.7</u>	<u>0.8</u>	<u>0.9</u>	<u>1.0</u>
TOE 1	-	-	-	<u>±</u>	+	+
TOE 13	-	-	-	-	-	-

+ designates healthy growth

- absence of growth

± some growth

on the colony forming ability of TOE 1 and TOE 13 is shown in Table 3.1.

A recA derivative of TOE 1 was constructed by mating TOE 1 with JC10-240 for 25 minutes at 30°C and plating onto oxid nutrient agar containing streptomycin (200 ug/ml) and tetracycline (20 ug/ml). Forty clones were tested, and all were found to be sensitive to nitrofurantoin (4 ug/ml) and to have an increased sensitivity to UV-irradiation. One clone was purified, checked for temperature sensitivity and designated TOE 1 recA.

Spot test complementation tests with TOE 1 recA gave identical results to those shown in Fig. 3.1. Lysogens of TOE 1 recA were constructed at 30°C with λ 16-2, λ ddl⁺ and λ FH16 and tested for temperature sensitivity on oxid nutrient plates. All lysogens were temperature resistant.

3.4 Physiological analysis of TOE 1

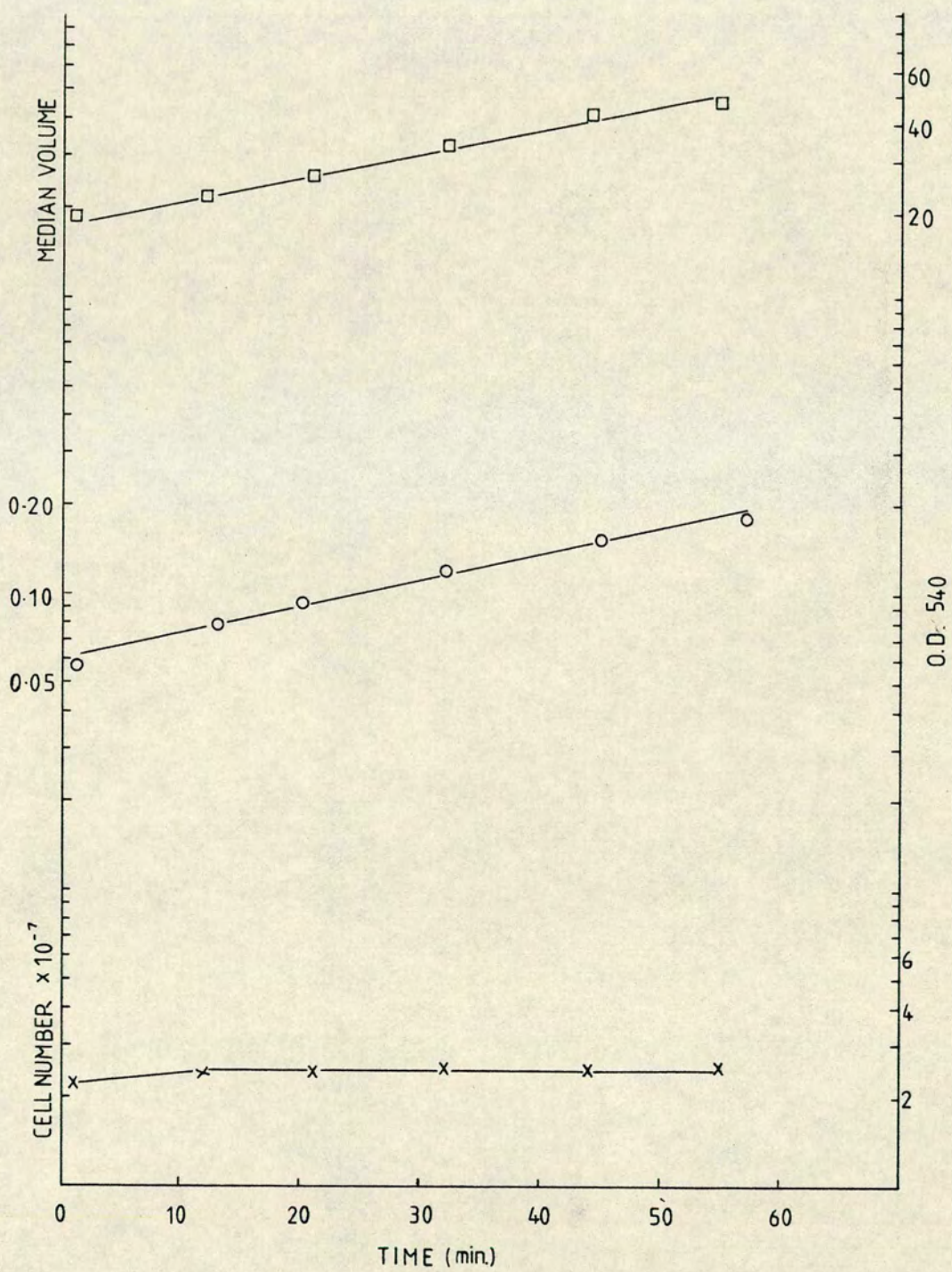
3.4.1 Kinetics of cell division during temperature shifts in asynchronous cultures

An overnight culture of TOE 1 in oxid nutrient broth was diluted into the same medium and grown at 30°C into exponential phase. (Microscopical examination revealed that in early log phase growth the mutant cells separate poorly and tend to grow as chains, although the culture becomes more homogeneous with better cell separation after 2-3 hours growth). On shifting this culture to 42°C cell division stopped immediately, although cell growth continued exponentially for at least three mass doublings resulting in the formation of long filaments (Fig. 3.2 and Fig. 3.3). These filaments were examined

Fig. 3.3. Kinetics of cell growth and division in a log-phase population of strain TOE1 after a shift from 30°C to 42°C

After shifting to the restrictive temperature, cell mass was followed as OD_{540} (○), and cell number (x) and median cell volume (□) measured in the Coulter Counter and Channelyzer.

Fig 3.3



microscopically on agar containing chloramphenicol (200 ug/ml) and 23% polyvinylpyrrolidone (Donachie et al. 1979), and observed to be multinucleate indicating that DNA replication and segregation were not affected. These results are consistent with the idea that the TOE 1 mutation is in a late acting gene involved specifically in cell division.

3.4.2 Kinetics of cell division following a temperature shift-down in synchronous culture of TOE 1

Small cells from a 400 ml starting culture of TOE 1 were prepared by sucrose gradient centrifugation as described in chapter 2, and inoculated into 35 mls of prewarmed oxid broth at 42°C. 5 mls samples were removed at times 0, 25, 30, 40, 45 and 50 minutes and incubated in separate flasks at 30°C. A culture was kept at 42°C throughout the experiment as a 42°C control culture. OD₅₄₀ readings and samples for cell size and number determination were taken every ten minutes from each culture. Fig. 3.4 shows the OD₅₄₀, cell number and median cell size plotted against time. A microscopical examination of the cultures indicated that after septum formation, cell separation was poor, as was observed in early log-phase asynchronous cultures. Cell number determination by the Coulter Counter does not therefore give a reliable assessment of the septation process, but only of cell separation. In order to quantitatively analyse the septation process the percentage of dividing cells was estimated by microscopically examining the formaldehyde fixed samples, and scoring the number of cell doubles (those containing a visible constriction). 100 cells from each sample were counted. The percentage of dividing cells in this experiment are shown in Table 3.2. The cell numbers corrected

Fig. 3.4. Kinetics of cell division following a temperature shift-down in synchronous cultures of TOE1

Aliquots of a synchronous culture of strain TOE1 were shifted from 42°C to 30°C at various time intervals after inoculation of the small cells into broth at 42°C. Cell mass was followed as OD₅₄₀ (○)^(x10⁻¹), and cell number (x10⁸) (▲) and median cell volume (□)^(x10⁻¹) measured in the Coulter Counter and Channelyzer.

The time that samples were shifted to 30°C is shown in a box at the top left of each graph.

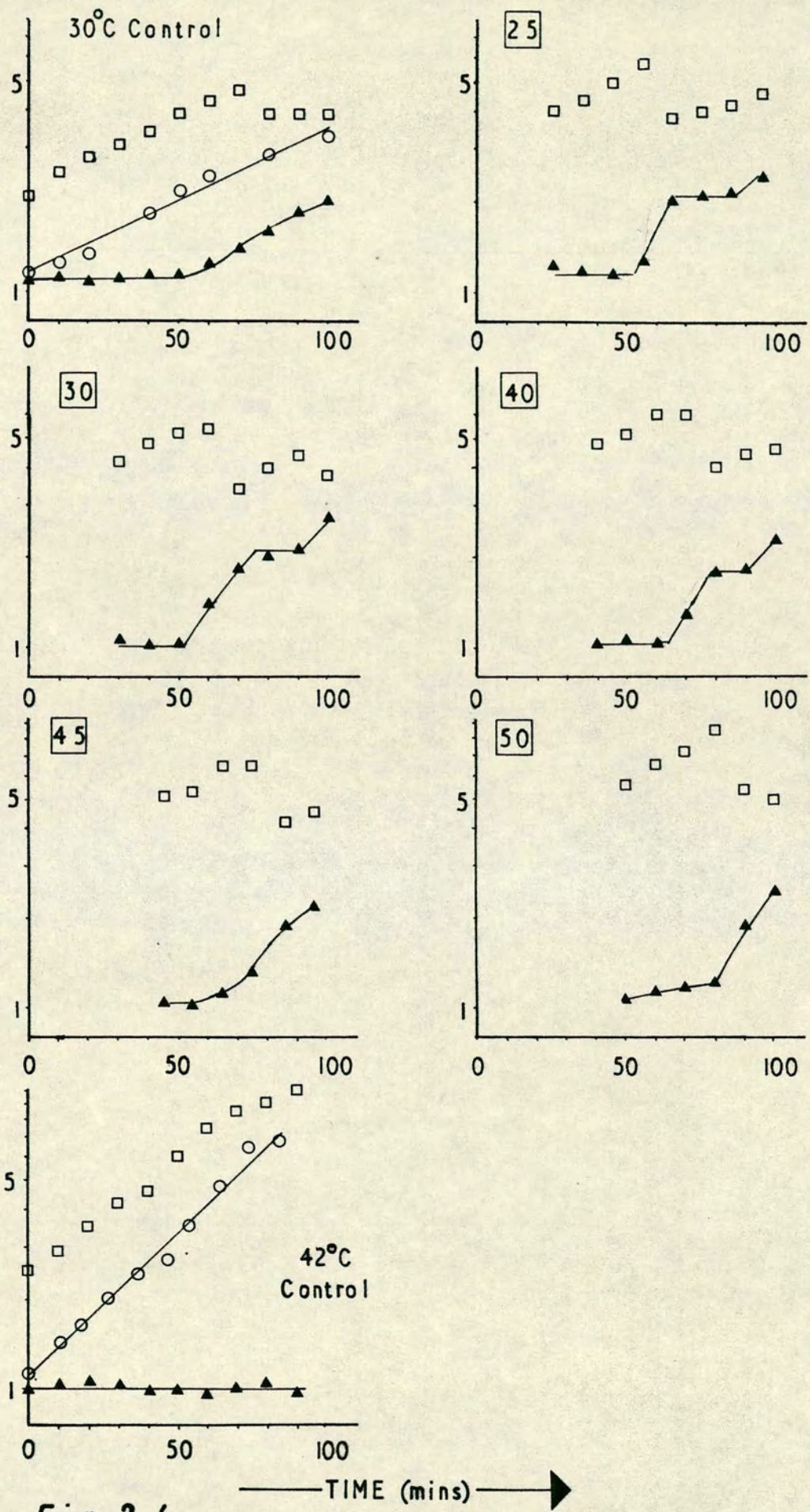


Fig 3.4

— TIME (mins) —→

Table 3.2. Percentage of cells containing a visible constriction in synchronous samples of TOE 1 shifted from 42°C to 30°C
(see Figs. 3.4 and 3.5).

<u>Shifted at 0 minutes (%)</u>		<u>Shifted at 25 minutes (%)</u>	
0'	4	25'	2
10'	4	35'	4
20'	2	45'	50
30'	2	55'	68
40'	14	65'	20
50'	28	75'	22
60'	42	86'	10
70'	62	96'	50
80'	50	96'	50
90'	32		
100'	34		

<u>Shifted at 30 minutes (%)</u>		<u>Shifted at 40 minutes (%)</u>	
30'	2	40'	2
40'	2	50'	6
50'	74	60'	66
60'	28	70'	66 (approx. 1/3 2nd cycle)
70'	6	80'	14
80'	10	90'	22
90'	40	100'	26
100'	28	100'	26

Table 3.2. (continued)

<u>Shifted at 45 minutes (%)</u>		<u>Shifted at 50 minutes (%)</u>	
45'	2	50'	2
55'	2	60'	2
65'	2	70'	42
75'	74 (approx. $\frac{1}{2}$ 2nd cycle)	80'	82 (approx. $\frac{2}{3}$ 2nd cycle)
86'	20	90'	38
96'	34	100'	16

Fig. 3.5. Corrected cell numbers in synchronous samples of TOE1 shifted from 42⁰C to 30⁰C

Cell numbers, as measured in the Coulter Counter in Fig. 3.4, were corrected for the percentage of cells containing a visible constriction. The corrected cell number (\blacktriangle) is shown here for each sample ($\times 10^8$).

The time that samples were shifted to 30⁰C is shown in a box at the top left of each graph.

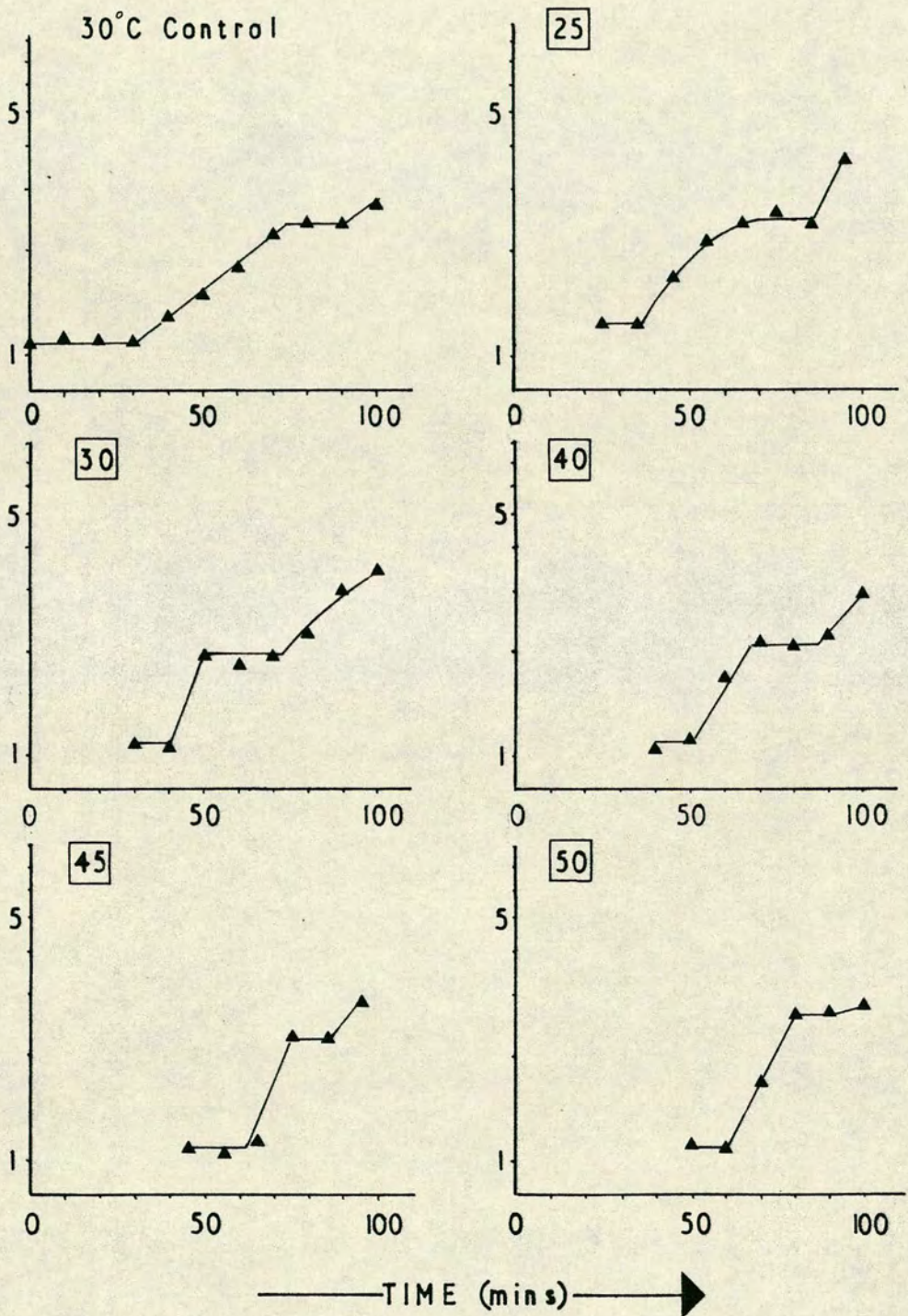


Fig 3.5

for the percentage of dividing cells are shown in Fig. 3.5.

3.4.3 Temperature pulsing of synchronous fractions of TOE 1

Small cells from a 400 ml starting culture of TOE 1 were prepared as in chapter 2, and inoculated into 45 mls of oxid nutrient broth, prewarmed at 30°C. At time 0 minutes 5 mls was removed into a separate flask prewarmed at 42°C and incubated throughout the experiment as a 42°C control culture. At times 0, 25, 30, 40, 45 and 50 minutes, 5 ml samples were removed into flasks prewarmed at 42°C and heat pulsed at 42°C for 8 minutes. Each sample was then incubated at 30°C for the remainder of the experiment. OD₅₄₀ readings and samples for cell number and size determination were taken at suitable intervals from each sample. Fig. 3.6 shows these parameters plotted against time for each sample. As in other experiments with TOE 1, cell separation was again poor and the percentage of dividing cells in each sample were estimated (Table 3.3). The corrected cell numbers are shown in Fig. 3.7.

Fig. 3.6. Kinetics of cell division following temperature pulsing of synchronous cultures of TOE1

Small cells from a sucrose gradient were inoculated into broth (30°C) at 0 minutes. Aliquots were shifted at various times for 8 minutes at 42°C and then returned to 30°C. Cell mass was followed as OD₅₄₀ (○)^(x10⁻¹), and cell number (▲)^(x10⁷) and median cell volume (□)^(x10⁻²) in the Coulter Counter and Channelyzer. There was insufficient volume of culture to follow the OD₅₄₀ beyond 80 minutes. OD₅₄₀ readings were not taken in samples other than the 0 minute pulse culture, and 30°C and 42°C control cultures.

The time that samples were shifted to 30°C is shown in a box at the top left of each graph.

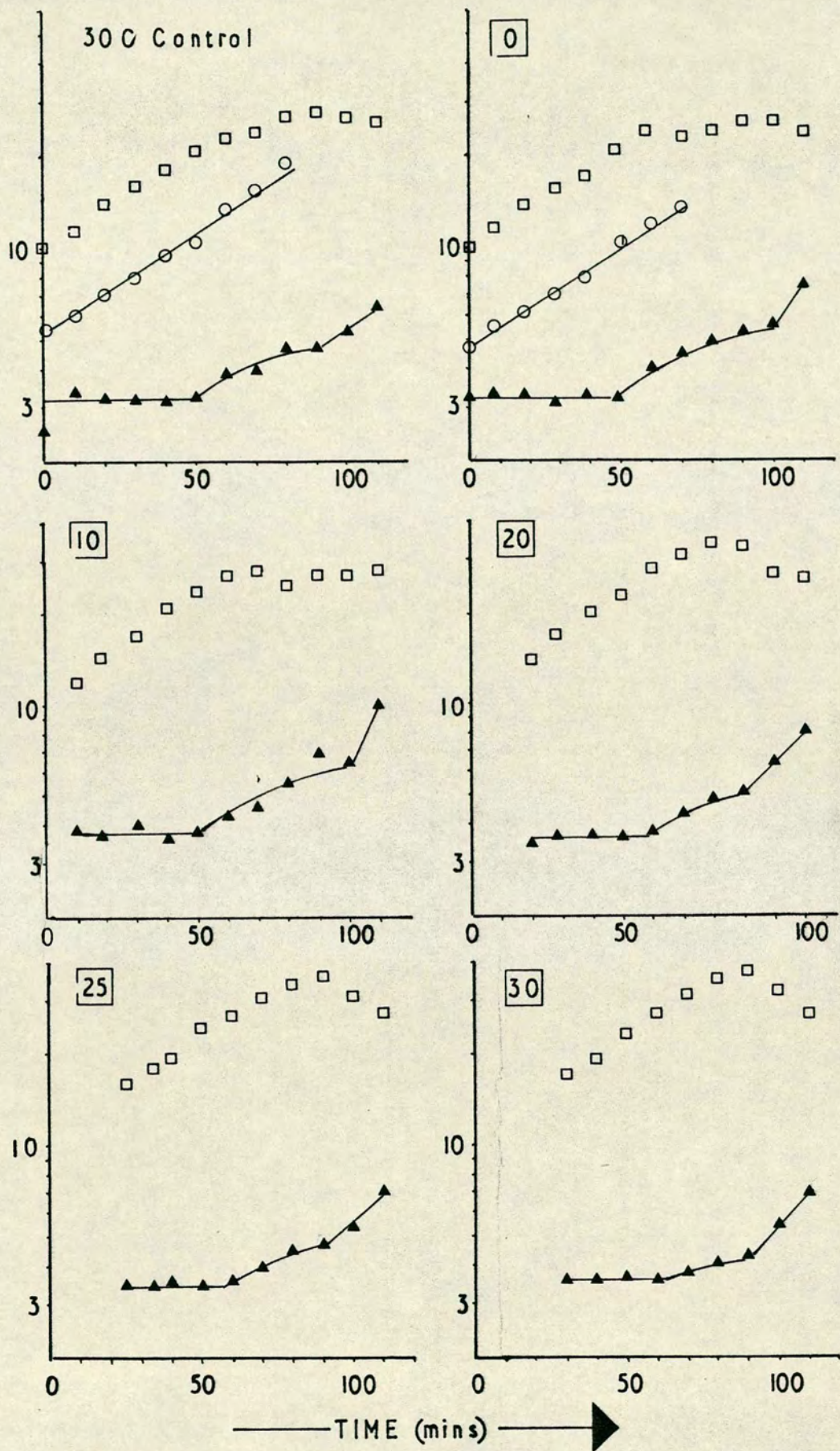
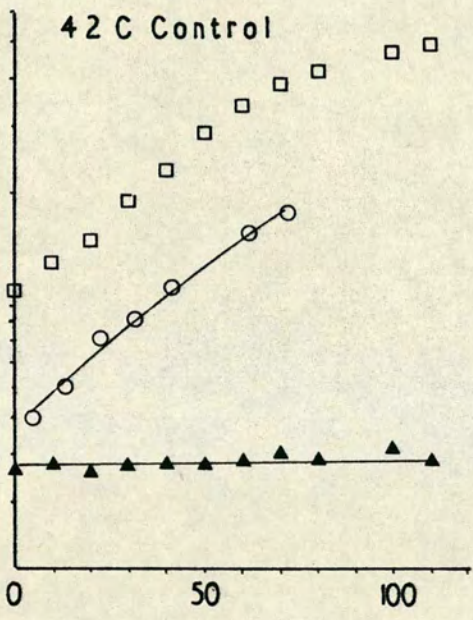
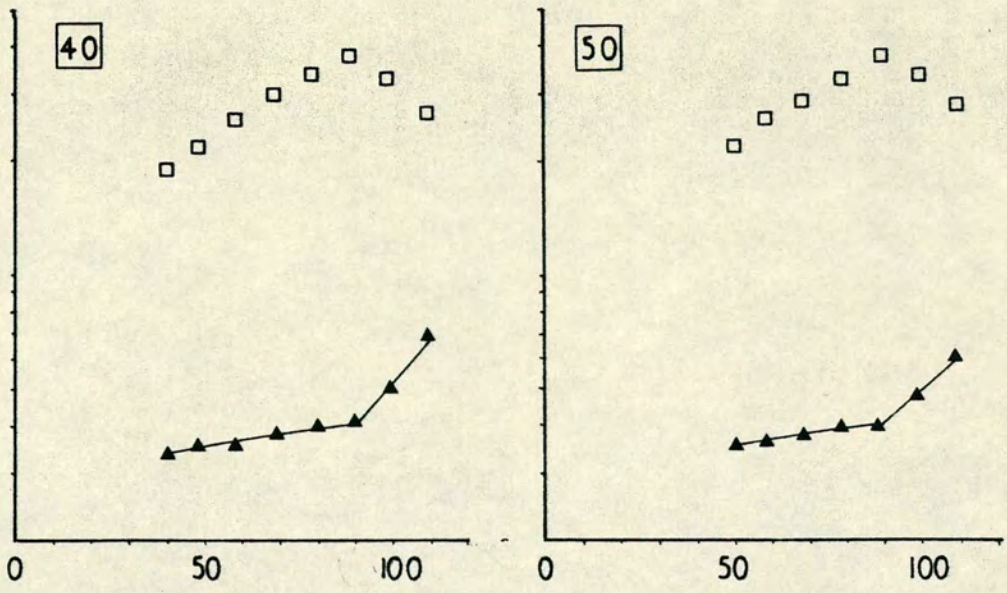


Fig 3.6



— TIME (mins) —>

Fig 3.6 (cont.)

Table 3.3. Percentage of cells containing a visible constriction in synchronous samples of TOE, temperature pulsed at 42⁰C
(see Figs. 3.6 and 3.7)

<u>30⁰C Control (%)</u>		<u>Pulse at 0' minutes (%)</u>	
0'	2	0'	2
10'	2	8'	2
20'	2	18'	2
30'	6	28'	2
40'	10	38'	30
50'	46	50'	42
60'	70	60'	52
70'	52	70'	44
80'	42	80'	36
90'	48	90'	60
100'	70	100'	68
110'	48	110'	58

<u>Pulse at 10 minutes (%)</u>		<u>Pulse at 20 minutes (%)</u>	
10'	2	20'	2
18'	2	28'	2
30'	2	40'	4
40'	10	50'	28
50'	64	60'	78
60'	84	70'	80
70'	64	80'	68
80'	34	90'	62

Table 3.3 (continued)

Pulse at 10 minutes (%)

90'	46
100'	68
110'	40

Pulse at 20 minutes (%)

90'	62
100'	58
110'	36

Pulse at 25 minutes (%)

25'	2
33'	2
40'	4
50'	28
60'	60
70'	64
80'	66
90'	62
100'	70
110'	52

Pulse at 30 minutes (%)

30'	2
38'	2
50'	14
60'	42
70'	78
80'	66
90'	74
100'	64
110'	42

Pulse at 40 minutes (%)

40'	14
48'	16
58'	18
68'	30
78'	54

Pulse at 50 minutes (%)

50'	44
58'	36
68'	42
78'	36
88'	78

Table 3.3 (continued)

<u>Pulse at 40 minutes (%)</u>		<u>Pulse at 50 minutes (%)</u>	
89'	70	98'	78
99'	68	108'	48
109'	56		

Fig. 3.7. Corrected cell numbers in synchronous samples of TOE1 temperature pulsed at 42⁰C

Cell numbers, as measured in the Coulter Counter in Fig. 3.6, were corrected for the percentage of cells that contained a visible constriction. The corrected cell number (\blacktriangle) is shown here for each sample ($\times 10^7$)

The time that samples were shifted to 30⁰C is shown in a box at the top left of each graph.

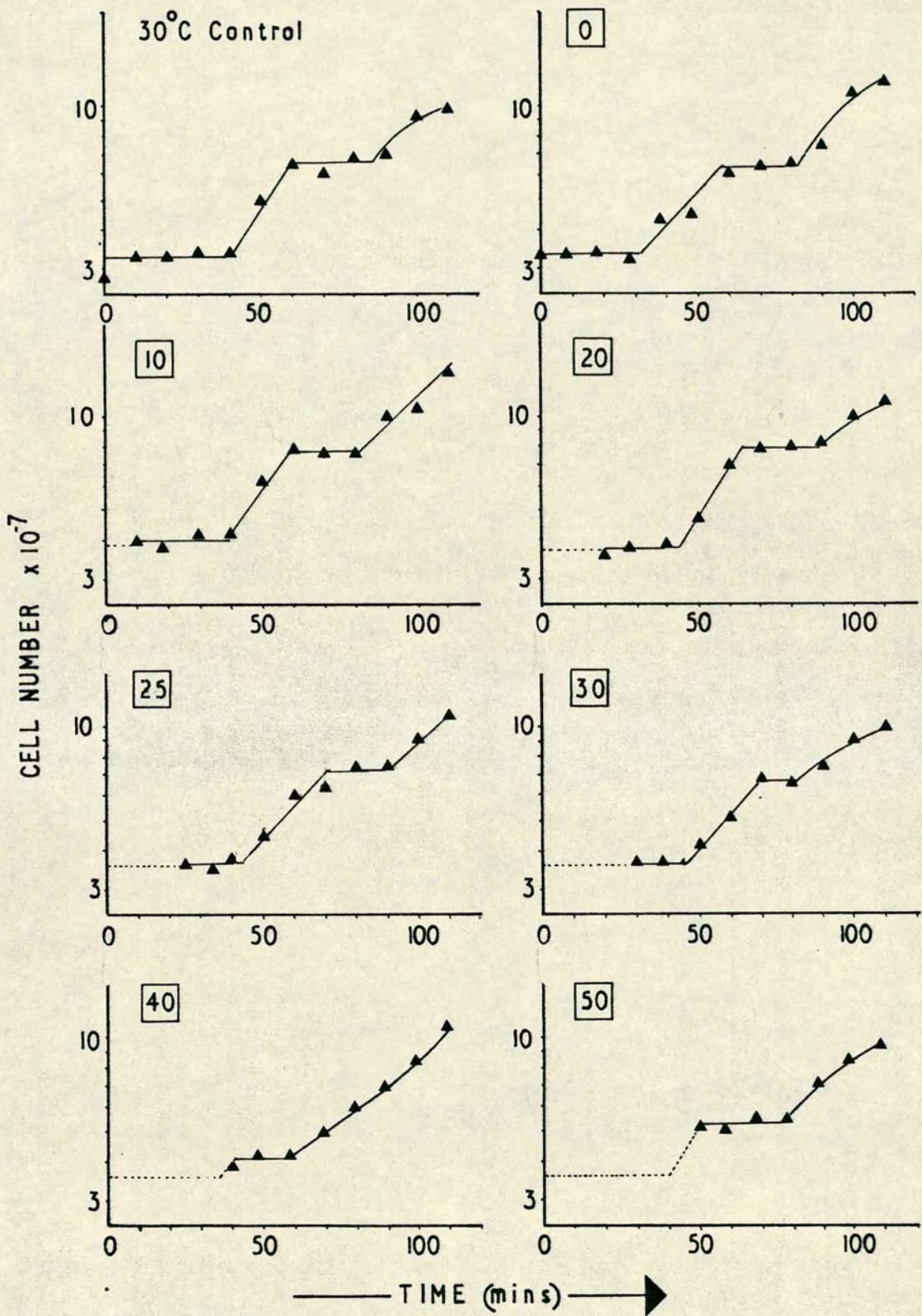


Fig 3.7

3.5 Discussion

The TOE selection procedure has been used to isolate mutants in essential cell division genes. The procedure was devised to isolate mutants in genes that were periodically required for cell division, although it is not yet clear if the selection enriches for these mutants above the total population of mutants in cell division genes. However, a more detailed analysis by Dr. Begg (in this laboratory) shows that of the 35 mutants so far isolated, 11 are linked to leu by P1 transduction, and 7 of the 35 mutants map in the ftsA gene. Two mutants (including TOE 1) have been isolated in the ftsQ gene. A control experiment is in progress (by Dr. Begg) to isolate total filament forming temperature sensitive mutants by filtration, and examine the proportion of mutants linked by P1 transduction to leu and the proportion of ftsA and ftsQ mutants. If the results from the control experiment differ from the TOE selection procedure, this would indicate strongly that "TOE" selection does enrich for mutants in periodically required genes.

The isolation of TOE 1 has been extremely useful. Complementation with the transducing phages clearly identifies this mutant as mapping in a previously unidentified gene, that we have designated ftsQ. This gene maps adjacent and anticlockwise to ftsA, but unlike all the ftsA mutants so far isolated, does not have invaginations in the filaments (at the restrictive temperature) and the temperature sensitive lesion can be reversed by increasing the salt concentration to 0.9%. The possibility that the TOE 1 mutation lies in the N-terminal part of the ftsA gene, and λ ddl⁺ complements by polypeptide complementation is considered unlikely. λ ddl⁺ carries nearly all of the ftsA gene (missing only 150-300 bps) but

doesn't complement any of the ftsA mutants so far isolated (e.g. TKF12, OV16). Complementation data in chapter 5 confirms that the mutation does not lie in ftsA. Complementation of TOE 1 and TOE 1 recA by the λ transducing phage as lysogens, or as a spot test where phage promoters are switched off (Reichardt and Kaiser 1971) would suggest that a promoter for ftsQ is present on the bacterial insert, and more specifically, on the 2.2kb EcoRI restriction fragment. This fragment also carries the ftsA gene, which codes for a 50 Kilodaltons protein (Lutkenhaus and Donachie 1979). Assuming an average amino acid weight of 115 daltons there is sufficient coding capacity on the 2.2kb EcoRI fragment to code for an additional 34 Kilodalton protein, although some of the coding capacity may be occupied by the N-terminal part of the ftsZ gene, and possibly the C-terminal part of the ddl gene. (Lutkenhaus and Wu 1980).

The physiological analyses of the TOE 1 mutant were designed to determine a) whether the ftsQ product was required throughout septation, or only in the initiation of septation, and b) whether the ftsQ product was required periodically.

The physiological analyses of TOE 1 are complicated by the failure of the cells to separate well in synchronous fractions. This failure to separate also occurs in asynchronously growing cultures of TOE 1. The mutation not only prevents septum formation at 42⁰C but also inhibits cell separation at 30⁰C. The estimation of cell number by the Coulter Counter is therefore difficult to interpret, as it is a function of both septum formation, and cell separation, and the degree of cell separation appears to vary greatly in different cultures, and cells in mid-log asynchronous growth cultures of TOE 1 actually separate quite

well. The problem of cell separation can be partially overcome by counting the percentage of cells that can be seen to be dividing and adjusting the Coulter Counter measurement of cell number accordingly. However, this introduces two new problems. Firstly, cells with a visible constriction are effectively scored as two cells, before they have completed septation, and this has the effect of apparently bringing forward the time of division. Secondly, it is assumed that those cells containing a visible constriction will go on to complete a division. In a cell division mutant which has spent some time at the restrictive temperature, this will be a false assumption in some cell samples. For those reasons, cell numbers, (Coulter Counter), the percentage dividing cells, and the corrected cell number are all presented.

There are three lines of evidence to suggest that the ftsQ product is required throughout septation. The filaments produced at the restrictive temperature are predominantly straight-sided and do not contain the invaginations that ftsA filaments do. Presumably the ftsQ product is required at a fairly early stage in the division process. However, cell separation is also affected by the ftsQ mutation, and this is reflected by the physiology of TOE 1 at 30°C, suggesting that the ftsQ product may be required right up to the last stages of division. Temperature shifting an asynchronous culture of TOE 1 from 30°C to 42°C (Fig. 3.3) results in an immediate stop to cell division, and this also suggests that the ftsQ product is required throughout cell division. If ftsQ was only required for the initiation of septation, then those cells that had initiated septa at the permissive temperature would go on to divide, and residual division would be observed. The quick stop

of division also suggests that the ftsQ product in TOE 1 is inactivated very quickly at the restrictive temperature.

The results in Fig. 3.4 show that the ftsQ product is not required at all in the first 25-30 minutes of the division cycle, as the timing of division does not appear to be significantly affected by a 25 or 30 minute period of growth at 42°C, and these samples divide at about the same time as the 30°C control culture (about 60 minutes into the cycle). The period at 42°C appears to improve the separation of cells after septation at 30°C. The correction for % dividing cells (Fig. 3.5) for these cultures, also suggests that the cultures shifted at 25 minutes and 30 minutes divide at the same time as the 30°C control. This therefore accords with the expected behaviour of a mutant in a gene that is required specifically for division.

In experiment 3.4.2, samples were also held for longer periods at 42°C, to determine if the ftsQ product is periodically required. The rationale for this experiment was that if the ftsQ product is not periodically required, then cells in samples held at 42°C when the first division was due to occur would be able to divide immediately they were returned to 30°C (or after a constant delay). If the ftsQ product was periodically required, then these samples would not be able to divide until they reached the next period in the second cycle.

The results in Fig. 3.4 and Fig. 3.5 indicate that in this experiment it is difficult to differentiate between a constant delay and the "missing" of a cell division. In Fig. 3.4, all the samples divide approximately 30 minutes after shifting back to 30°C. This may not be interpreted as a constant delay however, as an inspection of Table 3.2

shows that due to the greater growth rate at 42⁰C the 40, 45 and 50 minute samples all have a large number of second cycle divisions occurring by 70 minutes into the cycle.

Experiment 3.4.3 was devised to attempt to overcome the problems in the above experiment. Having defined the period when ftsQ is required in the cell cycle, synchronous cultures were pulsed for only 8 minutes at the restrictive temperature, so that the difference in growth rate between different samples would not be appreciable, and that if there was a constant delay after returning to 30⁰C (i.e. not periodically required) this would be distinguishable from entry into the second cycle.

Fig. 3.6 shows that all the samples in this experiment, including the 30⁰C control sample separated badly, and the cell numbers can not be interpreted without correction for the percentage dividing cells (Fig. 3.7). An examination of Fig. 3.7 shows that 8 minute pulses at 0, 10, 20 and 25 minutes into the cycle do not affect the time of the first division (about 50 minutes into the cycle), and they all septate at about the same time as the 30⁰C control. The second cycle divisions in the 0, 10, 20 and 25 minutes samples all occur at the same time (90 minutes), and slightly earlier than the 30⁰C control (100 minutes) presumably due to the slightly greater growth rate at 42⁰C.

The first cycle division is affected by temperature pulsing at 30, 40 and 50 minutes. In the 30 minute sample, the division is very slightly delayed, and in the 40 minute sample, although septum formation has already begun when shifted to 42⁰C, it is halted, and restarts about 10 minutes after shifting back to 30⁰C. In this sample, the delay in the first division causes it to run into the second cycle, so that there is

no plateau in cell number between the divisions. In the 50 minute sample, septum formation has already begun when shifted to 42°C. As in the 40 minutes sample, septum formation and cell separation stopped immediately, but cell number does not rise after returning to 30°C until 80-90 minutes when the second cycle has arrived. This physiological data would suggest therefore that the ftsQ product ~~may be~~ periodically required for cell division.* If the product is not active during the critical period, then septation can not occur, and only resumes when the critical period is reached during the next cell cycle. This periodicity could be^a periodic requirement for synthesis ^{as suggested} for ftsA, or there could be periodic competence for the cell to respond to active ftsQ product irrespective of when in the cycle the product was synthesised. Unfortunately, an amber mutant is not available in ftsQ to physiologically differentiate these two aspects.

The following conclusions can be made:

- (a) "TOE" selection procedure has been used to isolate cell division mutants, and amongst these are mutants in ftsA and a new gene ftsQ.
- (b) Physiological analyses with TOE 1 (ftsQ^{ts}) suggest that the ftsQ product may be periodically required.* This does not prove that "TOE" selection successfully enriches for mutants in periodically required genes, as the frequency with which these mutants are isolated in the control experiment is not yet known, and TOE 1 may have been selected for a much simpler reason, such as its very poor cell separation.

- (c) The gene ftsQ and its promoter map on a 2.2kb EcoRI restriction fragment, adjacent and anticlockwise to ftsA.

* An alternative explanation of the data is that partially formed septa are sensitive to interruptions, so that samples shifted during septum formation take longer to recover when returned to the permissive temperature. A periodic requirement for a product cannot be distinguished from sensitivity of septa during their formation.

CHAPTER 4

Cloning of the 2.2kb Eco RI Restriction Fragment

4.1 Introduction

The λ 16-2 transducing phage (Fig. 1.2) isolated by Lutkenhaus and Donachie (1979) contains a chromosomal insert of approximately 10.5kb and carries at least seven complete chromosomal genes. (Lutkenhaus and Wu 1980,^{Boyd} Hatfull and Donachie 1980. A detailed analysis of these genes (and in particular ftsA) can be simplified by the subcloning of restriction fragments from this phage (Lutkenhaus, Wolf-Watz and Donachie 1980). An attempt was made to subclone a restriction fragment that carried the ftsA gene.

Two different cloning systems were used to subclone the Eco RI fragments from λ 16-2: λ cloning vectors and non-conjugative copy number plasmids. λ transducing phage are convenient for complementation analysis and the mapping of chromosomal mutations, whilst restriction analysis and construction of small deletions is easier in small plasmid recombinants.

4.2 Subcloning into λ vectors

Two vectors were used for subcloning Eco RI restriction fragments: λ NEM616 and λ NEM607 (Fig. 4.1). λ NEM616 is a non-temperature sensitive immunity derivative of λ NEM816 (Wilson and Murray 1979), a replacement vector derived from λ plac5. It is integration proficient, immunity 21, and carries the entire lacZ gene and its promoter. Part of the lacZ gene (and the promoter) lies between the two Eco RI restriction sites and can be replaced by donor DNA to give recombinant

Fig. 4.1. Physical maps of λ phages used to clone the 2.2Kb Eco RI restriction fragment

λ NEM616 is a replacement vector, with immunity 21. The promoter and part of the structural gene of lac Z lie between the two Eco RI sites, so that replacement of this fragment gives the phage a lac⁻ phenotype. (Wilson and Murray, 1979).

λ NEM607 is an immunity insertion vector. There is a single Eco RI restriction site within the immunity 434 region, so that the insertion of Eco RI fragments into this site results in phage with a clear plaque morphology. (Murray, Brammar and Murray, 1977).

λ 16-2 Δ E is a deletion derivative of λ 16-2. The extent of the deletion is not known, although this phage has not lost any Eco RI or Hind III restriction sites. The open box represents the bacterial insert in this phage.

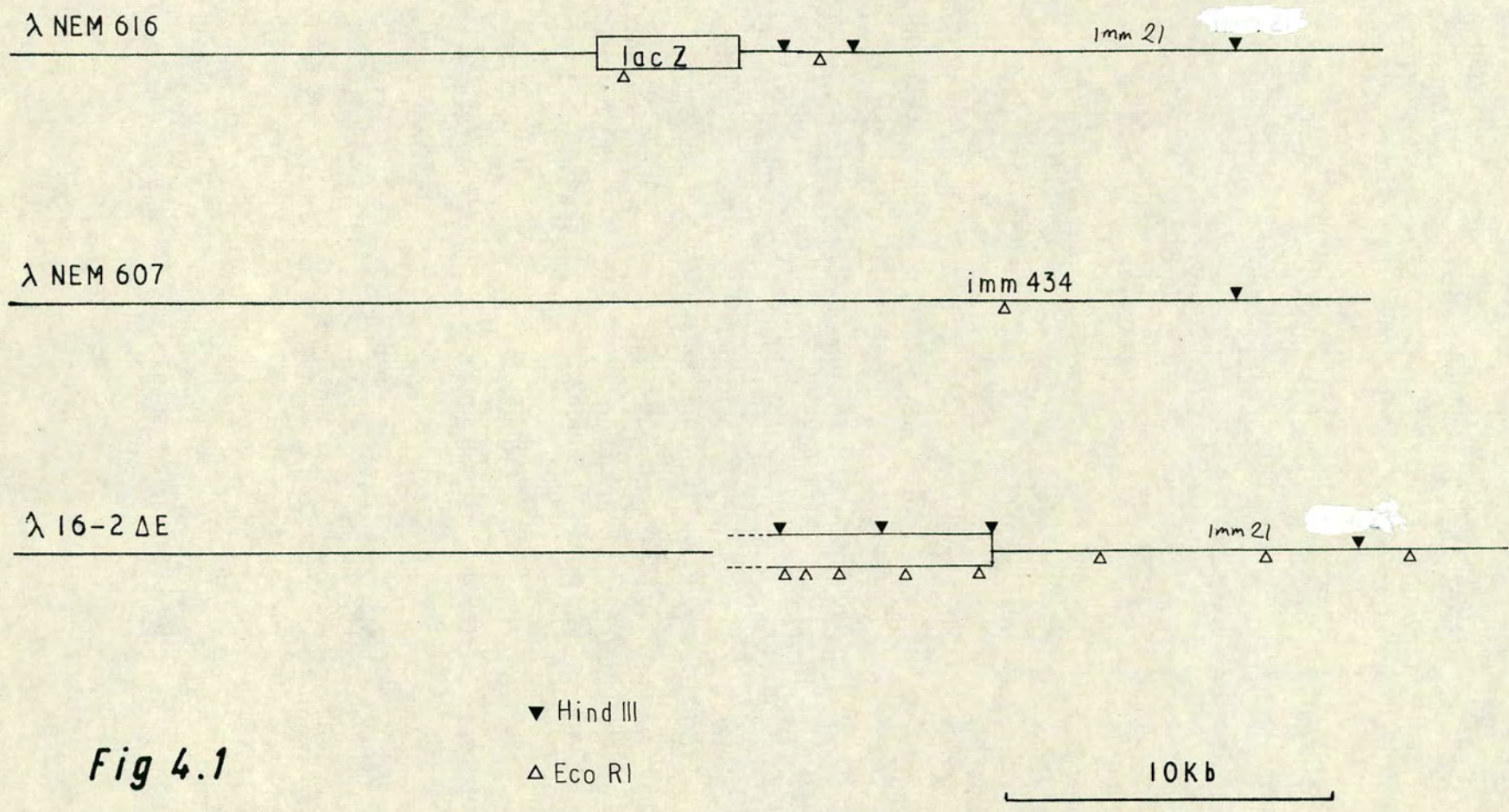


Fig 4.1

phage with a Lac phenotype. If there is no DNA inserted between the Eco RI sites then there is insufficient DNA to efficiently package into the phage heads, and the phage grow as very small 'pin-point' plaques. (Murray, Brammar and Murray 1977).

λ 16-2 Δ E (Fig. 4.1) was used to donate bacterial DNA, and is a deletion derivative of λ 16-2 (Lutkenhaus, Wolf-Watz and Donachie 1980). This phage differs from λ 16-2 in that bacterial DNA anticlockwise from the left-most Hind III site has been deleted, so that λ 16-2 Δ E does not complement mur C or mur G mutants. No Hind III or Eco RI restriction sites have been lost in the construction of λ 16-2 Δ E (see Fig. 1.4. for a comparison).

λ 16-2 Δ E was digested to completion with Eco RI, ligated with EcoRI digested λ NEM616 and plaques recovered by transfection into AA125. The transfection mixture was plated onto MacConkey lactose agar plates and 20 non-red plaques picked from the plates with sterile pasteur pipettes, into 1ml aliquots of phage buffer. Phage suspensions were sterilised with chloroform and tested for the ability to complement TKF12 (ftsA) at 42⁰C or D22 (envA) on agar containing 5 ug/ml Rifampicin (Normark 1970). Four phage transduced the envA marker, and four phage transduced the ftsA marker, although no phage complemented both markers. One of the recombinant phage that complemented envA was designated λ GH200, and shown to contain a 2.4kb Eco RI insert, but was not characterised further.

A recombinant phage that transduced the ftsA marker was designated λ GH16, and an Eco RI restriction digest showed that it contained a 2.2kb Eco RI fragment, inserted into the phage arms (Fig. 4.2). The right arm must come from λ NEM616 as this arm of λ 16-2 Δ E is cleaved

Fig. 4.2. 0.8% agarose gel of Hind III and Eco RI digestions of λ NEM616, λ GH616 and λ 16-2 Δ E

<u>Track</u>		
1	λ 16-2 Δ E	Hind III + Eco RI
2	λ 16-2 Δ E	Eco RI
3	λ 16-2 Δ E	Hind III
4	λ GH16	Hind III + Eco RI
5	λ GH16	Eco RI
6	λ GH16	Hind III
7	λ NEM616	Hind III + Eco RI
8	λ NEM616	Eco RI
9	λ NEM616	Hind III

Fig. 4.3. 0.8% agarose gel of Eco RI restriction digestions of λ GH16, λ NEM607 and λ NY-1

<u>Track</u>		
1	λ GH16	Eco RI
2	λ NEM607	Eco RI
3	λ NY-1	Eco RI

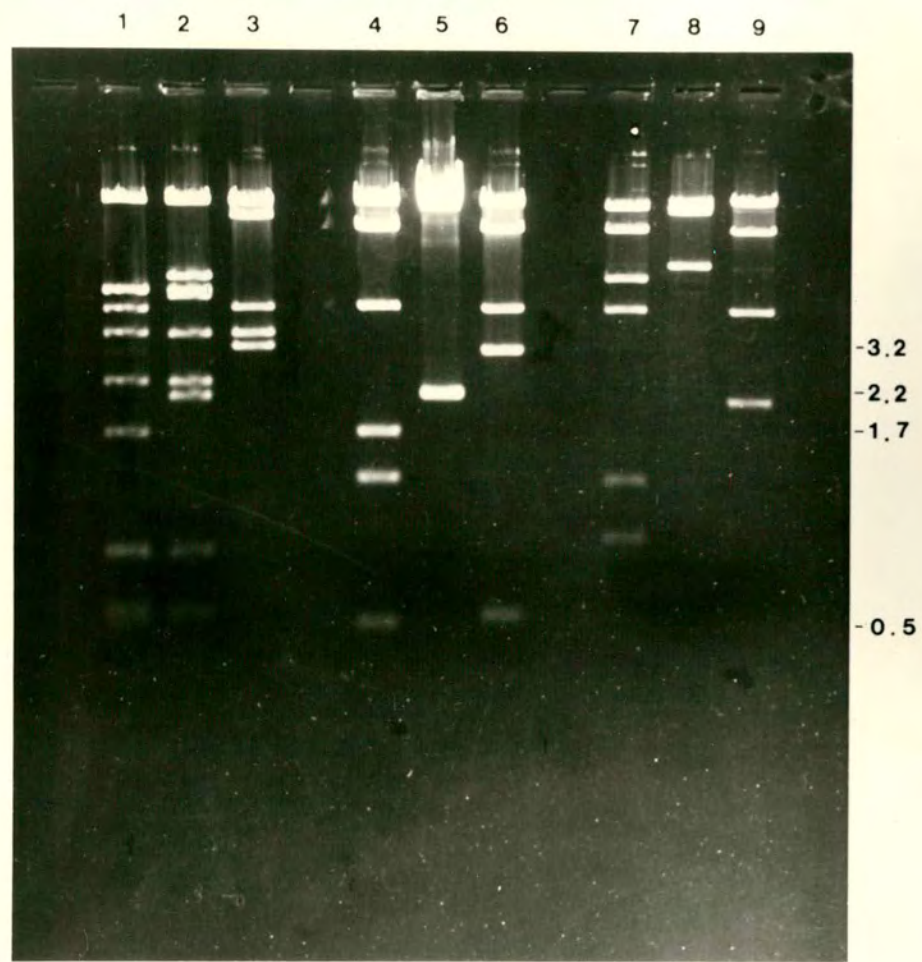


Fig 4.2

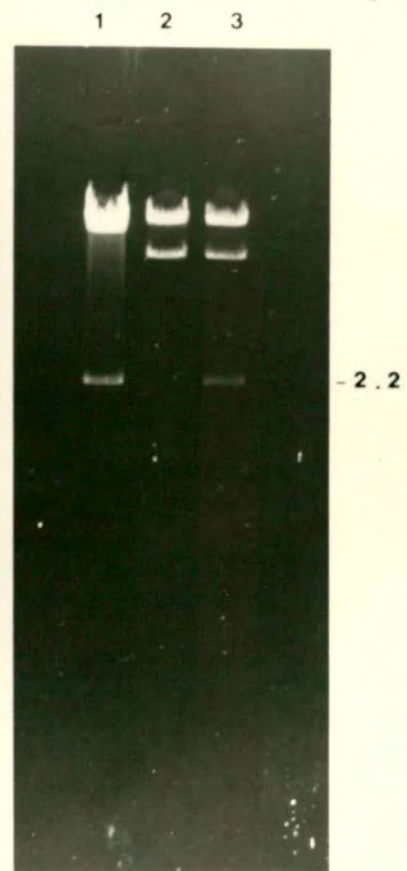


Fig 4.3

in several places by Eco RI. The Hind III digest and Hind III, Eco RI double digest of λ GH16 indicate however that λ GH16 has the left arm of λ 16-2E rather than λ NEM616 (Fig. 4.2). The Hind III digest yields a small fragment of approximately 0.6kb, and as the nearest Hind III to the cloning sites in λ NEM616 is 1.2kb away, either there are two Hind III sites within the 2.2kb Eco RI fragment or an additional Hind III site has been introduced on the phage left arm. The small Hind III fragment is cleaved by Eco RI close to one end (Hind III, Eco RI double digest, Fig. 4.2) and therefore both Hind III sites cannot be within the Eco RI fragment, and one Hind III site must be in the phage left arm close to the Eco RI site. The phage left arm in λ GH-16 (Fig. 4.4) therefore comes from λ 16-2AE and contains some chromosomal DNA internal to the murC gene as well as the 2.2kb Eco RI fragment. Lutkenhaus, Wolf-Watz and Donachie (1980) mapped the Eco RI and Hind III sites in λ 16-2, and the position of the sites in the murC gene confirms the construction of λ GH16.

For the purposes of complementation analysis it was desirable to reconstruct this phage so that it contained only the 2.2kb Eco RI fragment, and this was achieved in a two stage process. λ GH16 was digested with Eco RI, and ligated with λ NEM607 and plaques recovered by transfection into AA125. λ NEM607 (Fig. 4.1) (Murray, Brammar and Murray 1977) is an immunity insertion cloning vehicle, with a single Eco RI site within the C_I gene of immunity 434. Recombinant phages with a clear plaque morphology from the transfection were picked with sterile pasteur pipettes into 1ml aliquots of phage buffer. One of these phage was designated λ NY-1 and DNA from this phage was prepared. Eco RI digestion of λ NY-1 (Fig. 4.3) shows it to contain the 2.2kb Eco RI fragment inserted into the phage arms of λ NEM607. λ NY-1 was digested



Fig. 4.4. Physical maps of recombinant λ transducing phages carrying the 2.2Kb Eco RI restriction fragment

Single lines represent phage DNA, filled boxes the inserted DNA, and open boxes bacterial DNA other than the insert.

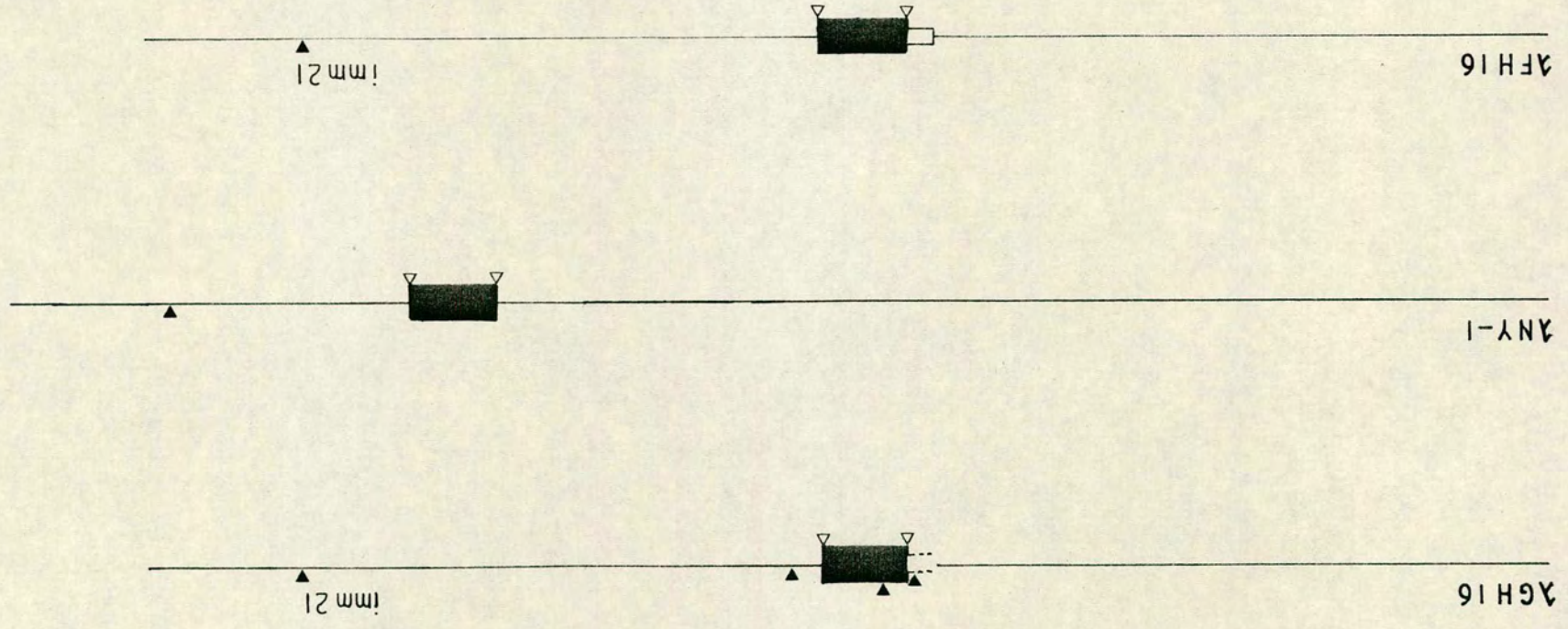
λ GH16 carries the 2.2Kb Eco RI restriction fragment within the left arm of λ 16-2 Δ E and the right arm of λ NEM616. The phage is immunity 21, and also contains DNA from within the mur C gene (in the dotted box).

λ NY-1 carries the 2.2Kb Eco RI fragment within the phage arms of λ NEM607. This phage has a clear plaque morphology.

λ FH16 carries the 2.2Kb Eco RI fragment with the arms of λ NEM616. The left arm also carries some DNA from the C-terminal end of the lac Z gene. This phage is immunity 21. The orientation of the insert has not been determined.

Fig 4.4

▲ HindIII
▽ EcoRI



with Eco RI, ligated with Eco RI digested λ NEM616 and plaques recovered by transfection into AA125. Turbid, non-red plaques from MacConkey lactose agar plates were tested for the ability to transduce the ftsA marker, and one such phage was designated λ FH16. (Fig. 4.4). This phage is integration proficient and immunity 21. After this phage was constructed, Lutkenhaus, Wolf-Watz and Donachie (1980) reported constructing a similar phage λ JFL41, which also complemented ftsA markers as do both λ GH16 and λ FH16.

As discussed in chapter 3, these phage ^(λ GH16, λ FH16) also complement TOE 1 (ftsQ^{ts}) as well as ftsA^{ts} mutants. When inserted as a lysogen in a recA derivative of TOE 1 the phage promoters P_L and P_R are not active (Reichardt and Kaiser 1971) and therefore expression of the ftsQ gene must be from a bacterial promoter.

4.3 Cloning into pBR325

pBR325 (Fig. 4.5) is a 6.0kb high copy number plasmid vector derived from pBR322 (Bolivar et al. 1977) by the insertion of the chloramphenicol acetyl transferase gene from Plcm^R (Bolivar 1978). This gene confers resistance to chloramphenicol and has a single Eco RI site within it. This is the only Eco RI site in pBR325 and cloning DNA fragments into it destroys the ability to confer resistance to chloramphenicol. Like pBR322, pBR325 also confers resistance to tetracycline and ampicillin.

λ NY-1 was digested with Eco RI and ligated with Eco RI digested pBR325. Ampicillin, tetracycline resistant clones were recovered by transformation into NEM259 and plating onto selective agar plates. Of 200 transformants that were tested, 20 clones had a recombinant phenotype

Fig. 4.5 Physical map of pBR325

This map shows relevant restriction sites and the approximate location of the ampicillin resistance gene (A_p^R), tetracycline resistance gene (Tc^R) and chloramphenicol resistance gene (Cm^R). (Bolivar, 1979).

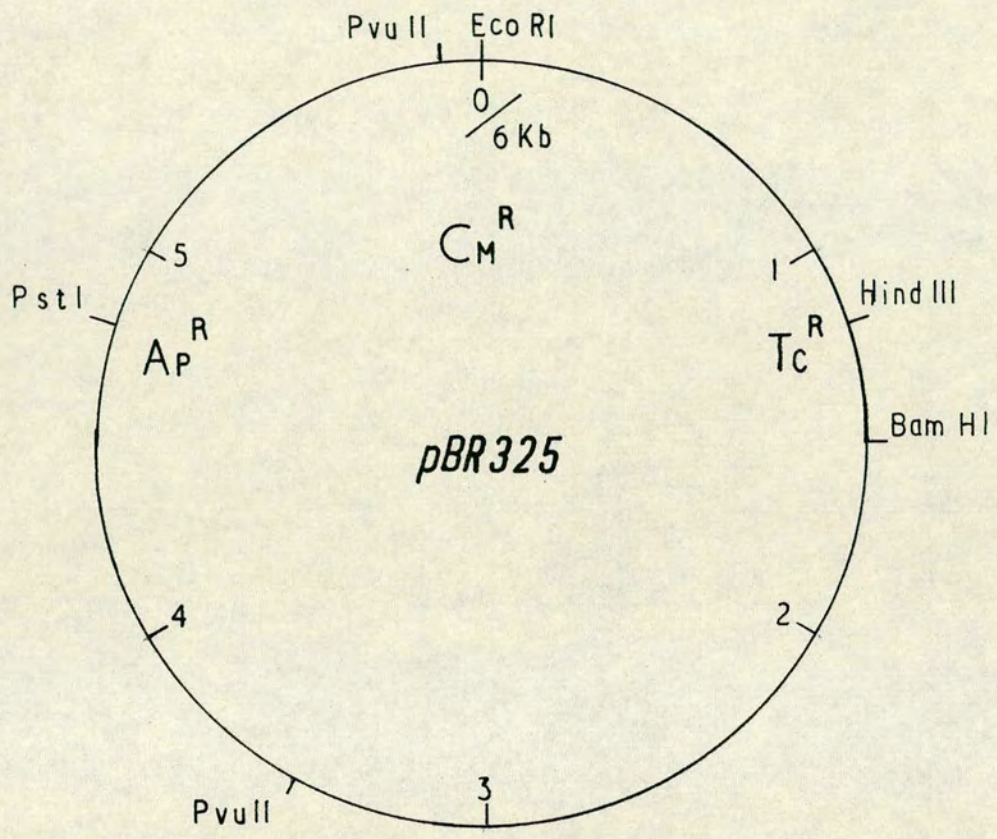


Fig 4.5

Fig. 4.6. 0.8% agarose gel of Hind III, BamHI and Eco RI restriction digestions of pBR325 and pGH-4

<u>Track</u>			
1	λ C _{I857}	Eco RI	Hind III
2	pBR325	Eco RI	
3	pBR325	Eco RI	Hind III
4	pBR325	Eco RI	Bam HI
5	pGH-4	Eco RI	
6	pGH-4	Hind III	
7	pGH-4	Bam HI	

Sizes of λ C_{I857} Hind III, Eco RI fragments are 21.7kb, 5.24kb, 5.05kb, 4.21kb, 3.41kb, 1.98kb, 1.90kb, 1.57kb, 1.32kb, 0.93kb, 0.84kb, 0.58kb.

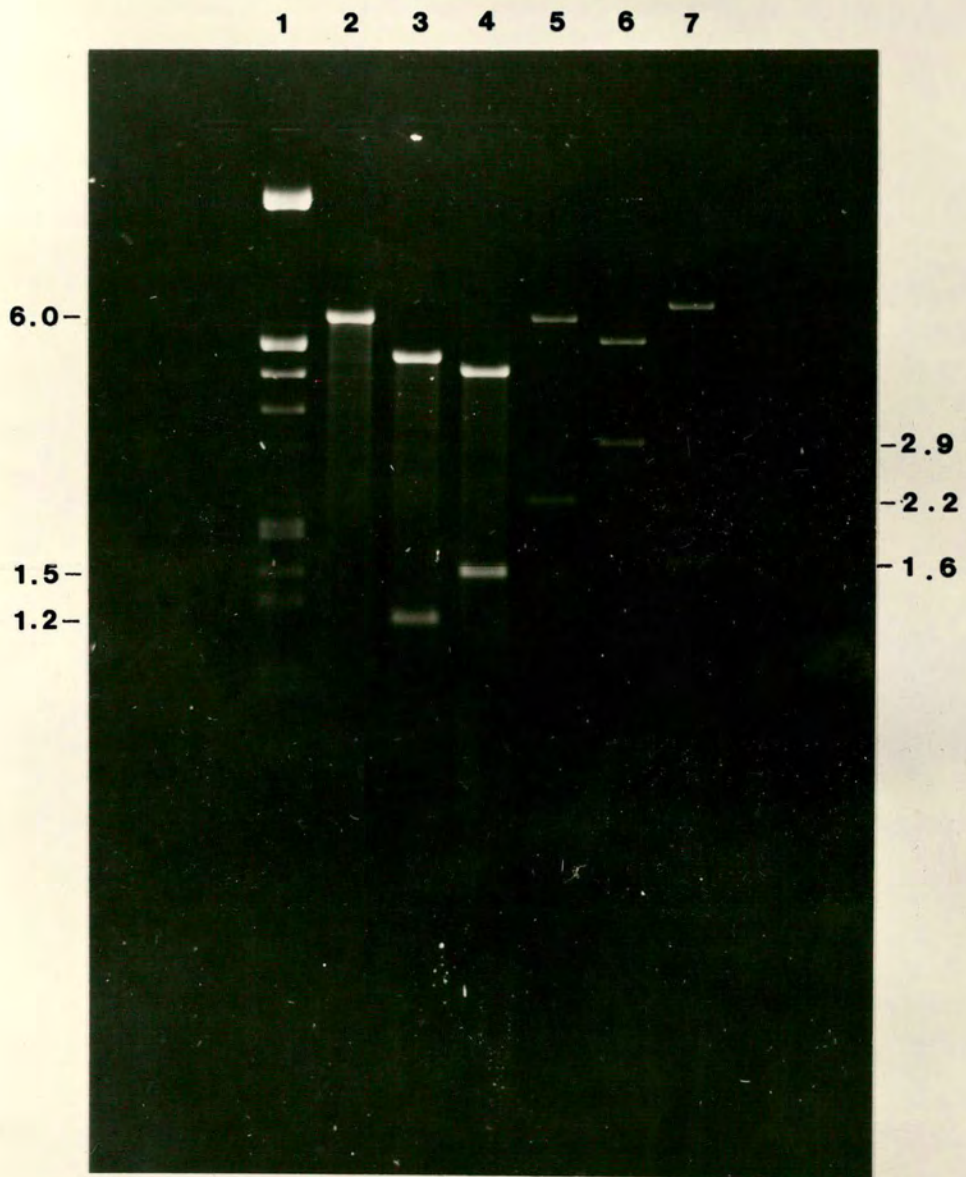


Fig 4.6

(Ap^R, Tc^R, Cm^S), and the Eco RI restriction patterns of Birnboim preparations of ten of these were examined by agarose gel electrophoresis. One of these clones yielded a 6.0kb fragment and a 2.2kb fragment upon Eco RI digestion, (Fig. 4.6) and this plasmid was designated pGH-4 (Fig. 4.7). Hind III digestion of pGH-4 (Fig. 4,6) yields two fragments, one of 5.3kb and one of 2.9kb, Eco RI, Hind III double digestion of pBR325 (Fig. 4.6) confirms that the vector Hind III site (in the Tc^R gene) is 1.2kb from the Eco RI cloning site, so that the Hind III site within the 2.2kb Eco RI insert must be 1.7kb from this site in pGH-4. The position of this asymmetrical Hind III site in the 2.2kb Eco RI fragment, correlates well with the Eco RI, Hind III double digestion of λGH16 which yields a 1.7kb fragment and a fragment of approximately 0.5kb.

Bam HI digestion also yields two fragments, one of 1.6kb and one of 6.6kb (Fig. 4.6). Bam HI, Eco RI double digestion of pBR325 (Fig. 4.6) confirms that the vector Bam HI site (in the Tc^R gene) is 1.5kb from the Eco RI cloning site, and therefore there is a Bam HI site in the 2.2kb Eco RI insert approximately 0.1kb from the Eco RI site. Lutkenhaus, Wolf-Watz and Donachie (1980) map the Bam HI site near to the C-terminal end of the ddl gene, and this defines the orientation of the 2.2kb Eco RI fragment in pGH-4. According to the data of Lutkenhaus and Wu (1980), ftsA would be transcribed in pGH-4 away from the Tc^R gene through the Hind III ^{site} within the DNA insert in pGH-4 as shown diagrammatically in Fig. 4.7.

4.4 Mapping of BglIII, Kpn I and Pvu II Restriction Sites

20 ug of pGH-4 was digested with Eco RI, ethanol precipitated,

Fig. 4.7. Physical map of pGH-4

The thick line represents the 2.2Kb Eco RI fragment which has been inserted into the Eco RI site of pBR325 (thin line). This plasmid has intact genes coding for ampicillin resistance (Tc^R) and tetracycline resistance (Tc^R) but does not confer resistance to chloramphenicol.

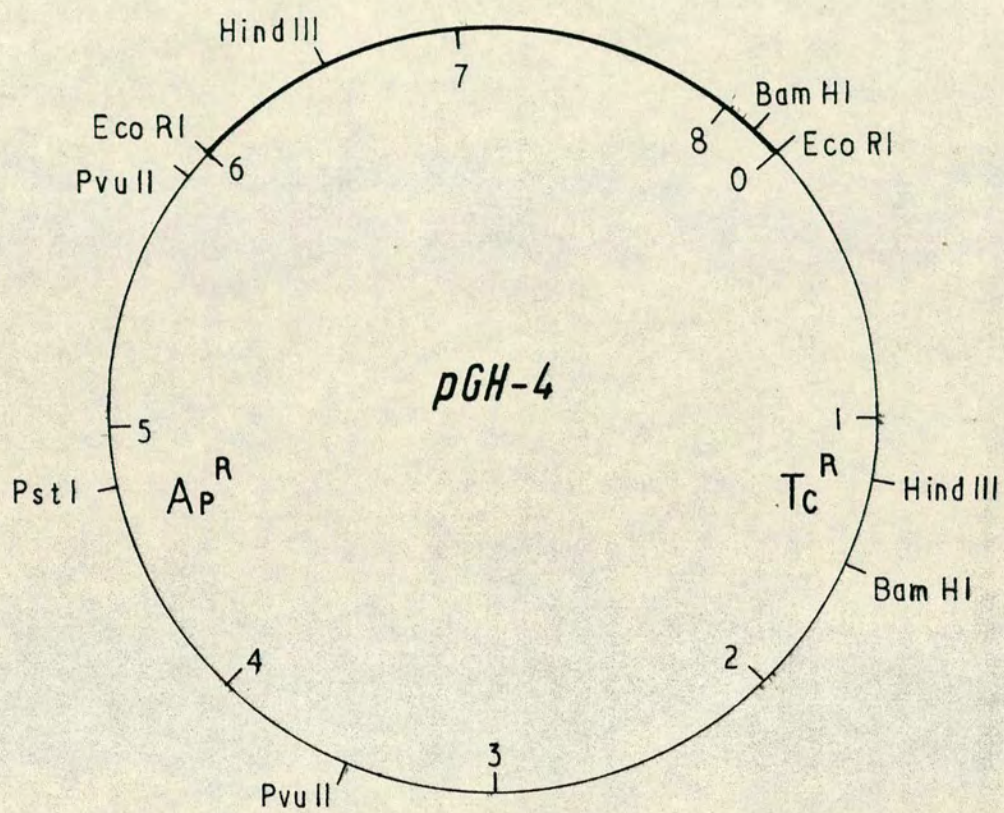


Fig 4.7

resuspended in water, and divided into 20 aliquots. Aliquots were digested with various restriction enzymes and analysed by agarose gel electrophoresis. A summary of this restriction ^{data} is shown in Table 4.1. The Bgl II, Kpn I and Pvu II sites were mapped by further restriction analysis.

Bgl II site : Eco RI, Bgl II double digestion of pGH-4 (Fig. 4.8) yields a 6.0kb vector fragment and two other fragments of 1.0kb and 1.2 kb. There is therefore an asymmetrical Bgl II site within the 2.2kb Eco RI fragment. Bgl II, Hind III double digestion of pGH-4 (Fig. 4.8) defines the position of the site more precisely, and yields fragments of 5.3kb, 2.2kb and 0.7kb. Therefore, the Bgl II site must be approximately 0.7kb from the Hind III site as shown in Fig. 4.9.

Kpn I sites : Kpn I digestion of pGH-4 (Fig. 4.8) yields a 7.6kb fragment and a 0.6kb fragment. There are no Kpn I sites within pBR325 and therefore there must be at least two Kpn I sites within the 2.2kb Eco RI insert. Kpn I, Hind III double digestion of pGH-4 (Fig. 4.8) yields fragments of 5.3kb, 1.5kb, 0.8kb and 0.6kb. Therefore one Kpn I site is 0.8kb from the insert HIII site, and the other 1.4kb away, approximately 0.3kb from the Eco RI site (Fig. 4.9).

Pvu II sites : pBR325 has two Pvu II sites, one very close and anticlockwise to the Eco RI site (about 0.1kb) and one 2.6kb anticlockwise to the Eco RI site (Fig. 4.5). Pvu II digestion of pGH-4 yields three fragments, a 4.2kb fragment, a 2.5kb fragment and a 1.3kb fragment. The 2.5kb fragment is internal to the vector and there must be at least one Pvu II site in the 2.2kb fragment. Double digestion of pGH-4 with Pvu II and Eco RI (Fig. 4.8) yields four

Fig. 4.8. 0.8% agarose gel for restriction digestions of pGH-4

<u>Track</u>			
1	λ C ₁₈₅₇	Hind III	+ Eco RI
2	pGH-4	Bgl II	+ Eco RI
3	pGH-4	Bgl II	+ Hind III
4	pGH-4	Kpn I	
5	pGH-4	Kpn I	+ Hind III
6	pGH-4	Pvu II	
7	pGH-4	Pvu II	+ Eco RI

Sizes of λ C₁₈₅₇ Hind III, Eco RI fragments are 21.7kb, 5.24kb, 5.05kb, 4.21kb, 3.41kb, 1.98kb, 1.90kb, 1.57kb, 1.32kb, 0.93kb, 0.84kb, 0.58kb.

Track 4 was run on a gel separate from these markers.

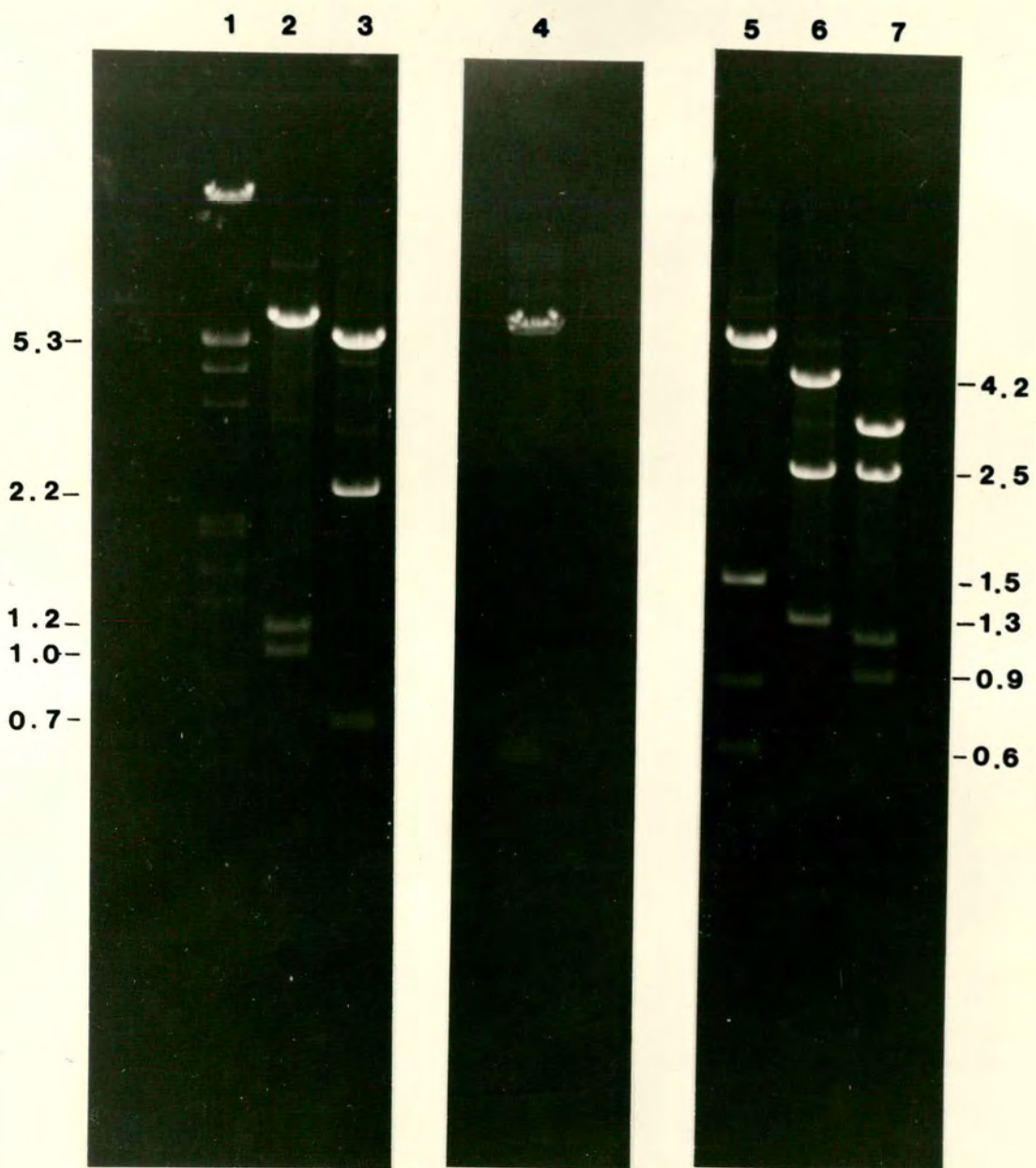


Fig 4.8

Table 4.1. Restriction enzyme sites in 2.2Kb Eco RI fragment

<u>Restriction enzyme</u>	<u>No. of sites in 2.2Kb EcoRI fragment</u>
Bam HI	1
Bgl II	1
Hind III	1
Kpn I	2
Pvu II	2
Hae II	several
Taq I	many
Msp I	many
Sau 3A	many
Sal I	none
Sma I	none
Sac I	none
Pst I	none
Xho I	none

Fig. 4.9. Restriction endonuclease map of the 2.2Kb Eco R1 restriction fragment

The fragment is represented so that the orientation is the same as it is in the bacterial chromosome, with fts A being transcribed towards the terminus. The position of the fts A is not exact.

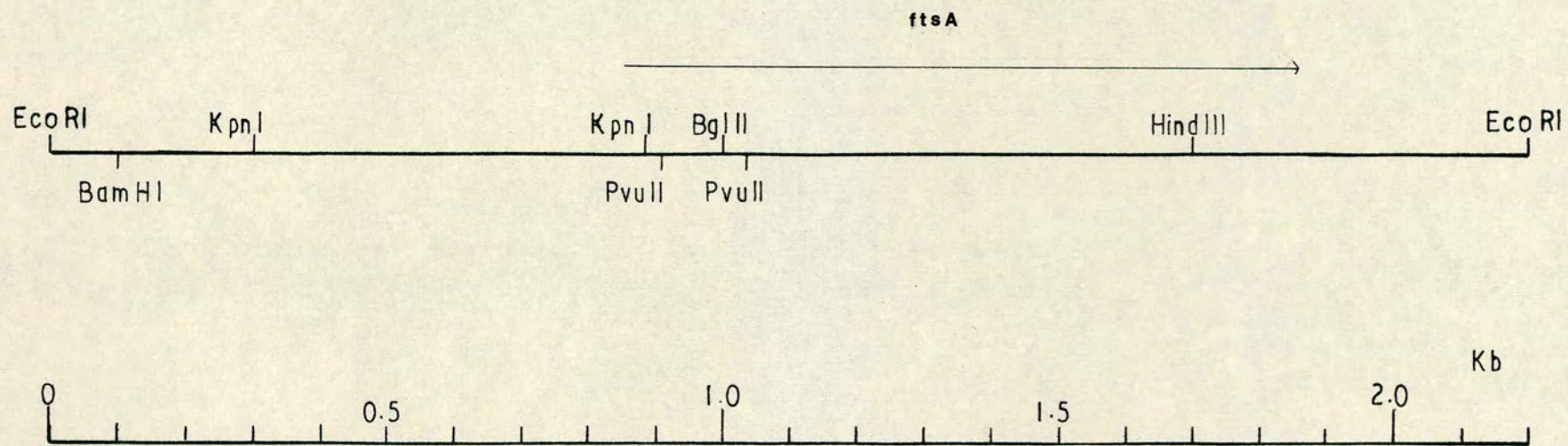


Fig 4.9

fragments, one of 3.3kb, one of 2.5kb, one of 1.2kb and one of 0.9kb. The 2.5kb Pvu II fragment is not cleaved by Eco RI as expected, but the 4.2kb and 1.3kb fragments are cleaved. As one Pvu II site in the vector is 0.1kb from the Eco RI site, the 1.3kb Pvu II fragment must be cleaved by Eco RI to yield the 1.2kb Eco RI-Pvu II fragment. There is therefore a Pvu II site in the 2.2kb Eco RI fragment, 1.2kb from the left most Eco RI in pGH-4. A 0.9kb fragment is also present in the Eco-RI-Pvu II double digest, and this suggests that there may be two Pvu II sites close together near the Bgl II site.

The restriction mapping with agarose gel electrophoresis cannot easily resolve two restriction sites that are very close together (E.g. within 50bps). The sites identified here will however be described as single restriction sites, unless any contradictory evidence is obtained to suggest that any of them are actually double sites. It should also be noted that with this type of mapping, the smaller fragments (1.0kb and less) can only be sized approximately, and polyacrylamide gels would have to be used if it was desirable to size these fragments more accurately.

4.5 Complementation of *fts* mutants with pGH-4

pGH-4 was transformed into TOE 1 (*fts*^{Q^{ts}) and TKF12 (*fts*^{A^{ts}) and tetracycline, ampicillin resistant clones selected on agar at 30°C. Transformants from each strain were checked for the presence of the plasmid by agarose gel electrophoresis of Birnboim preparations. Transformants were streaked out on selective agar plates and incubated at 30°C and 42°C, and also examined microscopically in exponentially growing cultures (in nutrient broth, ampicillin) at 30°C and 42°C.}}

TKF12 pGH-4 grows well on agar at both 30°C and 42°C, and in liquid culture grows as normal sized cells at both temperatures. pGH-4 therefore transduces the ftsA marker. TOE1 pGH-4 grows well at 30°C on selective agar plates, but very poorly at 42°C. In liquid culture, TOE1 pGH-4 is physiology^{at 30°C} similar to TOE1, with poor cell separation. At 42°C, TOE1 pGH-4 grows as long filamentous cells with some visible septa, and some normal sized cells. pGH-4 therefore appears to slightly improve cell division in TOE 1 at the restrictive temperature but complements the mutation very poorly.

CHAPTER 5

Transcription and Complementation analysis of the 2.2kb Eco RI Fragment5.1 Introduction

The 2.2kb Eco RI fragment carries at least two structural genes and ^{perhaps} their promoters, ftsQ and ftsA. Lutkenhaus and Wu (1980) determined that the control region and N-terminal end of the ftsZ gene is also present, and suggested that there may be an additional ftsZ promoter or a control site within the ftsA gene, on the N-terminal side of the Hind III site.

The 2.2kb Eco RI fragment was cloned into a plasmid where the promoters could be recognised by transcriptional fusion to an assayable product. Deletion derivatives were constructed so that each promoter on the fragment could be identified, and the position of the genes better defined.

The recently developed KO system (McKenney, Shimatake, Court, Scheissner, Brady and Rosenberg, in press) was used for constructing in vitro genetic fusions. In this system, plasmid and phage vectors have been constructed so that restriction fragments can be inserted, and promoter or terminator activity assessed by its influence on the galactokinase (gal K) gene. For studying the 2.2kb fragment and the fts genes it is desirable to use both the single copy phage systems and the multicopy plasmids systems, although the phage system is not yet available for use, and therefore in this study, only the plasmid system has been used, in particular pK0-1 (Fig. 5.1).

Fig. 5.1 Diagram of pK0-1

pK0-1 (3.9kb) contains single sites for Eco RI, Hind III and Sma I into which DNA fragments can be inserted, galK is expressed only weakly in pK0-1. The 150bp leader sequence and translation stop codons serve to prevent polar effects on galK expression, so that galK activity is equal to upstream promoter activity. The Pvu II site of pBR322 has been destroyed by cloning in galK. The Eco RI-Hind III fragment (290bp) is derived from the λ O gene.

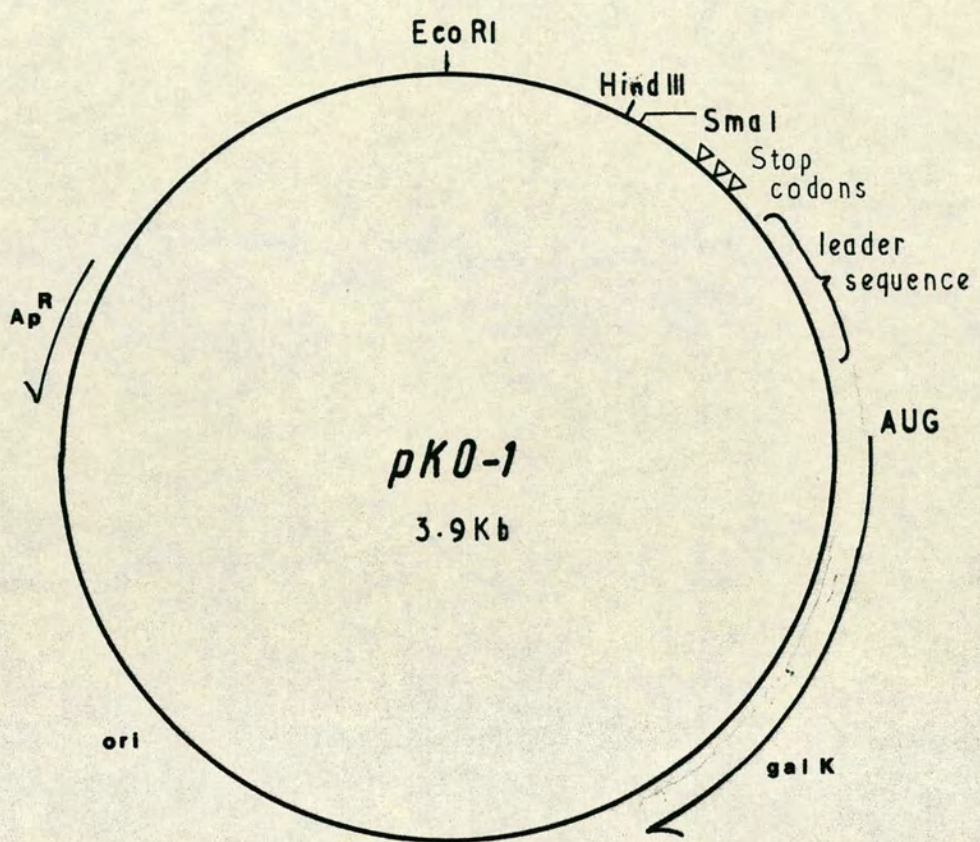


Fig 5.1

The origin of replication and ampicillin resistance gene in pK0-1 are derived from pBR322, the galK gene and its leader sequence from E. coli and the 290bp Eco RI-hind III fragment from the λ 0 gene. The entire nucleotide sequence of this plasmid is known apart from about 700bps internal to the galK gene. A galK⁻ strain carrying pK0-1 is white when growing on MacConkey galactose plates, as the galK gene in pK0-1 is only expressed very weakly. Restriction fragments that carry promoters can be inserted into the unique Eco RI, Hind III or Sma I sites, so that they now express the galK gene, and recognised as red colonies when harboured in a galK⁻ strain growing on MacConkey galactose plates. This system has specifically been designed so that neither transcriptional nor translational polarity affects the expression of galK, and the assayable level of galactokinase is equal to the strength of the promoter.

Translational polarity is avoided by the introduction of translation stop codons in all three reading frames upstream of the galK gene. Ribosomes that initiate translation on the mRNA synthesised from within the cloned fragment ^{cannot} approach the galK gene, and the galK translation initiation sequence is free for ribosome binding.

Transcriptional polarity has also been avoided in the pK0 system. The galK gene in vivo is located within a high molecular weight transcript (gal E, I and K), and McKenney et al (1981) reasoned that the natural boundary region between galT and galK ensures that galK is expressed independently from upstream RNA structures. For this reason the 150bp leader sequence to the galK gene was kept intact in the construction of pK0-1. McKenney et al (1981) inserted λP_L at different

distances from the galK gene and demonstrated that upstream mRNA structures will not influence the expression of galK.

The pK0 system has the additional advantage that galK can be both positively and negatively selected as a genetic marker. pK0-1 will not complement a host strain with a gal E⁺T⁺K⁻ genotype so that there is no growth on minimal agar containing galactose as sole carbon source. If the vector carries a promoter and expresses the galK gene then the cells can grow to form a colony. This positive selection can be used to isolate "up" promoter mutations which activate promoter function (McKenney et al. 1981).

A strain with a gal E⁻T⁻K⁺ phenotype does not grow in the presence of galactose due to the accumulation of the toxic intermediate galactose-1-phosphate. A recombinant pK0 plasmid that expressed galactokinase in a gal E⁻T⁻K⁻ host will therefore cause cell death if grown in the presence of galactose. This negative selection can be used to select for "down" promoter mutations which inactivate the promoter function (McKenney et al. 1981).

Other plasmids, such as pK0-4, pK0-5, pK0-6 and pK0-7 are also available which have other unique restriction sites for cloning promoters and extend the diversity of the pK0 system. Plasmids are also available that contain known promoters cloned into pK0 plasmids (such as pKG1800), and these can be used for cloning and recognising transcriptional terminators (McKenny et al. 1981). None of these plasmids have yet been used for the analysis of the 2.2kb Eco RI fragment other than pK0-1.

Several host strains are available for use with the pK0 system. The two used in this work are C600K⁻, a galK⁻ derivative of C600, and N100 a galK⁻ strain that is also recA⁻.

5.2 Construction of pK0 recombinant plasmids

5.2.1 Construction of pGH106 and pGH106/A

pGH-4 was digested with Hind III, ligated with Hind III digested pK0-1 and ampicillin resistant clones recovered by transformation into C600K⁻. About 5% of clones were red on MacConkey galactose plates. Birnboim preparations of eight of these clones were digested with Hind III and separated by agarose gel electrophoresis. A clone that yielded a 3.9kb fragment and a 2.9kb fragment (Fig. 5.2) was then digested with Eco RI to yield a 1.5kb fragment and a 5.3kb fragment (Fig. 5.2). This plasmid was designated pGH106 (Fig. 5.3). As Fig. 5.3 shows, in pGH106, the ftsA gene is transcribed towards the galK gene.

The pBR325 moiety and the 290bps Eco RI-Hind III fragment were removed from pGH106 by digesting with Eco RI, ligating in a diluted solution (10 ug/ml DNA) and ampicillin resistant clones recovered by transformation into C600K⁻. All clones were red on McConkey galactose agar plates. Birnboim preparations of four clones were digested with Eco RI and the DNA fragments separated by agarose gel electrophoresis. All four clones had lost the 1.5kb Eco RI fragment, and one of these was designated pGH106/A (Fig. 5.2 and Fig. 5.3). This plasmid now contains only the 1.7kb Eco RI-Hind III fragment and the 3.6kb pK0-1 vector Eco RI-Hind III fragment (Fig. 5.2).

Fig. 5.2 0.8% agarose gel of Hind III and Eco RI restriction digests of pGH-4, pKO-1, pGH106, pGH106/A, pGH110, pGH203

Track 5

1	λ C _I 857	Hind III	
2	pGH-4	Hind III	
3	pKO-1	Hind III	
4	pGH106	Hind III	
5	pGH106	Eco RI	(partial digest)
6	pGH106/A	Hind III	
7	pGH106/A	Hind III + Eco RI	
8	pGH110	Eco RI	
9	pGH110	Eco RI + Hind III	(partial digest)
10	pGH203	Hind III	
11	pGH203	Eco RI	(partial digest)

λ C_I Hind III marker fragments are 23.6kb, 9.64Kb, 6.64Kb, 4.34Kb, 2.26Kb, 1.98Kb, 0.56Kb.

Sizes of relevant fragments are shown in Kb. Tracks 6 and 7 were run on a gel separate from these markers.

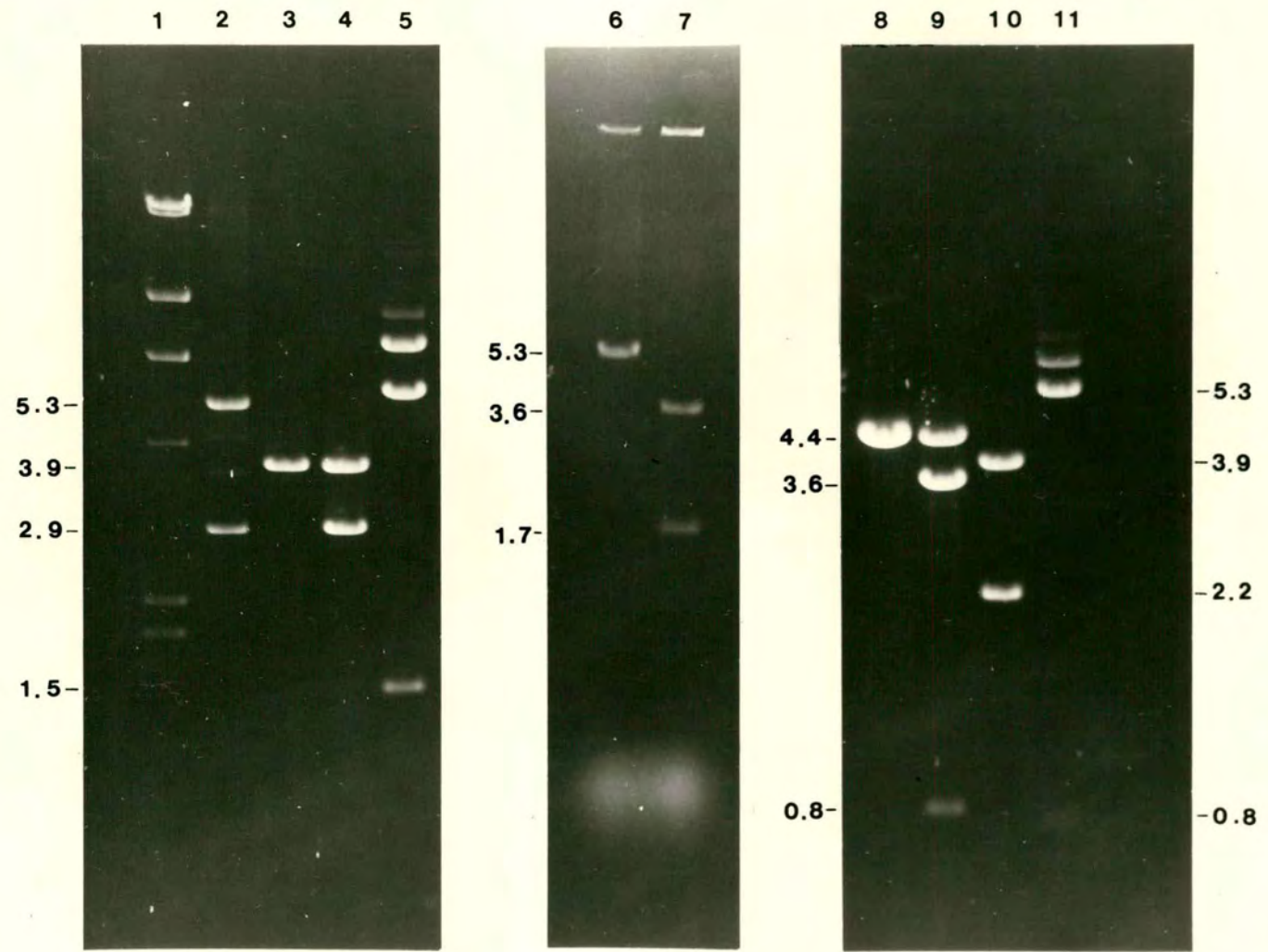


Fig 5.2

5.2.2 Construction of pGH110

As described in chapter 4, the 2.2kb Eco RI fragment contains a Bam HI and a BglII site, separated by about 0.9kb. Bam HI recognises the hexanucleotide sequence GGATCC, and BglII the hexanucleotide sequence AGATCT, although both leave a GATC 5' extension after cleavage. These sites may be ligated together to form a hybrid Bam HI-Bgl II site AGATCC which is not recognised by either enzyme.

The Bam HI-Bgl II 0.9kb fragment was removed from pGH106/A by restricting with both enzymes and ligating in a dilute DNA concentration (10 ug/ml). Ampicillin resistant clones were recovered by transformation into C600K⁻ and plating onto MacConkey galactose agar plates. Birnboim preparations of four ^{red} clones were digested with Eco RI and the products separated by agarose gel electrophoresis. All clones yielded only a 4.4kb fragment. One of these was designated pGH110 (Fig. 5.2 and Fig. 5.3). Eco RI-Hind III double digestion of pGH110 yields a 3.6kb vector fragment and a fragment of approximately 0.8kb (Fig. 5.2) showing that the 1.7kb Eco RI-Hind III site has been reduced in size by about 0.9kb.

5.2.3 Construction of pGH203

To prepare a purified sample of the 2.2kb Eco RI fragment, approximately 100ug of pGH-4 DNA were digested with Eco RI, and loaded onto a 10-20% sucrose gradient. After spinning for 24 hours at 22Krpm in an AH627 swing out rotor (10⁰C), the gradient was fractionated into 250ul fractions from the bottom of the tube. 10ul of each fraction were run on an agarose gel to determine which fractions contained the 2.2kb Eco RI fragment. These fractions were pooled, and dialysed against TE.

Fig. 5.3 Physical maps of pGH106, pGH106/A, pGH110 and pGH203

Bacterial DNA is shown as an open box, and vector DNA as single lines. DNA that has been deleted is shown as a gap. Restriction endonuclease sites for Hind III (\blacktriangledown), Eco RI (\triangle), Bam HI (\downarrow) and Bgl II (\downarrow) are shown. The direction of transcription of the ampicillin resistance gene (Ap^R) and galK are shown with respect to the bacterial insert.

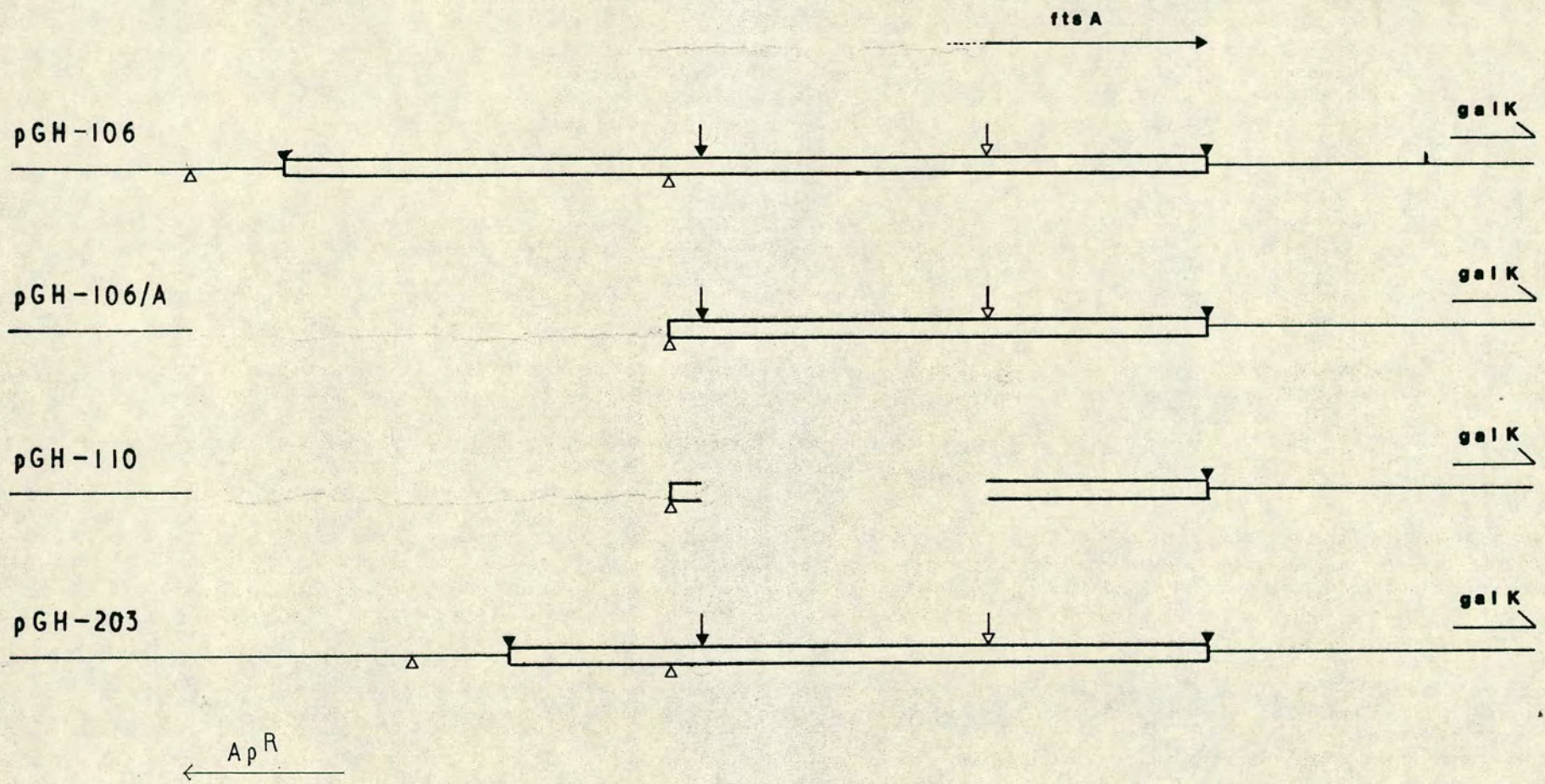


Fig 5.3

Fig. 5.4 Diagrammatic representation of construction of pGH203

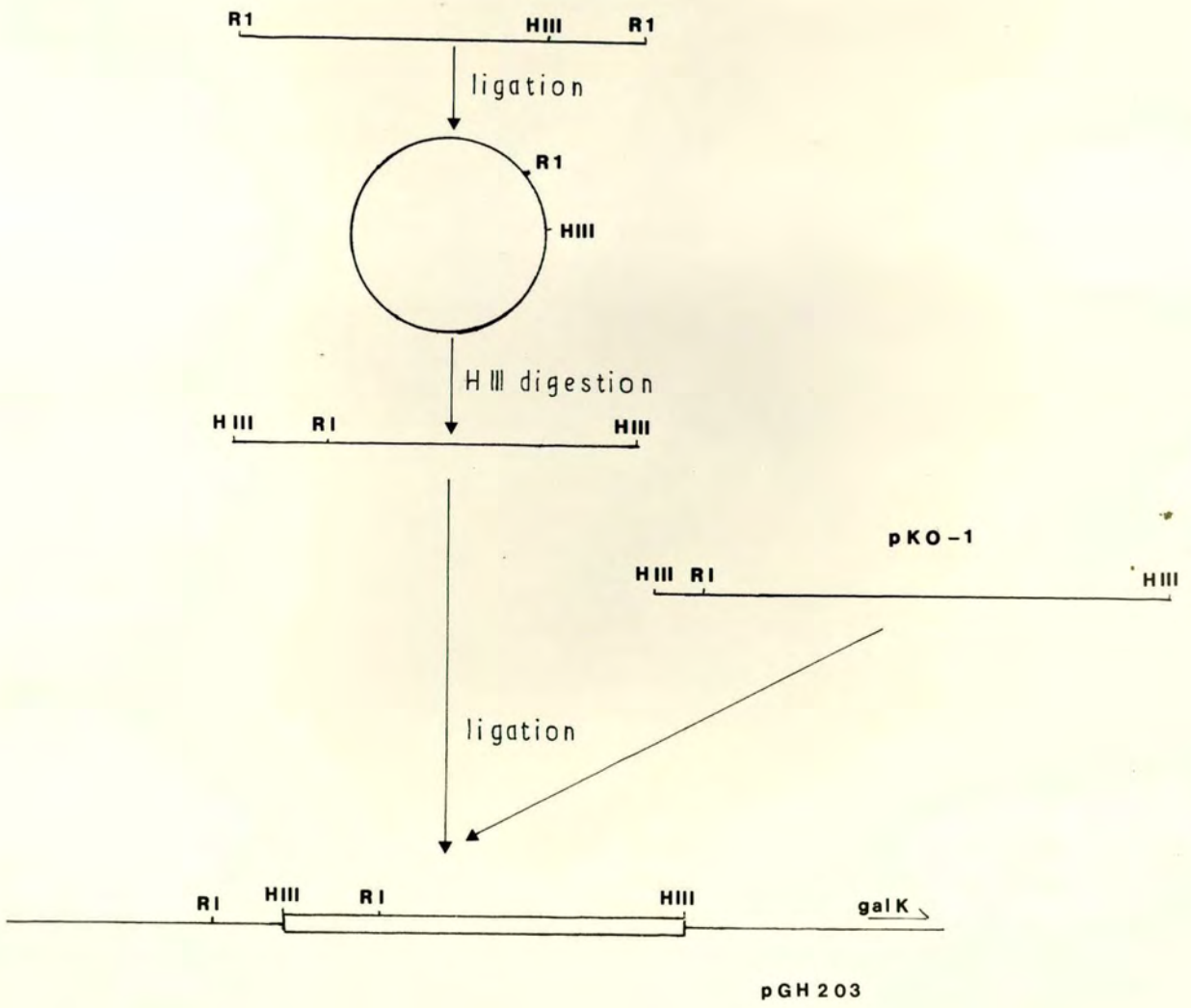
The top part of the diagram shows a 0.8% agarose gel of manipulation with the 2.2Kb Eco RI fragment.

Track

1	2.2Kb Eco RI fragment (purified)
2	Ligated fragment
3	Ligated fragment re-restricted with Eco RI
4	Ligated fragment re-restricted with Hind III

The lower part of the diagram illustrates the circularisation of the 2.2Kb Eco RI and re-restriction with Hind III to create a 2.2Kb Hind III fragment, with a single Eco RI site internal to it. This rearranged fragment was ligated with Hind III cleaved pK0-1 to construct pGH203. The direction of transcription of the ampicillin resistance gene (Ap^R) and galK are shown in pGH203 with respect to the bacterial insert (open box)

Fig 5.4



The DNA was ethanol precipitated, dried and resuspended in 400ul TE.

A 40ul aliquot of the purified Eco RI fragment was ligated by itself for 4 hours at 15°C and then divided into four aliquots. One aliquot was digested with Eco RI and one with Hind III and the products separated by agarose gel electrophoresis, along with one undigested aliquot, and unligated DNA (Fig. 5.4). Both Eco RI and Hind III digested aliquots yielded predominantly a 2.2kb fragment suggesting that most of the purified fragments had circularised upon ligation.

The remaining aliquot of ligated fragment was digested with Hind III and ligated with Hind III digested pK0-1. Ampicillin resistant clones were recovered by transformation into C600K⁻. Birnboim preparations of four red clones were digested with Hind III and the fragments separated by agarose gel electrophoresis. All of these clones yielded a 3.9kb vector fragment and a 2.2kb fragment (Fig. 5.2). One of these clones was designated pGH203 (Fig. 5.3). Eco RI restriction of pGH203 yields a 5.3kb fragment and a 0.8kb fragment (Fig. 5.2). Fig. 5.4 shows a diagrammatic representation of the construction of pGH203.

5.2.4. Construction of pGH301 and pGH300

10ul of the 2.2kb Eco RI fragment preparation was ligated with Eco RI digested pK0-1 and ampicillin resistant clones recovered by transformation into C600K⁻. Birnboim preparations of four red clones were digested with Eco RI and the fragments separated by agarose gel electrophoresis. One clone that yielded a 3.9kb vector

Fig. 5.5 0.8% agarose gel of Hind III and Eco RI restriction digestions of pGH301, pGH300, pGH305, pGH360, pGH370 and pGH350

<u>Track</u>		
1	λ C _I 857	Hind III
2	pGH301	Eco RI
3	pGH301	Hind III
4	pGH300	Eco RI
5	pGH300	Hind III
7	pGH305	Eco RI
6	pGH305	Eco RI + Hind III
9	pGH360	Eco RI
8	pGH370	Eco RI
10	pGH350	Eco RI
11	pGH350	Hind III
12	pGH350	Eco RI + Hind III

λ C_I857 Hind III marker fragments are 23.6Kb, 9.64Kb, 6.64Kb, 4.34Kb, 2.26Kb, 1.98Kb, 0.56Kb

Sizes of relevant fragments are shown in Kb.

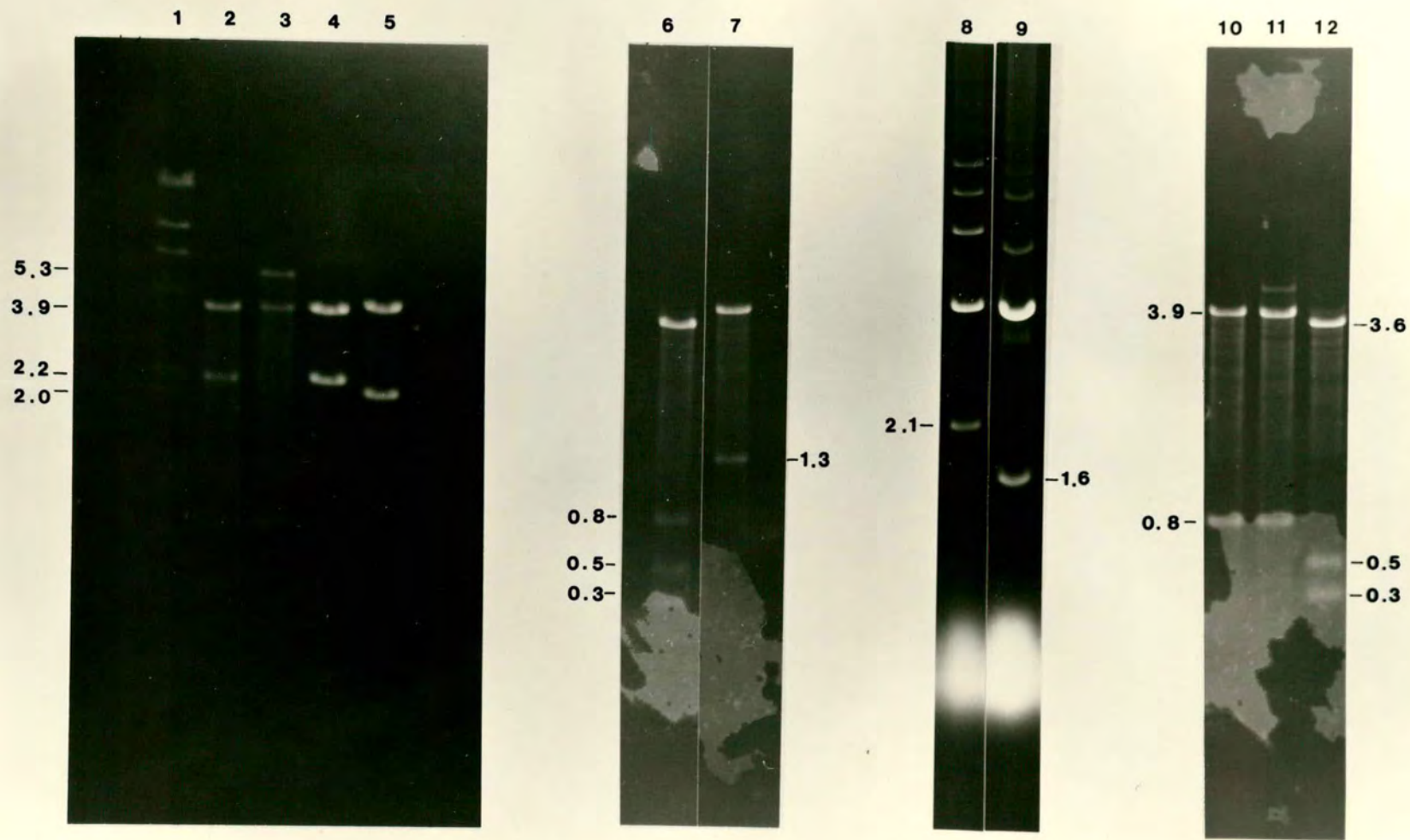


Fig 5.5

fragment, and a 2.2kb inserted fragment was designated pGH301 (Figs. 5.5 and 5.6). Hind III digestion of pGH301 (Fig. 5.5) yields a 5.3kb fragment and a 0.8kb fragment, and from the restriction map in ^{Fig} 4.9 must be inserted so that ftsA is transcribed towards galK. The Hind III digest also yields a 3.9kb fragment, so that in pGH301 there must be two copies of the vector present, in the same orientation.

Birnboim preparations of 10 other randomly chosen clones were digested with Eco RI and the fragments separated by agarose gel electrophoresis. One of these yielded a 3.9kb fragment and a 2.2kb fragment, and on Hind III digestion yielded fragments of 4.1kb and 2.0kb (Fig. 5.5). This *plasmid* was designated pGH300. The 2.2kb inserted fragment in this plasmid is inserted in the opposite orientation to that in pGH301 (Fig. 5.6).

5.2.5 Construction of pGH305

pGH301 was digested with Bam HI and Bgl II, ligated in diluted DNA solution (10ug/ml) and ampicillin resistant clones recovered by transformation into N100. All clones were red on MacConkey galactose agar plates. Birnboim preparations of four red clones were digested with Eco RI and the fragments separated by agarose gel electrophoresis. One of the clones that yielded a 3.9kb fragment and a 1.3kb fragment was designated pGH305 (Fig. 5.5 and 5.6). Hind III, Eco RI double digestion yields fragments of 3.6kb, 0.8kb, 0.5kb and 0.3kb as would be predicted from deletion of the Bam HI-BglIII fragment.

5.2.6 Construction of pGH360

The restriction analysis in chapter 4, showed that there are two Kpn I restriction sites in the 2.2kb Eco RI fragment. pK0-1 has no Kpn I restriction sites, and the 0.6kb Kpn I fragment from the 2.2kb Eco RI fragment can be removed by restriction and religation.

pGH301 was digested with KpnI and religated at a DNA concentration of 10ug/ml. Ampicillin resistant clones were recovered by transformation into N100. All clones were red on MacConkey galactose plates. Birnboim preparations of four clones were digested with Eco RI and the fragments separated by agarose gel electrophoresis. One of these clones yielded a 3.9kb vector fragment and a 1.6kb fragment. This clone was designated pGH360 (Fig. 5.5 and Fig. 5.6).

5.2.7 Construction of pGH370

The restriction analysis in chapter 4 and the sequence analysis in chapter 7 has shown that there are two Pvu II sites very close to the centre of the 2.2kb Eco RI fragment. pK0-1 does not contain any Pvu II sites and therefore the small (150bps) Pvu II fragment can be removed from the 2.2kb Eco RI fragment by restriction with Pvu II and religation.

pGH301 was digested with Pvu II and then religated at a DNA concentration of 10ug/ml. Ampicillin resistant clones were recovered by transformation into N100. All clones were red on MacConkey, galactose plates. Birnboim preparations of three of these, were digested with Eco RI and the fragments separated by agarose gel electrophoresis. One of these clones yielded a 3.9kb vector fragment and a 2.1kb fragment. This plasmid was designated pGH370 (Fig. 5.5 and Fig. 5.6).

Fig. 5.6 Physical maps of pGH301, pGH300, pGH305, pGH360, pGH370, pGH350

Bacterial DNA is shown as open boxes and vector DNA is single lines. DNA that has been deleted is represented as a gap. Restriction endonuclease sites for Hind III (\blacktriangledown), Eco RI (\triangle), Bam HI (\blacktriangledown), BglIII (\blacktriangledown), Pvu II (\blacktriangle) and KpnI (\blacktriangle) are also shown. The direction of transcription of the ampicillin resistance gene (AP^R) and galK are shown with respect to the bacterial insert.

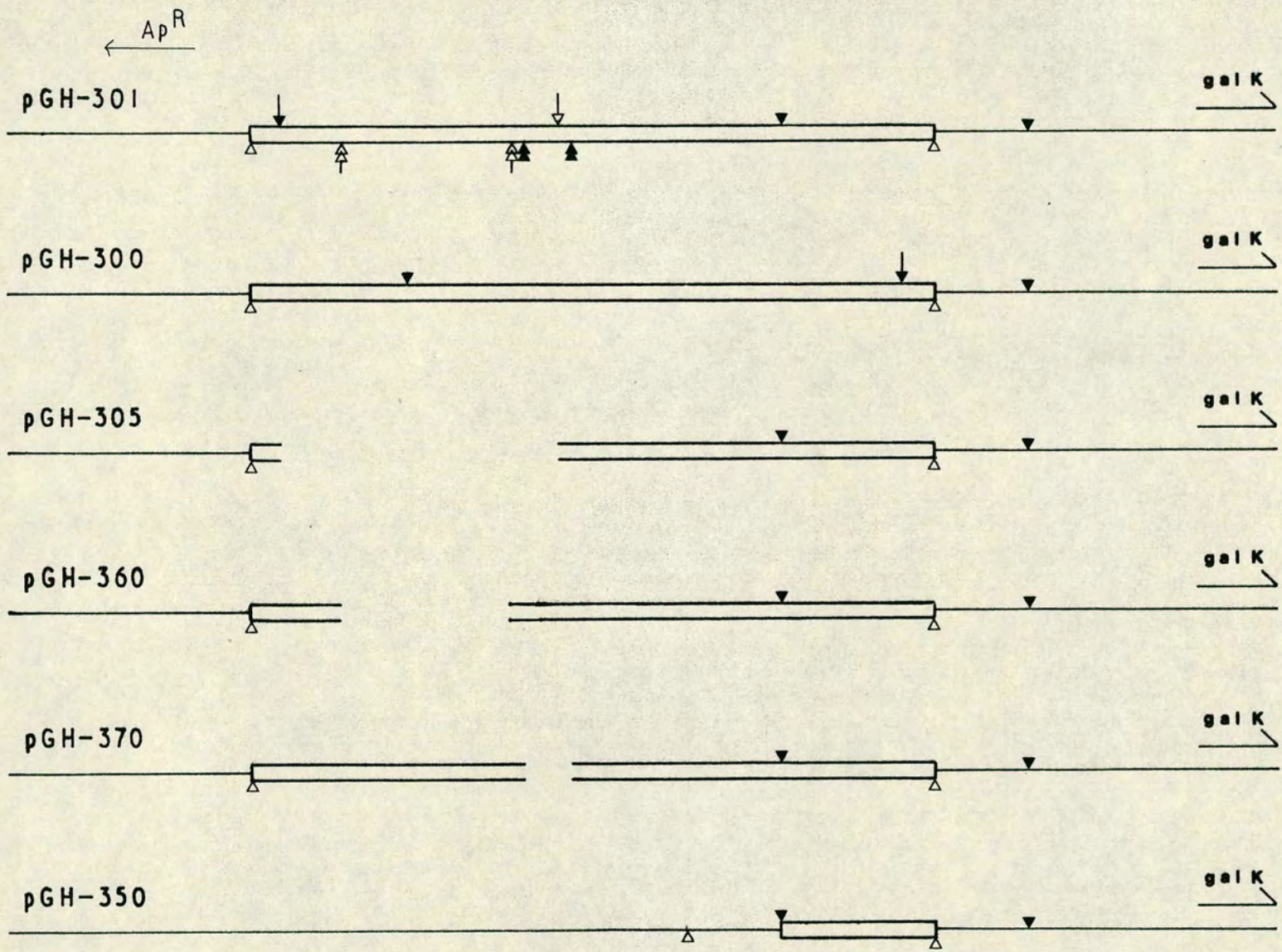


Fig 5.6

Fig. 5.7 Densitometry trace of Hind III, Eco RI double restriction digest of pGH350

The densitometry trace of the Hind III, Eco RI double digestion (Fig. 5.5) shows three peaks, one for the 3.6Kb fragment, one for the 0.5Kb fragment, and one for the 0.3Kb fragment. However the tops of the peaks cannot be joined by a straight line (as they would be, if in equimolar ratios), indicating that there is more than one copy of the 0.3Kb fragment in this digestion.

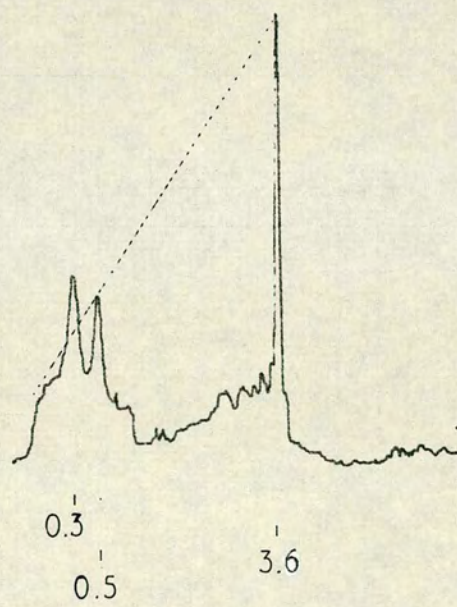


Fig 5.7

5.2.8 Construction of pGH350

pGH301 was digested with Hind III, and then with Pst I to destroy the vector molecules. These fragments were ligated with Hind III digested pK0-1 and ampicillin resistant clones recovered by transformation into (C600K⁻). Birnboim preparations of four red clones were digested with Hind III and the fragments separated on an agarose gel. All these clones yielded a 3.9kb vector fragment and a 0.8kb fragment (Fig. 5.5). One of these was designated pGH350. This clone yields a 0.8kb fragment on digestion with Eco RI (Fig. 5.5). Double digestion of pGH350 with Eco RI and Hind III yields only the 3.6kb vector fragment and 0.5kb and 0.3kb fragments. Therefore the 0.8kb Hind III fragment has an Eco RI site within it, and the 0.8kb Eco RI fragment has a Hind III site within it. The structure of pGH350 as shown in Fig. 5.5 is confirmed by the densitometer trace which shows that the fragments are not in equimolar ratios (Fig. 5.7), the 0.3kb fragment being present more than once in the Hind III, Eco RI double digest.

5.3 Complementation analysis of pK0 recombinant plasmids

pK0-1 and the recombinant plasmids described above were transformed into TOE 1 and TOE 13, selecting for ampicillin resistant clones at 30°C on MacConkey galactose agar plates. pK0-1 and pGH300 both gave white clones, whereas all the other plasmids gave red clones, in both TOE 1 and TOE 13. This is the same colour reaction as seen with N100 and C600K⁻ (Table 5.2).

Transformants were grown at 30°C in nutrient broth with ampicillin, until in early exponential phase. An aliquot of each culture was transferred to prewarmed flasks at 42°C and incubated in a water bath

Table 5.1. Growth ability and physiology of plasmid harbouring strains of TOE 1 and TOE 13 at the restrictive temperature

Plasmid	TOE 1		TOE 13	
	Agar 42°C	Liquid 42°C	Agar 42°C	Liquid 42°C
pK0-1	-	Filaments	-	Filaments
pGH106/A	+	Normal	-	Filaments
pGH110	-	Filaments	-	Filaments
pGH203	+	Normal	-	Filaments
pGH301	+	Normal	+	Normal
pGH300	-	Filaments	+	Normal
pGH305	-	Filaments	-	Filaments
pGH360	-	Filaments	-	Filaments
pGH370	+	Normal	-	Filaments
pGH350	-	Filaments	-	Filaments
pGH106	+	Filaments and some normal cells	-	Filaments

Table 5.2 Galactokinase activity in strains N100 and C600K⁻ harbouring plasmids

<u>Plasmid</u>	<u>N100</u>	<u>C600K⁻</u>	<u>Colour</u>
No. Plasmid	0	0	White
pK0-1	15.8 [±] 2.8	22.8 [±] 1.1	White
pGH301	101.9 [±] 0.7	167.0 [±] 26.0	Red
pGH300	0.6 [±] 0.2	0.5 [±] 0.7	White
pGH106/A	71.3 [±] 9.4	104.4 [±] 47.6	Red
pGH110	50.1 [±] 16.0	78.4 [±] 28.5	Red
pGH305	95.3 [±] 11.1	112.8 [±] 29.4	Red
pGH350	33.1 [±] 3.6	51.9 [±] 5.3	Red
pGH360	130.4 [±] 29.8	—	Red
pGH370	133.5 [±] 50.7	170.9 [±] 15.9	Red

Each activity is the average of two separate assays. The standard deviation is shown. The colour of either strain harbouring the plasmids growing on MacConkey galactose plates is shown in the right-hand column.

for a further three hours. All cultures were examined microscopically. The results are shown in Table 5.1. Transformants were also streaked out onto oxoid nutrient agar plates with ampicillin and incubated at 42°C, incubated overnight and then checked for complementation, as growth at the restrictive temperature. The results are shown in Table 5.1.

5.4 Galactokinase assays of pK0 recombinant plasmids

Stationary phase cultures of N100, and C600K⁻ strains harbouring pK0 recombinant plasmids were diluted 1 in 50 into fresh nutrient broth containing ampicillin, and incubated at 37°C with shaking, until OD₆₅₀ 0.3-0.5. 1ml samples were removed from each and assayed as in 2.20. Each culture was assayed on two separate occasions. The average of the two results for each culture, and the colour of the strains on MacConkey galactose plates, are shown in Table 5.2.

5.5 Discussion

Complementation analyses with the deletion derivatives of the 2.2kb Eco R1 fragment has defined more precisely the location of the ftsQ gene. pGH370 has a small deletion within it of about 150bps, and this results in loss of the ability to complement ftsA, without affecting ftsQ. It is likely then that ftsQ lies between the left most Pvu II site and Eco R1 site and that the deletion in pGH 370 interrupts the integrity of the ftsA structural gene. Assuming an average amino acid weight of 115 daltons, the maximum size of a protein that could be synthesised from the Eco R1-Pvu II fragment (0.9kb) is about 34.5 kilodaltons. pGH360 also fails to complement ftsA, so the ftsA gene probably extends further, past the KpnI site that is close to the

Fig. 5.8 Graph showing comparison between plasmid galactokinase activities in strain 600K⁻, and plasmid galactokinase activities in N100

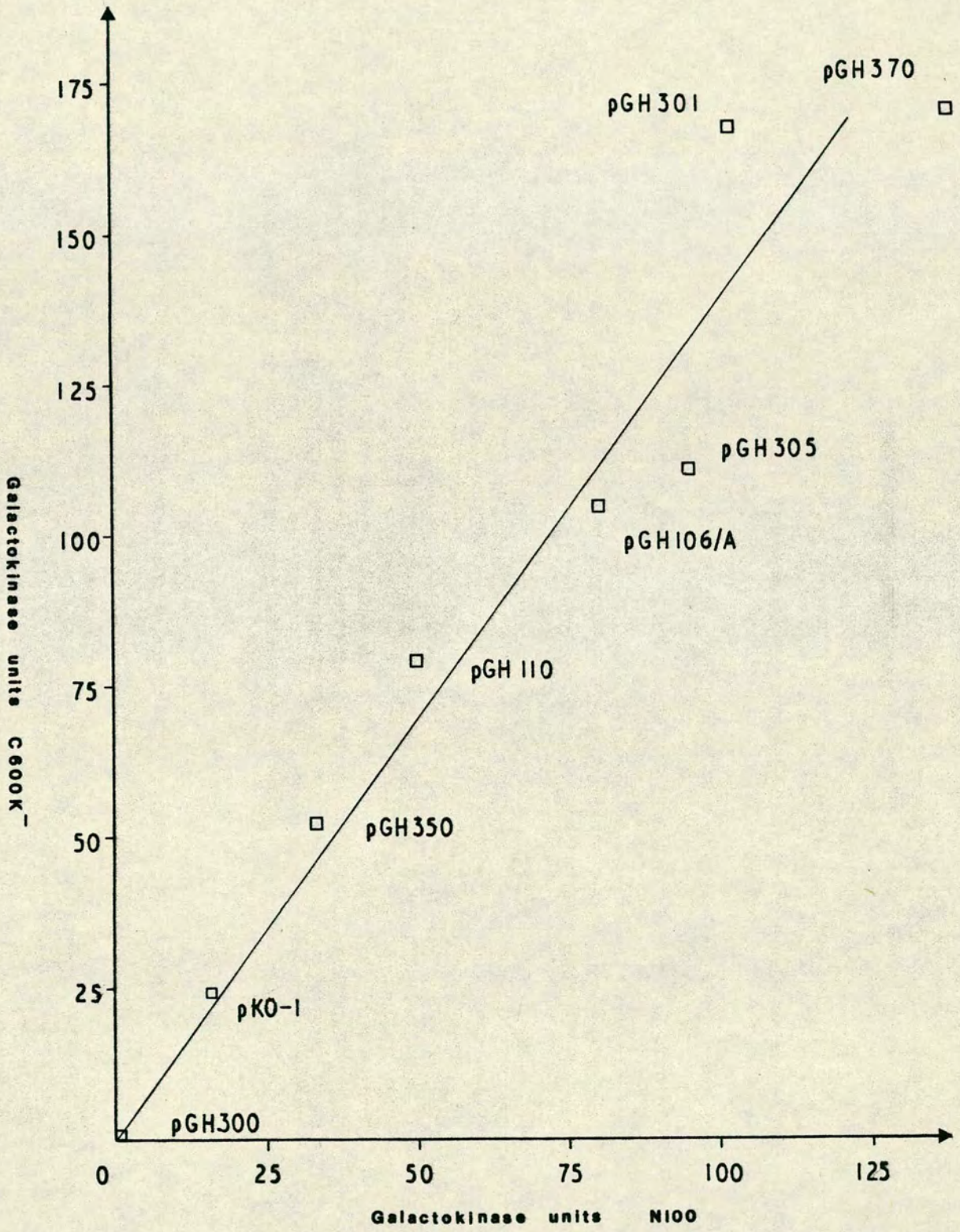


Fig 5.8

PvuII site.

If the ftsQ gene lies in this fragment of DNA, then why does pGH300 fail to complement the ftsQ mutation, and pGH106 complement very badly (as does pGH-4), when pGH301 complements very well? One possible explanation is that part of the ftsQ gene is absent from the Eco RI fragment, so that the DNA sequence of the vector adjacent to the cloning site determines whether or not an active ftsQ product is synthesised. As the 2.2kb Eco RI fragment complements TOE 1 when cloned in the λ vectors, as well as the plasmid vectors would suggest that only a small part of the gene is missing, more probably from the control region rather than from the structural gene itself. However, the fact that pGH300 complements ftsA but not ftsQ suggests strongly that these two genes are separately expressed.

Table 5.2 shows the amount of galactokinase (in units) produced by each fusion plasmid, with the standard deviation between two results. The assays in C600K are consistently higher than in N100. (The linear relationship in Fig. 5.8) but with a higher standard deviation, probably due to recombination between plasmids and the chromosome, which does not occur in the recA strain N100. It is therefore only these assays that will be further discussed.

The standard deviations in these results, and in particular pGH360 and pGH370, indicate that the assay is rather variable. The results would be more reliable if an average was taken of about ten independent assays, an exercise which time did not permit in this study. However, there are some interesting points about these fusion assays.

pGH300 consistently gives very little galactokinase activity, and a level below that of the pK0-1 vector. There is therefore no transcription from the 2.2kb Eco RI fragment through the Eco RI site nearest to the galK gene in pGH300. The residual galK activity in pK0-1 must therefore arise upstream of the Eco RI site, and not from DNA between the Eco RI site and galK gene. This residual activity in pK0-1 is presumably reduced in pGH300 due to opposing transcription of the ftsA gene.

pGH350 has about twice the galactokinase activity of pK0-1. As the location of any terminators in the 0.5kb Hind III-Eco RI is not known, it is unclear whether the activity in pGH350 is solely due to an inserted promoter, or a combination of an inserted promoter and the residual pK0-1 activity. However, there must be a promoter on this 0.5kb Hind III-Eco RI fragment. Lutkenhaus and Wu (1980) found that transducing phage carrying the ftsZ gene with this Hind III-Eco RI fragment (but no DNA anticlockwise to the Hind III), complemented ftsZ mutants only under certain conditions, and synthesised the 45 kilodalton gene product very poorly. It is likely that the promoter identified on the 0.5kb Hind III-Eco RI fragment is responsible for this expression of the ftsZ gene.

Lutkenhaus and Wu (1980) also found that the addition of DNA anticlockwise to the Hind III site improves the expression of the ftsZ gene. The galactokinase activity in pGH301 is approximately three times the activity measured in pGH350, although again it is not known if the residual pK0-1 activity is contributing to this. The increased transcriptional activity in pGH301 above pGH350 could be either because DNA sequences anticlockwise to the Hind III site are

stimulating the promoter downstream, on the 0.5kb Hind III-Eco RI fragment, or because transcripts from upstream in the DNA insert are not terminated in the 2.2kb Eco RI fragment, and galactokinase activity in pGH301 results from several different transcripts.

pGH370, pGH360 and pGH305 all have deletions in the 2.2kb Eco RI fragment, which do not significantly decrease the galactokinase activity from that in pGH301. pGH360 and pGH370 have rather high galactokinase activities, but the standard deviation is also high, so the increase in activity above that in pGH301 may not be significant. pGH305 has a larger deletion than either pGH360 or pGH370, with approximately 0.9kb deleted between the Bam HI and Bgl II site. Additional activity above that in pGH350 must therefore arise from the DNA fragments Eco RI-Bam HI (approximately 0.1kb) and Bgl II-Hind III (0.7kb). This is confirmed by promoter activity in pGH110 which has only these two fragments inserted into pK0-1. The ftsA promoter (provided that ftsA is transcribed separately to ftsQ, as is suggested) is probably absent from pGH110, and promoter activity must arise from either within the ftsA structural gene (Bgl II-Hind III fragment) or from the Eco RI Bam HI fragment, and the question therefore arises as to whether the ftsQ promoter is on this 0.1kb fragment.

pGH106/A may have more promoter activity than pGH110. This would suggest that there maybe an additional promoter on the Bam HI-Bgl II fragment, possibly the ftsA promoter although this is considered to be rather weak evidence, and the Bam-Bgl II fragment will have to be cloned to assess its promoter activity.

The following conclusions can be made about transcription of the fts genes:

- a) There is a promoter on the 0.5kb Hind III-Eco RI fragment, that reads through the Eco RI ^{site} and this is most likely the ftsZ promoter.
- b) There is a promoter ~~on the 0.1kb Eco RI-Bam HI fragment,~~ on the 0.7kb Bgl II-Hind III fragment which is internal to the ftsA structural gene, or *created at DNA junction(s)*.
- c) Transcription through the Eco RI site in ftsZ probably results from several different transcripts. No evidence has been found to suggest that there is a control site within the ftsA gene that stimulates the ftsZ promoter.

It is essential for similar fusions to those described above to be constructed in the single copy system when this is available, in order to observe any copy number effects and titration of any factors that are essential for the expression of these genes.

CHAPTER 6Protein Synthesis directed by pK0 recombinant plasmids6.1 Introduction

Lutkenhaus and Wu (1980) observed six gene products which were synthesised from the chromosomal insert in $\lambda 16-2$. Each product was assigned to a gene by analysing protein synthesis and complementation patterns of $\lambda 16-2$ deletion derivations. Other gene products may be synthesised from the $\lambda 16-2$ bacterial insert which cannot be observed in the phage systems, either because they are small and cannot be discerned from phage directed proteins, or are too weakly expressed from their own promoters to be observed on infection of a homoimmune lysogen. An example of the latter, is the 30 kilodalton protein identified by Lutkenhaus and Wu (1980) as the ddl gene product. This protein cannot be observed at all on infection of $\lambda 16-2$ into a homoimmune lysogen (Lutkenhaus and Wu 1980).

We reasoned that to identify the ftsQ gene product these problems may be overcome by using the minicell system for identifying plasmid-coded proteins. Minicells produced by mutant strains of E. coli were originally characterised by Adler et al. (1967) as small inviable DNA-less cells. Levy (1971) and Roozen et al (1971) demonstrated that minicells are capable of RNA and protein synthesis if the strain harbours a plasmid that is segregated into the minicells. Minicells can be easily separated from their parental cells by sucrose gradient centrifugation (Frazer and Curtis 1975; Levy 1974) and the proteins that are being synthesised, radioactively labelled. A preincubation

period allows degradation of existing chromosomal mRNA, so that all protein synthesis is plasmid coded. It was hoped that a comparison of proteins synthesised by the recombinant plasmids described in chapter 5, and the vector, would reveal the ftsA gene product, which has been identified as a 50 kilodalton protein (Lutkenhaus and Donachie 1979), and the previously unidentified ftsQ gene product. An analysis of deletion derivatives of the 2.2kb Eco RI fragment should help to discriminate these products from other vector-coded products.

6.2 Preparation and labelling of minicells

Plasmid DNA of pK0-1, pGH301, pGH300, pGH106/A pGH360 and pGH203 was transformed into the minicell producing strain DS410, and transformants selected on nutrient agar plates containing ampicillin. Minicells from these transformants and from the strain DS410 without a plasmid, were separated from the parental cells by three rounds of sucrose gradient centrifugation as described in chapter 2. Minicell preparations were labelled with ^{35}S -methionine and the proteins separated by electrophoresis on a 7-20% gradient polyacrylamide gel. The labelled proteins were visualised by autoradiography, and one such autoradiogram is shown in Fig. 6.1.

6.3 Discussion

All the minicell preparations that contain a plasmid synthesise a 30 kilodalton protein, a 32 kilodalton protein and a 27 kilodalton protein. The 30 kilodalton protein is β -lactamase, product of the ampicillin resistance gene (Dougan and Sherratt 1977), the 32 kilodalton protein a precursor of this, and the 27 kilodalton protein a degradation

Fig. 6.1 7-20% gradient polyacrylamide gel separating proteins synthesis in minicells

The minicell producing strain DS410, and DS410 strains harbouring plasmids were labelled with ^{35}S -methionine and the labelled proteins separated by polyacrylamide gel electrophoresis.

<u>Track</u>	
1	DS410 pK0-1
2	DS410
3	DS410 pGH301
4	DS410 pGH106/A
5	DS410 pGH203
6	DS410 pGH360
7	DS410 pGH300

Unlabelled marker proteins were run in tracks either side of the labelled samples, and the sizes of relevant proteins calculated, and shown in kilodaltons.

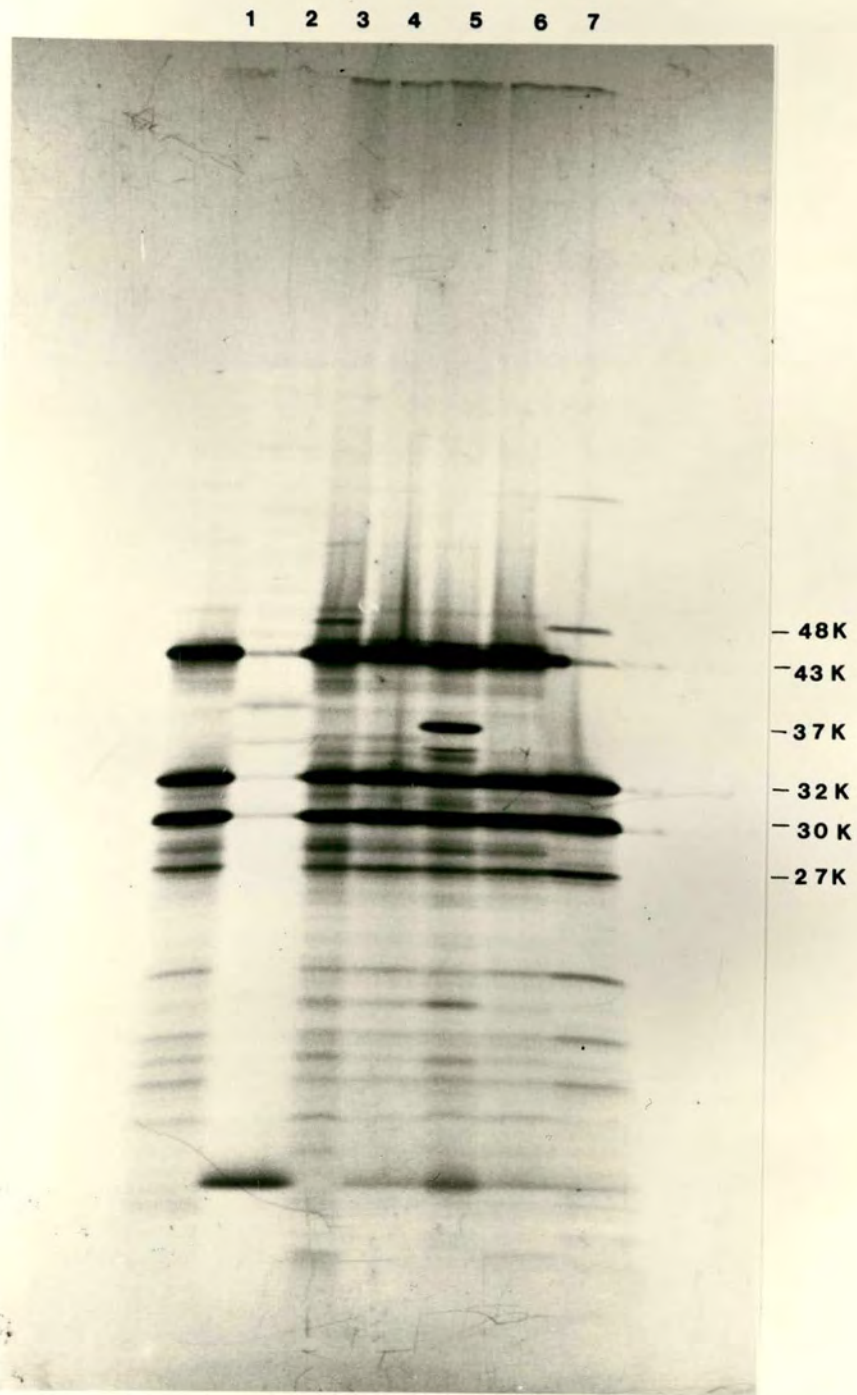


Fig 6.1

product.

A 43 kilodalton protein is synthesised in the plasmid-containing minicells preparations, although rather less from pGH300 than the other plasmids, and it is probably the galK gene product, which has previously been identified as a 40 kilodalton protein (Lewin, Gene Expression Vol. 1). It is known from the transcription analysis that galK is only weakly transcribed in the pK0-1, although there appears to be a lot of the 43 kilodalton protein in this sample.* It is possible that the galK product is very stable and accumulates throughout the labelling period. pGH300 transcribed galK weakly, if at all, and this correlates with the 43 kilodalton protein being the galK product.

pGH301 and pGH300 also synthesise a 48 kilodalton protein, which is not synthesised by pK0-1, pGH106/A, pGH203 or pGH360. pGH301 and pGH300 both complement ftsA mutants which the other plasmids do not, and it seems likely that the 48 kilodalton protein is the ftsA gene product. Darby (Ph.D thesis) ^{Univ. Leicester, 1981} also identified the ftsA gene product as a 48 kilodalton protein, and the size difference to the 50 kilodalton protein identified by Lutkenhaus and Donachie (1979) is probably a function of the different gel systems and marker proteins that were used.

pGH106/A and pGH203 should both synthesise a truncated or hybrid ftsA product, as in both plasmids the ftsA gene is interrupted at the Hind III site at the C-terminal end. This hybrid protein would terminate at one of the stop codons prior to the galK gene, so that it would be smaller than the ftsA gene product. It is possible that the hybrid protein is masked by other proteins in the gel such as the galK product.

Both pGH300 and pGH301 synthesise very little of the ftsA product
 * It is also possible that differences of the levels of this protein are concealed by the intensity of the bands.

in comparison to the galK and β -lactamase products. There are several reasons why this may be so:

- a) the ftsA promoter may be very weak,
- b) chromosomal macromolecular synthesis may be necessary for synthesis of ftsA product,
- c) translation of the ftsA gene may be very inefficient,
- d) the high plasmid copy number may titrate out factors that are required for expression of the ftsA gene, so that only a few copies of the gene are expressed,
- e) the ftsA product may be very unstable.

It is not yet clear which of these factors is affecting expression of the ftsA gene.

There are no additional proteins produced by pGH301 and pGH106/A that could be the ftsQ gene product. However pGH203 strongly synthesises a 37 kilodalton protein, and less strongly a 35 kilodalton protein. The structure of pGH203 differs from pGH106/A in that the 0.5kb Hind III-Eco RI fragment has been inserted at the Eco RI site of pGH106/A (Fig. 5.3), this fragment carries the ftsZ promoter (chapter 5) and the N-terminal part of the ftsZ gene (Lutkenhaus and Wu 1980). The 37 kilodalton protein may originate from within the 0.5kb fragment and be synthesised across the Eco RI site to produce a hybrid ftsZ-ftsQ product.

Lutkenhaus and Wu (1980) identified the ddl gene product as a 30 kilodalton protein. This was deduced from the observation that the 3.2kb Hind III of λ 16-2 synthesised this protein (Fig. 1.5) and that ddl was the only known gene on this fragment. This identification

however now comes into question as another gene ftsQ maps on this same restriction fragment. The 30 kilodalton protein is not synthesised from a λ transducing phage (JFL41) that carries the 2.2kb Eco RI fragment (Lutkenhaus and Wu 1980), although this does not discount the possibility that the 30 kilodalton protein is the ftsQ gene product. For example, as discussed on chapter 5, the entire ftsQ gene may not be carried on this fragment, and the fragment in λ JFL41 may be inserted in the "wrong" orientation for the expression of the 30 kilodalton protein.

An additional 30 kilodalton protein would not be seen in the minicell preparations, as the strongly expressed β -lactamase is also 30 kilodaltons, and would obscure additional proteins of the same size. However, the results with pGH203 are compatible with the idea that the ftsQ product is a 30 kilodalton protein, so that the 37 kilodalton would be a ftsZ-ftsQ hybrid protein.

Experiments are in progress (in this laboratory) to separate the minicell proteins by 2D-electrophoresis, so that an additional^a 30 kilodalton protein with a different iso-electric-focusing point could be observed. Immuno-precipitation with a β -lactamase specific antibody could also be used, to remove the β -lactamase, and permit observation of similarly sized proteins.

CHAPTER 7DNA sequencing of the 2.2kb Eco RI fragment7.1 Introduction

The nucleotide sequence of DNA is of obvious biological importance. The position of genes can be predicted from open reading frames, the amino acid sequence of the proteins calculated, and with the use of mutations, various control elements can be recognised. It also provides a detailed and accurate restriction map, which is invaluable for further genetic manipulations. It was reasoned, that with the transcriptional assays described in chapter 5, the DNA sequence of the 2.2kb Eco RI fragment would prove a powerful tool in the determination of the control of the fts genes.

Of the DNA sequencing methods that have developed so far, the most rapid for relatively large DNA fragments is the M13 dideoxy method developed by Sanger, and this was used to begin the sequencing of the 2.2kb Eco RI fragment. The methodology of the chain terminating reactions is rather involved, and will not be discussed here, as it is adequately described in the literature (Sanger, Nicklen and Coulson 1977, Sanger and Coulson 1978, Smith 1980, Schreier and Cortese 1979, Anderson, Gait, Mayol and Young 1980, Messing, Crea and Seeburg 1981). The principle of the M13 dideoxy method, is not complicated and will be briefly discussed (see Fig. 7.1).

Restriction fragments of the DNA to be sequenced are cloned into specially constructed ^{phage}M13 vectors (M13mp2, M13mp2/Bam, M13mp2/Hind and M13mp7). The sites available for cloning in these vectors are shown

Fig. 7.1 Diagrammatic representation of DNA sequencing, using the M13 dideoxy method

Stage 1. Cloning into M13 and preparation of single stranded templates:

The double stranded replicative ~~form~~ form of M13 is cleaved with a restriction enzyme, and ligated with restriction fragments of the DNA to be sequenced. Plaques are recovered by transfection, and then grown in liquid culture on a bacterial host. Phage are precipitated from the supernatant, with PEG6000, phenol extracted and the SS DNA recovered.

Stage 2. Dideoxy sequencing reactions:

A single stranded template is primed by boiling with a 26bp primer and allowing to cool slowly. The primed template is mixed with a [$\alpha^{32}\text{P}$] dNTP and Klenow polymerase and divided into four aliquots. Each aliquot is incubated with the four dNTP's, and one of the four ddNTP's, so that each aliquot has a different ddNTP.

Stage 3. Denaturing polyacrylamide gel electrophoresis:

The partially synthesised, labelled DNA chains each having a common 5' terminus, but a base-specific 3' terminus are separated on a thin, denaturing polyacrylamide gel. The smallest DNA chains migrate to the bottom of the gel, whilst the longer chains migrate more slowly and remain further up the gel.

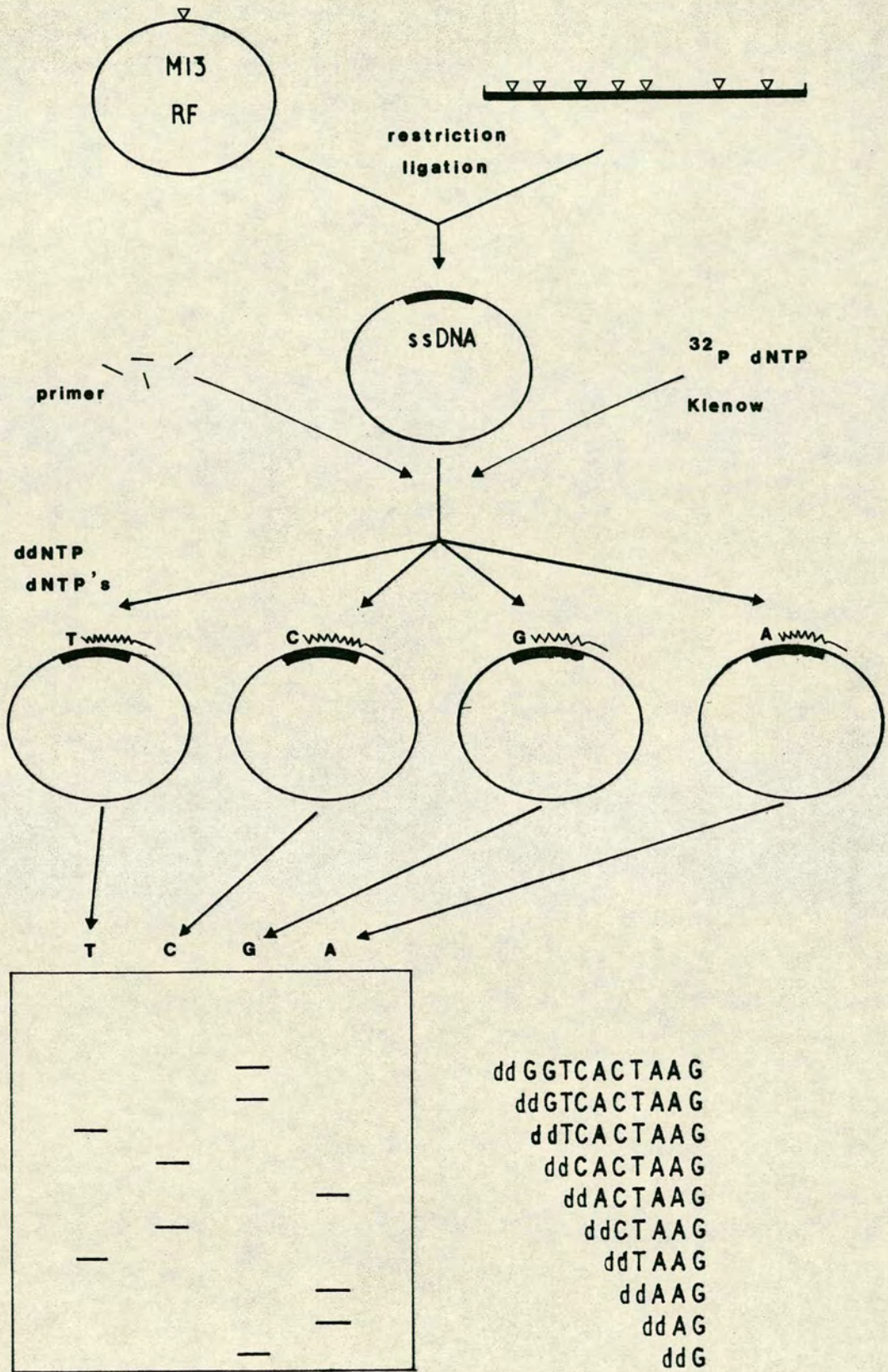


Fig 7.1

in Fig. 7.2. Fragments from different restriction enzyme digests are cloned, so that all the required DNA is represented by overlapping restriction fragments. Recombinant M13 phage are recognised as white plaques on XG plates, whereas the vectors form blue plaques. The filamentous phage from these recombinants can be easily prepared and the single stranded DNA within them rapidly purified. A short primer (26 bps) that is complementary to vector DNA close to the cloning sites is used to prime complementary strand synthesis from these single strand DNA templates using the large (Klenow) fragment of DNA Polymerase I.

A primed template is divided into four reaction vessels, each of which contains Klenow polymerase, the four deoxyribonucleotides (dCTP, dGTP, dATP, dTTP) one of which is ^{32}P labelled, and one of four dideoxyribonucleotides (ddCTP, ddGTP, ddATP, ddTTP). These dideoxyribonucleotides terminate DNA synthesis when inserted into the elongating chain, and if the deoxyribonucleotide: dideoxyribonucleotide ratio is correct, then different sized ^{32}P labelled DNA chains will be synthesised, that have a common 5' end, but a base-specific 3' end. The four reaction mixtures containing partially synthesised chains can be separated by size on a denaturing polyacrylamide gel and the DNA chains visualised by autoradiography. The DNA sequence can then be read from the shortest chains (5' end) at the bottom of the gel to the larger chains (3' end) at the top of the gel (Fig. 7.3). The number of bases that can be determined is limited only by the resolution of the gel. The required DNA sequence can be obtained by overlapping sequence data from different M13 clones.

Fig. 7.2 DNA sequence around the cloning sites in M13 vectors

M13mp2, M13mp2/Bam, M13mp2/Hind III and M13mp7 all have lac DNA (promoter, operator and first 145 amino acid residues of β -galactosidase gene) inserted into the intergenic region between genes IV and II. The cloning sites in these vectors all lie within the lac DNA, so that insertion of DNA fragments results in a lac^- phenotype. The direction of transcription of the lac DNA is shown. The 3' terminus of the "universal" 26bp primer is complementary to the lac DNA so that DNA synthesis proceeds towards the lac promoter.

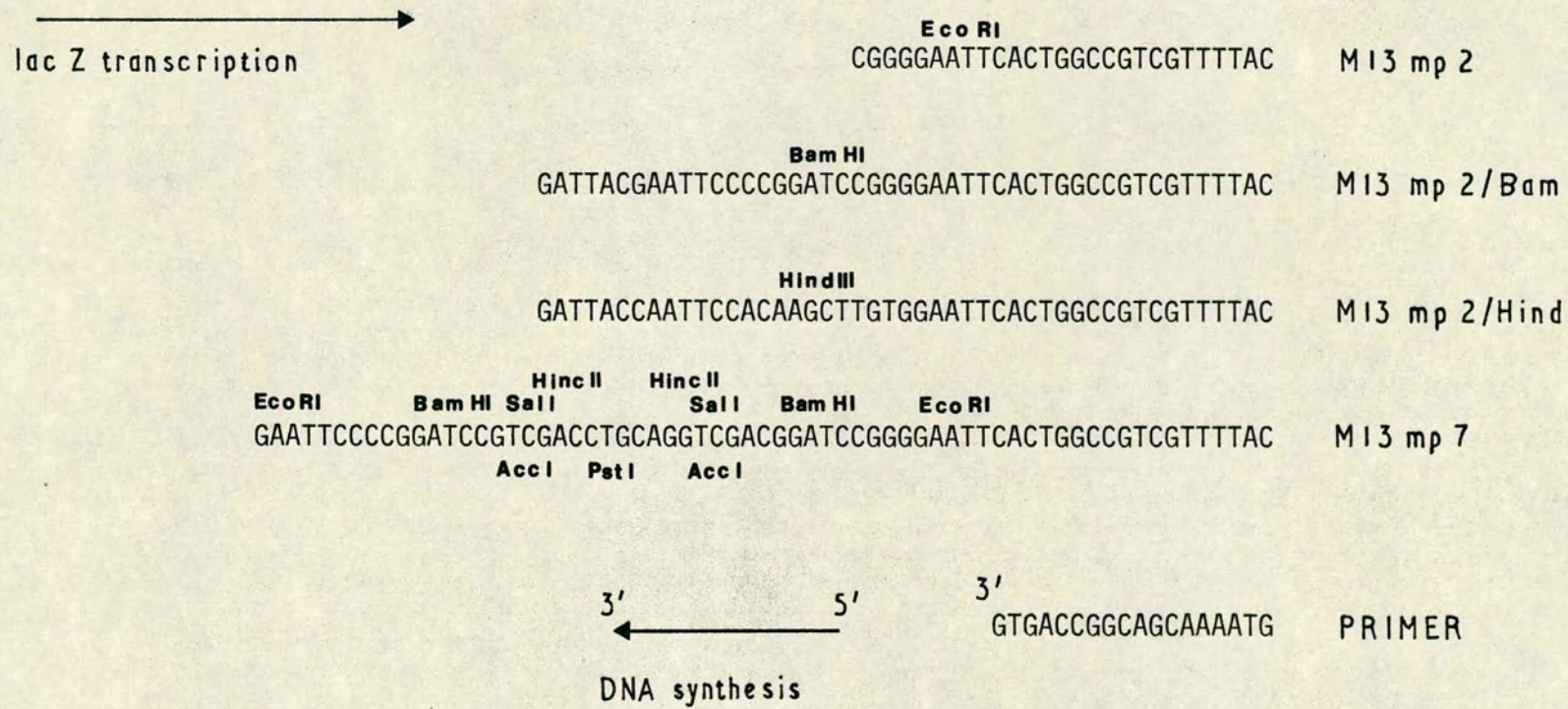


Fig 7.2

Fig. 7.3 Sequencing gel (6% acrylamide) of Bgl 4 template

The dideoxyribonucleotide used in each of the four aliquots of primed template are shown above the gel. This^s a typical sequencing gel, with the sequence being read from the bottom of the gel (5') to the top (3'). This particular sequence is from the Bgl II site within the 2.2Kb Eco RI fragment, reading towards the Bam HI site.

T C G A

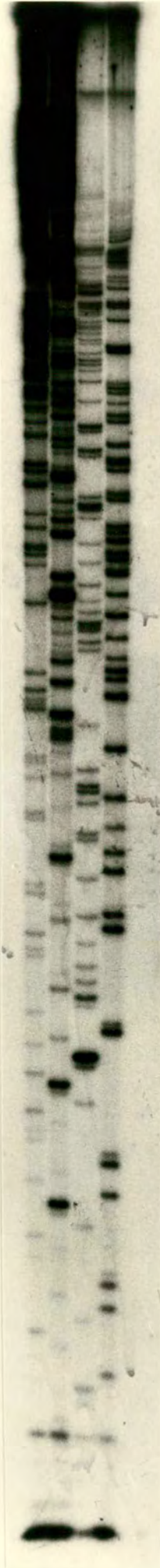


Fig 7.3

7.2 Sequencing strategy

It was decided to sequence the 2.2kb Eco RI fragment in two stages. First restriction fragments were inserted into the M13 vectors using the known restriction sites, i.e. Eco RI, Bam HI, Bgl II and Hind III. Sequence data from these clones should yield about 600-800 bps, and these defined clones permit the identification of which DNA strand has been cloned. To do this, different M13 clones are hybridised at 65°C, and analysed by agarose gel electrophoresis for the presence of double stranded DNA (which migrates slower than single stranded DNA). Double stranded DNA will only be formed if the two clones contain complementary inserted DNA (i.e. opposite strands).

In the second stage, restriction fragments produced by digestion with tetranucleotide recognition restriction enzymes were cloned into M13 so that random sequencing could provide the remainder of the data. Ideally, both DNA strands should be sequenced, although this is particularly true for data which is obtained from the top of a sequencing gel, where the sequence is most liable to be misread.

7.3 Cloning into M13 vectors (see Fig. 7.4)

7.3.1. Cloning into M13mp2

A preparation of purified 2.2kb Eco RI fragment was ligated with Eco RI digested M13mp2, and plaques recovered by transfection into JM101. White recombinant plaques were recognised on nutrient agar plate with IPTG (200ug/ml) and XG (200ug/ml). Avoiding the large plaques (which often have deletions), white plaques were picked and single stranded template DNA prepared from them. On hybridisation, two clones

designated R1 and R7 were determined as having opposite strands cloned into them. These strands were arbitrarily designated R1 (+) and R7 (-).

7.3.2.1 Cloning into M13mp 2/Bam

A preparation of the purified 2.2kb fragment was ligated and redigested with Bam HI as described in Fig. 5.4 for the construction of pGH203, to produce a 2.2kb Bam HI fragment. This was ligated with Bam HI digested M13mp2/Bam and white plaques picked after transfection into JM101, and single stranded templates prepared. By hybridisation to R1 and R7 templates, two clones of each strand were identified and designated Bam 1 and 2 (+), and Bam 3 and 4 (-).

7.3.2.2 The 2.2kb Eco RI fragment was rearranged to form a 2.2kb Bgl II fragment, as described above, and ligated with Bam HI digested M13mp 2/Bam. White plaques were recovered from transfection into JM101, and single stranded templates prepared. By hybridisation to R1 and R7, two clones, one of each strand were identified and designated Bgl4 (+) and Bgl 6 (-).

7.3.2.3 Purified 2.2kb Eco RI fragment was digested with Sau 3A and ligated with Bam HI digested M13mp 2/Bam. White plaques were recovered by transfection into JM101 and single stranded templates prepared. The percentage of white plaques was rather high (about 20%) and hybridisation of some of these clones to both R1 and R7 templates indicated that many of these clones had more than one insert.

7.3.3 Cloning into M13mp2/Hind III

The 2.2kb Eco RI fragment was rearranged into a 2.2kb Hind III

fragment as described in Fig. 5.4 and ligated with M13mp2/Hind III. White plaques were recovered by transfection into JM101, and single stranded templates prepared. Only one clone (Hind 3) hybridised to R1 and this has the (-) strand cloned.

7.3.4 Cloning into M13mp7

Purified 2.2kb Eco R1 fragment was digested with either Taq I or Msp I and ligated with Acc I digested M13mp7. White plaques were recovered by transfection into JM101 and single_{-stranded} templates prepared.

7.4 Sequencing of the single stranded templates

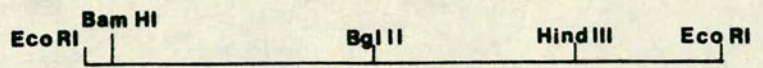
The success of the chain terminating sequencing reactions was found to be very variable, so that some templates produced better autoradiograms than others. There was particular difficulty with the M13mp7 recombinant clones. Filter hybridisation to ³²p labelled 2.2kb Eco R1 fragment (data not shown) showed that these clones do contain inserts from this fragment, but a sequence could only be obtained from a few clones. This variability proved to be the limiting factor in the rate with which sequence data could be accumulated.

Template R7 was sequenced, and about 120 bases of reliable sequence data obtained. More sequence data can be read from the gels, but becomes rather unreliable beyond about 120 bases unless the resolution is particularly good. An examination of this data reveals a Bam HI site, 105 bases from the Eco R1 site. This sequence must therefore come from the ftsQ end of the 2.2kb Eco R1 where a Bam HI has already been located (chapter 4), 0.1kb from the Eco R1 site. This was confirmed by

Fig. 7.4 List of templates used for sequencing the 2.2Kb Eco RI fragment

The point of origin of each template on the 2.2Kb Eco RI fragment is represented as a circle and the direction of DNA synthesis is shown by an arrow. The (+) and (-) signs arbitrarily represent the DNA strand that has been cloned into M13.

Template



RI	+								←○
R7	-		○→						
Bam1,2	+		—○						←
Bam3,4	-		○→						
Bgl4	+					←○			
Bgl6	-							○→	
Sau6	-							○→	
Hind3	-							○→	

Fig 7.4

sequencing the Bam 1 and Bam 3 templates. Bam 1 was identified by hybridising to the R7 template, as having cloned the (+) strand, and when sequenced, revealed an Eco R1 site 105 bases from the Bam H1 site. The DNA sequence between the Bam H1 and Eco R1 sites was exactly complementary to that determined in R7, without exception. Bam 3 was determined by hybridisation to have cloned the (-) strand, and the first 20 bases of its sequence were identical to those obtained from the R7 template. A further 80 bases were determined. Therefore about 200 bases of the 2.2kb Eco R1 fragment have been reliably determined at the ftsQ end of the fragment, and these are shown in Fig. 7.5. The sequence of only one strand is shown, from 5' to 3' progressing from the Eco R1 site into the 2.2kb Eco R1 fragment. By convention this is the antisense strand of genes that would be transcribed into the 2.2kb fragment (i.e. in the same direction as ftsA). Fig. 7.5 also shows some restriction sites which have been identified in the sequence, and a list of translation stop codons and initiation codons, in both DNA strands.

It proved difficult to obtain reliable sequence data from the R1 template. This was partly due to a G-C rich region about 20-30 bps into the fragment which proved difficult to resolve.

Sequence data was obtained from the Bgl 4 and Bgl 6 templates. 100 bases of reliable sequence were obtained from the Bgl 6 template, and 150 bases from the Bgl 4 template. The Bgl 4 template has the (+) strand cloned into it, so that the sequence data obtained is from the opposite strand to that of the sequence in Fig. 7.5. For convenience, the complementary strand of Bgl 4 is shown in Fig. 7.6 along with the sequence data from Bgl 6. Therefore this sequence in Fig. 7.6 is the same strand as shown in Fig. 7.5. Sequence data must be obtained across

Fig. 7.5 DNA sequence of the region around the Bam HI restriction site in the 2.2Kb Eco RI fragment

The sequence proceeds from the 5' end to the 3' end so that the strand shown is the antisense strand for genes transcribed from left to right. This strand is synthesised from template R7, (which has the (-) strand cloned) so the strand shown is the complementary (+) strand.

The tables below, show the position and frame of translation initiation and stop codons, in both strands. The position number for each codon is the number of bases from the middle base of the codon to the 5' terminus of that strand. The frame number is arbitrary.

Fig. 7.5

```

0 EcoRI GAATTCTGGA ACTGGCGGAC TAATATGTCG CAGGCTGCTC TGAACACGCG 50
51 AAACAGCGAA GAAGAGGTTT CTTCTCGCCG CAATAATGGA ACGCGTCTGG 100
101 BamHI CGGGGATCCT TTTCTGCTG ACCGTTTTAA CGACAGTGTT GGTGAGCGGC 150
151 TGGGTCGTGT TGGGCTGGAT GGAAGATGCG CAACGCCTGC CGCTCTCAAA 200
  
```

In the above (+) strand, translation initiation and stop codons appear at:

<u>Stop Codons (TGA, TAA or TAG)</u>		<u>Start Codons (ATG or GTG)</u>	
<u>Position</u>	<u>Frame</u>	<u>Position</u>	<u>Frame</u>
22	1	26	2
42	3	87	3
85	1	147	3
120	3	158	2
129	3	170	2
144	3	177	3

In the complementary (-) strand, translation initiation and stop codons appear at:

<u>Stop Codons (TGA, TAA, TGA)</u>		<u>Start Codons (ATG or GTG)</u>	
<u>Position</u>	<u>Frame</u>	<u>Position</u>	<u>Frame</u>
4	1	155	3
73	1		
180	2		

Fig. 7.6 DNA sequence of the region around the Bgl II restriction site
in the 2.2Kb Eco R1 fragments

The sequence proceeds from the 5' end to the 3' end so that the strand shown is the antisense strand for genes transcribed from left to right. The strand is the same strand as shown in Fig. 7.5 (i.e. the (+) strand). The tables below show the position and frame of translation initiation and stop codons in both strands. The position number for each codon is the number of bases from the middle base of a codon to the 5' terminus of that strand. The frame numbers are arbitrary.

The sequence is compiled by sequencing in both directions from the Bgl II, so it is not known if the sequence is contiguous through the Bgl II site.

Fig. 7.6

```

0 GAAGTTCTGC CCGACGGTAT GGTCAATATC ATTGGCGTGG CAGCTGCCCC 50
51 TCGCGTGGTA TGGATAAAGG CGGGGTGAAC GACCTCGAAT CCGTGGTCAA 100
101 GTCCTACAAC GCGCCATTCA TTGACCAGGC AGAATTGATG GCAGATTGTC 150
151 BgIIAGATCTCTTC GGTATATCTG GCGCTTTCTG GTAAGCACAT PvuIICAGCTGCCAG 200
201 AATGAAATTG GTATGGTGCC TATTTCTGAA GAAGAAGTGA CGCAAGAAGA 250
    
```

In the above (+) strand, translation initiation and stop codons appear at:

<u>Stop Codons (TGA, TAA, TAG)</u>		<u>Start Codons (ATG, GTG)</u>	
<u>Position</u>	<u>Frame</u>	<u>Position</u>	<u>Frame</u>
66	3	38	2
77	2	56	2
123	3	61	1
137	2	76	1
183	3	94	1
204	3	139	1
228	3	203	2
239	2	214	1
		217	1
		238	1

In the complementary (-) strand, translation initiation and stop codons appear at:

<u>Stop Codons (TGA, TAA, TAG)</u>		<u>Start Codons (ATG, GTG)</u>	
<u>Position</u>	<u>Frame</u>	<u>Position</u>	<u>Frame</u>
30	3	62	2
60	3	64	1
101	2	131	2

Fig. 7.6 continued

<u>Position</u>	<u>Frame</u>	<u>Position</u>	<u>Frame</u>
132	3	135	3
153	3	220	1
221	3		
227	2		

Fig. 7.7 DNA sequence around the Hind III site in the 2.2Kb Eco RI fragment

The sequence proceeds from the 5' end to the 3' end so that the strand shown is the antisense strand for genes that are transcribed from left to right. The strand is the same as that shown in Figs. 7.5 and 7.6 (+ strand). The tables below show the position of translation initiation and stop codons in each strand. The position of each codon is the number of bases from the middle base of a codon to the 5' terminus of that strand. The frame numbers are arbitrary.

Fig. 7.7

```

0  Sau3A
   TaqI GATCGAGCCG CGCTATACCG AGCTGCTCAA CCTGGTCAAC GAAGAGATAT 50
51  HindIII
   TGCAGTTGCA GGAAAAGCTT CGCCAACAAG GGGTTAAACA TCACCTGGCG 100
101 MspI
   GCAGGCATTG TATTAACCGG TGGCGCACGC Sau3A
   TaqI AGATCGAAGG TCTTGCAGCC 150
151 TGTGCTCAGC GCGTTGTTTC ATACGCAAGT
  
```

In the above (+) strand, translation initiation and stop codons appear at:

<u>Stop Codons (TGA, TAA or TAG)</u>		<u>Start Codons (ATG or GTG)</u>	
<u>Position</u>	<u>Frame</u>	<u>Position</u>	<u>Frame</u>
86	2	121	1
115	1	153	3

In the complementary (-) strand, translation initiation and stop codons appear at:

<u>Stop Codons (TGA, TAA or TAG)</u>		<u>Start Codons (ATG or GTG)</u>	
<u>Position</u>	<u>Frame</u>	<u>Position</u>	<u>Frame</u>
11	2	10	1
24	3	54	3
67	1	74	2
89	2	88	1
96	3	91	1
144	3		
153	3		
167	2		

the Bgl II site before the Bgl 4 and Bgl 6 sequences can be determined to be contiguous with each other. Fig. 7.5 also shows some restriction sites which can be predicted from the sequence, and a list of translation^{initiation} and stop codons in both strands.

The Hind 3 template containing the (-) strand was sequenced and 110 bases reliably obtained. One of the Sau 3A clones, Sau 6 was sequenced, and found to have a Hind III recognition site, and sequence data identical to that in Hind 3. Therefore the (-) strand of this Sau 3A fragment has been cloned into Sau 6. The sequence around the Hind III site is shown in Fig. 7.7 and is the same strand as that shown in Fig. 7.5 and 7.6. Fig. 7.7 also shows some restriction enzyme sites that can be predicted[†] from the sequence, and a list of translation initiation and stop codons in both reading frames.

Approximately 500 bases have also been sequenced from Sau 3A, Msp I and Taq I fragments, although some of this data overlaps with that shown in Figs. 7.5, 7.6, or 7.7, and will not be discussed further.

7.5 Interpretation of sequence data

Largely due to the difficulty experienced with sequencing the templates of M13mp 7 clones, the entire sequence of the 2.2kb Eco RI has not yet been obtained. It is hoped that these problems can be overcome by repeating the cloning of the Taq I and Msp I fragments into two newly constructed vectors M13mp8 and M13mp9 (Messing, unpublished data). However, until the entire sequence is obtained the exact position of the genes on the fragment cannot be determined. The data that has been obtained represents a significant start to the sequencing project, and some useful information can be extracted from each set of sequence data shown in

Figs. 7.5, 7.6, 7.7

7.5.1 Sequence about the Bam HI site

A study of the sequence data shown in Fig. 7.5 reveals that both strands have one open reading frame throughout the sequence. More sequence data is required though to assess whether either or both of these is actually being used in vivo for polypeptide synthesis. Transcriptional assays in chapter 5 suggested that the 105bp Eco RI-Bam HI fragment could (if the BglII-Hind III fragment does not) have promoter activity, so this sequence can be examined for promoter-like sequences.

In their review, Rosenberg and Court (1979) have collated 46 known promoter sequences and determined the similarities, and differences between them. There appears to be at least two regions of DNA that are involved in the binding of RNA polymerase, a sequence around -10 from the mRNA start point, often called a "Pribnow box", and a sequence at -35 nucleotides from the mRNA start point. The Pribnow box is a heptameric region, TATAATG, to which most promoter sequences are generally homologous. However, the only residue which appears to be invariable is the T residue in the sixth position. The T and A in the first and second positions are strongly conserved, and T, A and A in the third, fourth and fifth positions respectively, considerably less so. The residue in position 7 is weakly conserved, as are the 6-9 nucleotides downstream of it towards the mRNA start point. The mRNA start point is usually a purine, and an A more often than a G. (Rosenberg and Court 1979).

The second region, the -35 region exhibits some homology among the promoters that have been studied, although the position of the homology fluctuates ± 2 bps in respect to the mRNA startpoint. A

trinucleotide TTG is strongly conserved in this region, and adjacent to this and downstream is a weakly conserved trinucleotide ACA.

Promoter sequences that are under the influence of a positive regulatory molecule (λ PRE, gal P₁ and ara BAD) appear to have very poor -35 homologies (Rosenberg and Court 1979).

Because of the variability in sequences of known promoters it is obviously not possible to unequivocally recognise a promoter from a nucleotide sequence, and this can only be done with a biological assay. However, the sequence can be studied for promoter like sequences as a basis for further study. In the Eco RI-Bam HI 105 bp region there is only one possible "Pribnow box" like sequence in the strand shown in Fig. 7.5, and none in the complementary strand. This sequence occurs around position 23 and is TAATATG. This sequence has the strongly conserved TA in positions 1 and 2, and the invariable T in the sixth position as well as the weakly conserved G in the seventh position, and A in the fifth position. The sequence only differs from the standard "Pribnow box" in the third and fourth weakly conserved positions. There are also A and G residues at positions 32 and ^{33,34} 34 respectively which could act as mRNA start points. The -35 region of this putative promoter would be cleaved by Eco RI and therefore outside the 2.2kb Eco RI fragment.

If this putative promoter should be active in vivo then the question arises as to whether the DNA sequences are present to allow ribosome binding and initiation of polypeptide synthesis. As frames 1 and 3 are closed by several stop codons (Fig. 7.5) translation could only be in reading frame 2 unless initiated downstream of position 85 in frame 1, or downstream of position 144 in frame 3.

Polypeptide synthesis is initiated at the codons ATG or GTG, although ATG is the more commonly used of the two. Shine and Dalgarno (1974) identified a region upstream of the start codon which is required for ribosome binding, and which has homology to the 3' terminus of the 16S RNA of the ribosome, 3' AUUCCUCCACUAG^{5'}. Several mRNA sequences have been studied, and found to have sequences homologous to this, at around 8 residues upstream from the start codon. However, this homology can vary from 3 residues (in TrpE mRNA) up to 9 residues (in λP_R). (This includes G.U. pairing which does occur in RNA). (Shine and Dalgarno 1974).


As the extent of these homologies varies greatly, it is again very difficult to predict ribosome initiation points from a nucleotide sequence. There is a sequence that does have homology to the 16S RNA around position 161 in Fig. 7.5 upstream of the start codon at position 170. The sequence ~~ATGGG~~ has good homology to the 16S RNA, and is located perfectly for initiation at the ATG codon (position 170), although obviously further experimentation is required to determine if this is used in vivo.

7.5.2 Sequence around the Bgl II site

In the interpretation of this sequence, it must first of all be remembered that although the sequence is presumed to be contiguous through the Bgl II site, this has not yet been proven.

From the sequence shown in Fig. 7.6 one reading frame is open in both strands for the entire sequence. The complementation analysis in chapter 5 would indicate that this DNA is internal to the ftsA

gene, and it is most likely then that the ftsA gene is translated in the first reading frame which is not closed in the sequence, using the strand shown as the antisense strand.

This sequence is most useful in determining the precise location of the Pvu II  restriction sites. The sequence clearly shows two Pvu II sites, 150bps apart. This therefore defines the deletion in pGH370. The deletion of 150bps will not cause a frameshift if the ftsA gene is translated in the first reading frame, but this sequence must be essential for the function of the ftsA gene product as pGH370 does not complement ftsA mutants.

7.5.3 Sequence around the Hind III site

The Sa_{u} 6 template is most useful as it defines the presence of only one Hind III site. All the sequence in Fig. 7.7 is therefore contiguous. The complementation data in chapter 5 suggests that this sequence is internal to the ftsA gene. It is unlikely then that the complementary strand to that shown in Fig. 7.7 is also being used, and it does have stop codons within all three reading frames. Lutkenhaus and Wu (1980) determined that the ftsA gene must extend at least 135 nucleotides past this Hind III site. This suggests that ftsA is transcribed in frame 3, in Fig. 7.2, as both frames 1 and 2 have stop codons before this point. The C-terminal end of the ftsA gene has therefore not yet been sequenced in this study.

CHAPTER 8General Discussion

The isolation of the mutant TOE 1, has identified a new gene ftsQ which maps in the cluster of cell division genes at 2 minutes on the E. coli K12 genetic map (Bachmann and Brooks Low 1980). This gene maps adjacent and anticlockwise to ftsA, and its product may be required periodically in the cell cycle as is the ftsA gene product. Both genes map on a 2.2kb Eco RI restriction fragment, as does the ^{promoter} proximal part of the ftsZ gene and its promoter (Lutkenhaus and Wu 1980). The precise organisation of the genes on this fragment has not been determined, but the following model is proposed to explain the observations in this study.

1) ftsQ, ftsA and ftsZ all have their own promoters :

The 0.5kb Hind III-Eco RI fragment, when cloned into pK0-1 has promoter activity, and this accords with the previous suggestion that the ftsZ promoter, and proximal part of the structural gene lie within this fragment (Lutkenhaus and Wu 1980). However, the ftsZ gene may be translated from more than one type of transcript, as mRNA synthesis from upstream of the Hind III does not appear to terminate within the 0.5kb Hind III-Eco RI fragment.

The ftsA promoter has not been identified in the fusion plasmids that have been constructed, but there are two lines of evidence for its existence. a) ftsA must be transcribed independently of ftsQ as some plasmid recombinants (pGH300, pGH-4) complement ftsA mutants, but not ftsQ mutants and b) Promoter activity ^{be} may be greater in the 1.7kb Eco RI-

Hind III fragment (in pGH106/A), than in pGH110 where the 0.9Kb Bam HI-Bgl II fragment has been deleted, and this would suggest that a promoter ~~lies~~ lies within the Bam HI-Bgl II fragment.

pGH110 carries only the Eco RI-Bam HI (0.1kb) and Bgl II-Hind III (0.7kb) fragments, but has promoter activity. This activity may arise from within the 0.1kb Eco RI-Bam HI fragment where a promoter type sequence has been identified. A sequence similar to a "Pribnow box" has been located approximately 23 base pairs from the Eco RI site. This site would cleave the essential -35 region of this RNA polymerase recognition sequence. The sequence of the 5-10 nucleotides adjacent to the cloning site in the vector would therefore dictate the activity of this promoter. The identification of this region as the ftsQ promoter would correlate with complementation analyses of genetic recombinants, some of which complement TOE 1 (ftsQ^{ts}) well (pGH301, λ FH16, λ GH16), some rather poorly (pGH-4, pGH106) and some not at all (pGH300). The sequence of the vector DNA close to the cloning site is known in several of these recombinants (Fig. 8.1). None of these have the -35 sequence TTGACA observed in other -35 sequences (Rosenberg and Court 1979), although pGH301 does have two T residues, four bases from the Eco RI site in the vector. pGH-4 has only one of these T residues, and pGH300 neither of them. It is possible that it is these T residues (corresponding to the first two residues of the sequence TTGACA) that determine the activity of this promoter. Alternatively, ftsQ may be under positive control (like gal P₁), so that pGH301 has fortuitous similarity to the required sequence for ftsQ expression.

Fig. 8.1 DNA sequence around the Eco RI cloning site in pGH300,
pGH301 and pGH-4

The DNA sequence adjacent to the Eco RI site in pGH300 derives from
the λ 0 gene (McKenney et al 1981) in pGH301 from pBR322
(McKenney et al 1981)

and in pGH-4 from pBR325 (personal communication from N. Willetts).

The sequences below show the Pribnow box (Rosenberg and Court 1979)

and -35 sequence.

Eco RI
pGH-4 TTGCCCATACGGAATTCTGGAACTGGCGGACTAATATG
pGH 300 GGATTCGCCAGAATTCTGGAACTGGCGGACTAATATG
pGH301 TCGTCTCAAGAATTCTGGAACTGGCGGACTAATATG

TTGACA

TATAATG

-35

Pribnow

Fig 8.1

2) ftsQ may code for a 30 kilodalton protein

Lutkenhaus and Wu (1980) determined that the 3.2kb Hind III fragment carried on the λ 16-2 transducing phage synthesised a 30 kilodalton protein, and deduced that as ddl was the only gene identified on this fragment, that the 30 kilodalton protein is the ddl gene product. Their confirmation for this deduction was that the phage λ JFL41 (which carries the 2.2kb Eco RI fragment) does not synthesise this protein. The observations in the present study however, suggests that the 30 kilodalton protein may be the ftsQ gene product, rather than the ddl gene product. For example, the phage JFL41 may not synthesise the 30 kilodalton protein because the 2.2kb fragment is inserted so that the ftsQ promoter is not active and the phage promoters interfere with expression of the 30 kilodalton protein. There is sufficient coding capacity on the 2.2kb Eco RI fragment to code for an additional 30 kilodalton protein as well as the 48 kilodalton protein (ftsA gene product).

pGH203 synthesises a 37 kilodalton protein, which is most probably synthesised from the ftsZ promoter and translation initiation sequence, across the Eco RI site into the 2.2kb Eco RI fragment. The 37 kilodalton protein is probably an ftsZ-ftsQ hybrid protein, reading in the open reading frame identified in the sequence around the Bam HI site. The start codon ATG and ribosome binding site 160-170 bases from the Eco RI site may be the translation initiation sequence for the ftsQ gene which would synthesise a 30 kilodalton protein. There is another start codon (ATG) around 26 bases from the Eco RI site, within the "Pribnow box". From a calculation of the amino acid sequence, from the DNA sequence between the start codons, a protein synthesised from this start codon would be approximately 6 kilodaltons larger than the ftsQ gene product.

Fig. 8.2 Protein synthesis directed from pGH203

This diagram shows the hypothetical origin of the proteins synthesised by pGH203. The 30 kilodalton and 43 kilodaltons are presumed sizes for proteins, which would be "masked" by other plasmid coded proteins in the gel and have not been observed as yet. The promoters are shown by a letter P. The left-most promoter is the ftsZ promoter, and the rightmost the ftsA promoter.

(see note added in proof)

pGH 203

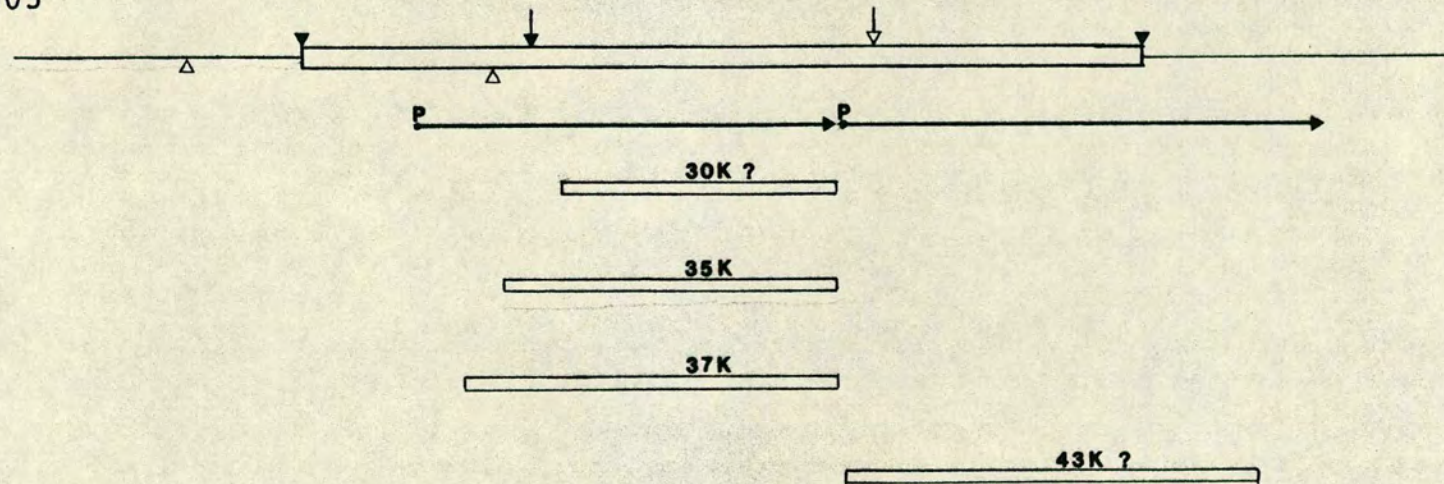


Fig 8.2

Fig. 8.3 Model for the organisation of the genes *ftsQ*, *ftsA* and *ftsZ* on the 2.2kb Eco RI fragment

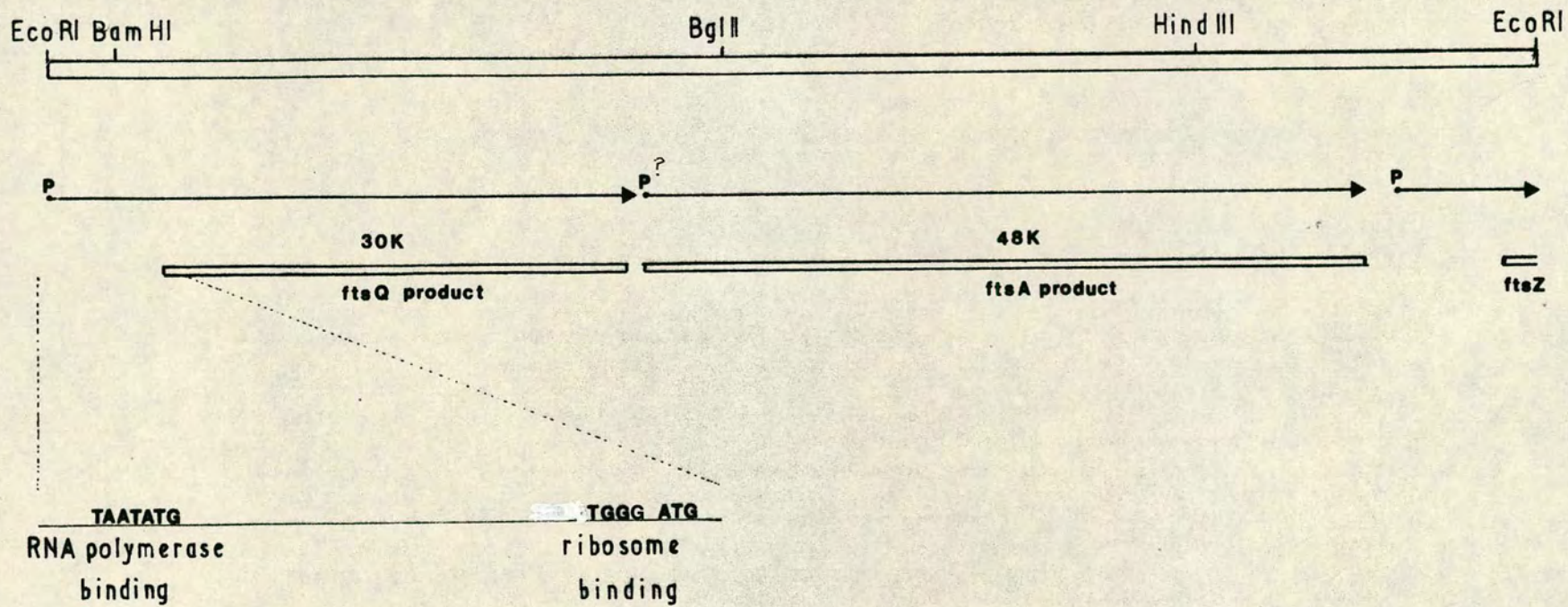


Fig 8.3

This protein could be the 35 kilodalton protein that is synthesised from pGH203 (Fig. 8.2). Fig. 8.3 shows the proposed model for the organisation of the fts genes.

The interpretation of the sequence data is speculative but does correlate well with the observations in this study. The gene organisation and control could be verified by further experimentation. For example, the completion of the DNA sequence of the 2.2kb Eco RI fragment, and the DNA upstream of this fragment, should clarify the precise location of the fts genes in this region. The restriction map predicted from this sequence will be used for cloning each fts promoter into pKO fusion plasmids, and control elements detected by the introduction of mutations by in vitro mutagenesis (Humphreys et al 1976). The mRNA mapping technique of Berk and Sharp (1977) could also be used to identify the mRNA start point within the region.

It is hoped that these studies will lead to the elucidation of the control of these genes, and their role in cell division.

Note added in proof

A recent experiment by Vicky Derbyshire and myself has shown from two dimension^{al} polyacrylamide gel electrophoresis that pGH203 synthesises an additional 43 kilodalton protein with a different isoelectric focusing point to that of the galK gene product. In this analysis pGH203 also synthesises an additional 30 kilodalton protein which has a different isoelectric focusing point to that of the 30 kilodalton β -lactamase protein.

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Identification of New Genes in a Cell Envelope-Cell Division Gene Cluster of *Escherichia coli*: Cell Division Gene *ftsQ*

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We report the identification, cloning, and mapping of a new cell division gene, *ftsQ*. This gene formed part of a cluster of three division genes (in the order *ftsQ ftsA ftsZ*) which itself formed part of a larger cluster of at least 10 genes, all of which were involved in some step in cell division, cell envelope synthesis, or both. The *ftsQAZ* group was transcribed from at least two independent promoters.

In *Escherichia coli* there is a large cluster of genes, all of which are involved in some aspect of cell envelope synthesis, cell division, or both (5-8, 11, 12; H. J. W. Wijsman, *Genetics* 74: S296, 1973). So far, eight different genes have been placed unambiguously within this region by the use of specialized transducing phages (5-7), and a number of other mutations of similar phenotype have been located less precisely within or in the neighborhood of this cluster (see reference 1). The purpose of this and the accompanying note (9) is to report the discovery and positions within the cluster of two new genes which, like those described hitherto, appear to be concerned specifically with cell envelope growth, septum formation, or both.

Previous studies on the *ftsA* gene have shown that it is located within this cluster (6, 10, 12), that the action of the *ftsA* protein (a polypeptide of molecular weight 50,000 [6]) is required only during septum formation (11), and that the synthesis of this protein appears to be required only during a short period immediately before the onset of septum formation (4). If *ftsA* protein is not made at the correct time in one cell cycle, then the formation of a septum is blocked until after the synthesis of the protein at the equivalent time in the next cycle (4). A search was therefore made for division genes which had a single required time of action in each cycle. To do this, an asynchronous population of UV-irradiated cells was grown at 30°C and shifted to 42°C for periods of 5 min at each successive mass doubling of the culture. After three such cycles, the cells were passed through a membrane filter (pore size, 14 μm) to enrich for abnormally long cells. Those few cells which were trapped by the filter were subjected to a further three cycles of growth at 30°C with 5-min pulses at 42°C as before and then filtered once more. The cells which were trapped on the filter were then plated on nutrient agar (Oxoid nutrient broth plus 50 μg of thymine per ml) at

30°C, and the resultant clones were screened for temperature-sensitive mutants by replica plating at 30 and 42°C. A number of such mutants (designated "TOE" mutants for "temperature-oscillation enrichment") were obtained.

TOE-1 obtained in this way formed colonies on Oxoid nutrient agar at 30°C but not at 42°C. This temperature sensitivity was abolished by 1% sodium chloride (as is that of several of the already known *fts* mutants [8]). TOE-1 grew and divided in liquid medium at 30°C at a rate similar to that of its parent (*E. coli* K-12 AB2497), although the cells were somewhat larger on the average than those of the parent strain. When an asynchronous population of TOE-1 was shifted from 30 to 42°C, all cell division stopped immediately (Fig. 1), but exponential cell growth continued for a minimum of three mass doublings so that long, filamentous cells were produced (Fig. 2). These filaments were multinucleate (data not shown), indicating that DNA replication and segregation were not affected at the temperature which was restrictive for division. The phenotype of TOE-1 is therefore that of an *fts* mutant in that it appears to be affected specifically in septum formation (8, 10).

The mutation responsible for the TOE-1 phenotype was found by P1 transduction to be 75% cotransducible with *leu* at 1.6 min on the genetic map of *E. coli* (1). This made it likely that the mutation was within the known cluster of division-related genes located at about 2 min (1, 5, 12, 13; H. J. W. Wijsman, *Genetics* 74:S296, 1973). This was confirmed, and the locus was determined precisely by the use of specialized transducing phages for this region (6, 7; G. Hatfull and J. F. Lutkenhaus, unpublished data). These plaque-forming, integration-proficient λ phages were constructed in vitro by cloning restriction endonuclease fragments of the chromosome and extending or deleting these in vivo or in vitro so as to contain various fragments of

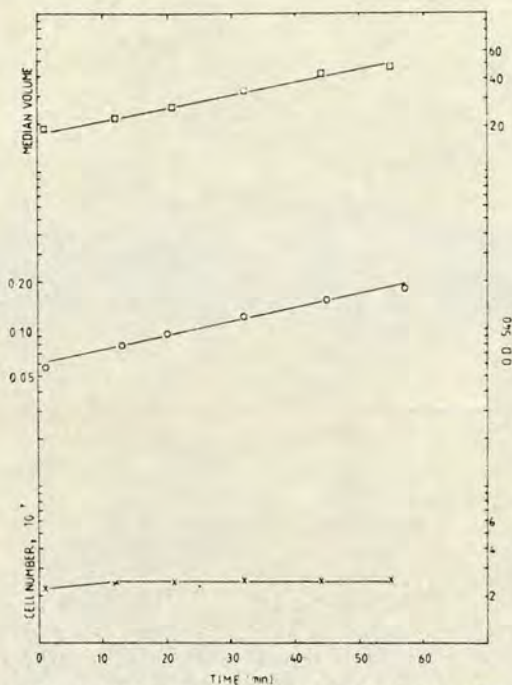


FIG. 1. Kinetics of cell growth and division in a log-phase population of strain TOE-1 [*ftsQ*(Ts)] after a shift from 30 to 42°C at 0 min. Increase in total cell mass was followed as optical density (OD) at 540 nm (○). Cell number was measured with a Coulter electronic particle counter (×), and median cell volume (□) was measured with a Coulter Channelyzer, as described previously (2). Cell division stopped almost immediately after the shift, but cell growth continued.



FIG. 2. A cell of strain TOE-1 [*ftsQ*(Ts)] after four generations of growth at 42°C. The cell is approximately 64 μ m in length, or about 10 to 20 times as long as cells grown at 30°C. Unlike the filamentous cells of *ftsA*(Ts) strains under comparable conditions, there is no sign of periodic indentations at presumptive septal sites (4, 6, 11).

the region. These cloning vectors are especially convenient in that stable lysogens can be easily obtained (3). TOE-1 was lysogenized with a number of these at 30°C, and the lysogenic clones were tested for temperature sensitivity. The results obtained with two of the transducing phages (λ *ddl*⁺ and λ FH16; Fig. 3) served to locate the TOE-1 mutation. Lysogenization with

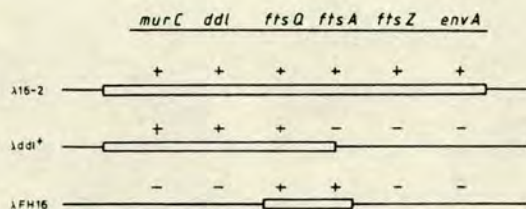


FIG. 3. The order of genes in the *murC-envA* section of the *E. coli* chromosome (top) and the chromosomal inserts (boxes) cloned into the three specialized transducing phages, λ 16-2, λ *ddl*⁺, and λ FH16. The "+" signs indicate that the gene directly above is complemented by the particular phage. The lengths of the inserts in the phages are approximately (from top to bottom) 9.77×10^3 , 6.51×10^3 , and 2.1×10^3 nucleotide base pairs. The region contains a minimum of three independent promoter sites.

either of these two phages rendered TOE-1 temperature insensitive. The phenotypes of other lysogens made with phages carrying neighboring or overlapping regions were consistent with this location. When temperature-resistant lysogens were cured of their phages by the use of λ b2 (6) the cured cells were once again temperature sensitive. The right-hand end (as written) of the chromosomal insert in λ *ddl*⁺ is defined by a *Hind*III site within the *ftsA* gene (6, 7) so that mutations within *ftsA* are not complemented by this phage. The chromosomal fragment cloned in λ FH16 was bounded by two *Eco*RI restriction sites (Fig. 3) and was already known to carry the entire *ftsA* gene (G. Hatfull, unpublished). The mutation in TOE-1 must therefore lie within this fragment in a new gene to the left of *ftsA*. Confirmation that this new mutation did not lie within the *ftsA* gene was obtained by lysogenizing TOE-1 with the phage λ 16-4, which carries the *ftsA12*(Ts) mutation on a larger fragment which covers the entire region in question (Fig. 3). Lysogenization of TOE-1 with λ 16-4 conferred a temperature-resistant phenotype. It could be argued that TOE-1 carries a missense mutation in *ftsA* and that λ *ddl*⁺ produces a peptide fragment that confers temperature resistance by intragenic complementation. However, as λ *ddl*⁺ did not complement any of the five *ftsA* alleles that we have tested, this is thought to be an unlikely possibility. Thus, we may conclude that TOE-1 carries a missense mutation in a new gene, which we designate *ftsQ*. The length of the chromosomal fragment cloned in λ FH16 is about 4.6% of lambda, or about 2.14×10^3 base pairs. The *ftsA* gene codes for a polypeptide of molecular weight 50,000 (6) which would require about 1.36×10^3 base pairs of DNA. The maximum length of the new gene (including its promoter) is about 700 to 800 base

pairs, giving a maximum molecular weight for the *ftsQ* product of about 20,000 to 30,000. It is not yet known whether *ftsA* shares this promoter or has one of its own. However, another independently expressed gene, *ftsZ*, has been located adjacent to *ftsA* on the other side (7) (Fig. 3), and this also is expressed on cloned fragments which do not carry a complete *ftsA* gene. Thus, *ftsA* may possibly share a common promoter with either *ftsQ* or *ftsZ*, but these latter two genes must have independent promoter sites. The known mutations in these three adjacent genes have very similar phenotypes in that they specifically affect late stages in septum formation. Nevertheless, it is clear that they form a minimum of two transcriptional units, each of which can be expressed efficiently when transposed away from its neighbors to an abnormal chromosomal location (*att*^λ). The functional reason, if any, for the close clustering of these cell division genes therefore remains unknown.

We thank Susan Wilkie for skilled assistance with these experiments.

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