Characterization of the *ftsK* gene of *Escherichia coli*

G. C. Draper

A Thesis presented for the Degree of Ph.D.

Institute of Cell and Molecular Biology
University of Edinburgh, Scotland

February 1998

To my parents and Harriet

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Declaration

I declare that the composition of this Thesis was all my own work except where stated

G. C. Draper

February 1998

Acknowledgements

Many thanks to my parents for making sure I finished the work that they started and for their constant belief and encouragement. Thank you Harriet for becoming my wife and enabling me to put things into perspective. I would also like to express my gratitude to Stephen, my son, for so many stress relieving trips to the park.

Thanks go to Willie for taking on a uncouth Englishman and sending forth a slightly older even more-Englishman. Thanks go to the members of the lab., past and present. Dave Boyle for tab break philosophy and too many drinks to remember, Medhat Khattar for the good advice and sound friendship and Neil McLennan for all things red, white, weird and unpronounceable. Also, thanks to Uta, Kenny, Sean, Guowen, Richard, Dawn and the Tiga for making my time in 820 more enjoyable.

To Jo, Anthony, Darren and Simone I wish luck in their futures and my thanks for the good times we had in the past. May our friendship continue beyond the confines of grants and deadlines.

Many thanks to the folks in Photography, who I've bothered and badgered and who have helped with suggestions and curses.

Abstract

Cell division in *Escherichia coli* requires the concerted action of at least nine division-specific gene products. These proteins are responsible for formation of the septum and have been predicted to form a macromolecular complex. The biochemical functions of three of the division proteins, FtsZ, FtsA and PBP3 are known at least in part. The biochemical function of the six remaining gene products is not known. Their involvement in the division process has been inferred from mutational and overexpression studies.

The division process can be described as having three distinct phases. The early stage involves the formation of a ring of FtsZ molecules at the mid-point of the cell. This process might involve a second protein, FtsW. During the late stage other division proteins are recruited to the mid-cell, and the cytoplasmic membrane, peptidoglycan sacculus and outer membrane invaginate at right angles to the long axis of the cell forming a septum. During the very late stage of cell division the septum is completed and daughter cells separate. One cell division gene, *ftsK*, has been implicated in this late stage of division.

The *ftsK44* mutant shows a temperature-sensitive block to cell division and forms filamentous cells at elevated temperatures. The nucleoid distribution within these filaments was unaffected. This work describes the cloning of *ftsK* and the characterisation of both overexpression and deletion of *ftsK*.

Overproduction of FtsK blocks cell division by either directly or indirectly blocking FtsZ ring formation. Deletion of the N-terminal membrane spanning region of FtsK resulted in a lethal block to cell division. This effect can be reversed by expressing the N-terminal 225 aa of FtsK. Examination of the nucleoids in the filaments that result when FtsK is depleted showed that chromosomal segregation was impaired. The N-terminus of FtsK is dispensable when FtsN, an essential cell division protein, is overproduced. Disruption of *ftsK* at bp 677 did not effect cell division *per se* but did result in a proportion of cells forming chains. Examination of chromosomal DNA within these chains revealed nucleoids trapped by the invaginations. FtsK was identified and was shown to migrate anomalously during SDS–PAGE.

Abbreviations

aa	-amino acid(s)
Amp	-ampicillin
APS	-ammonium persulphate
ara	-arabinose
ATP	-adenosine 5'-triphosphate
bp	-base pair(s)
BSA	-bovine serum albumin
cAMP-3', 5'	-cyclic adenosine monophosphate
Cmp	-chloramphenicol
CTAB	-hexadecyltrimethyl ammonium bromide
CTP	-cytidine 5'-triphosphate
DAPI	-4,6-diamidino-2-phenylindole
(d)dATP	-2'(3'-di)-deoxyadenosine-5'-triphosphate
(d)dTTP	-2'(3'-di)-deoxythymidine-5'-triphosphate
(d)dGTP	-2'(3'-di)-deoxyguonosine-5'-triphosphate
(d)dCTP	-2'(3'-di)-deoxycytidine-5'-triphosphate
DMSO	-dimethylsulphoxide
DNA	-deoxyribonucleic acid
DNase	-deoyxyribonuclease
EDTA	-Diaminoethanetetra-acetic acid
fts	-filamentous
GTP	-guanosine 5'-triphosphate
HEPES	-N-(2-hydroxyethyl) piperazine-N'-(2-ethanesulphonic acid)
IPTG	-isopropyl-β-D-thiogalactoside
Kan	-kanamycin
kb	-kilobase pair(s)
kDa	-kilodalton(s)
m.o.i.	-multiplicity of infection
mRNA	-messenger RNA
NAcGluc	-*N*-acetylglucosamine
NAcMur	-*N*-acetylmuramic acid
Nal	-nalidixic acid
OD	-optical density
OLB	-oligo-labelling buffer
ONPG	-*O*-nitrophenyl-β-D-galactoside
PCR	-polymerase chain reaction
PEG	-polyethylene glycol
PIPS	-penicillin-insensitive peptidoglycan synthesis
PIPES	-piperazine-N,N' bis (2-ethanesulphonic acid)
R	-resistant
rbs	-ribosome binding site
Rif	-rifampicin
RNA	-ribonucleic acid
RNase	-ribonuclease
rpm	-revolutions per minute

SDS	-sodium dodecyl sulphate
SDS–PAGE	-sodium dodecyl sulphate polyacrylamide gel electrophoresis
Spec	-spectinomycin
STE	-salt Tris-EDTA
SSC	-salt sodium citrate
Str	-streptomycin
TAE	-tris-acetate EDTA
TE	-tris-EDTA
TEMED	-N,N,N',N',tetramethylethylethylenediamine
Tet	-tetracycline
Tmp	-trimethoprim
TOE	-temperature oscillation enrichment
tRNA	-transfer RNA
Ts	-temperature-sensitive
TTP	-thymidine 5'-triphosphate
UTP	-uridine 5'-triphosphate
UV	-ultraviolet
v/v	-volume by volume
w/v	-weight by volume
X-gal	-5-bromo-4-chloro-3-indolyl-β-D-galactoside
Tris	-2-amino 2-hydroxymethyl-1,3-propandiol

CHAPTER 1
INTRODUCTION

Chapter 1. The cell cycle of *Escherichia coli*

In order for *Escherichia coli* to proliferate it has to elongate, replicate and partition its chromosomes and finally divide across the long axis of the cell (see Figure 1.1). The *E. coli* cell cycle can be defined as a number of integrated processes that are linked together by a host of controls and cues. This introduction aims to present a review of cell division, peptidoglycan biosynthesis, chromosome replication and partition.

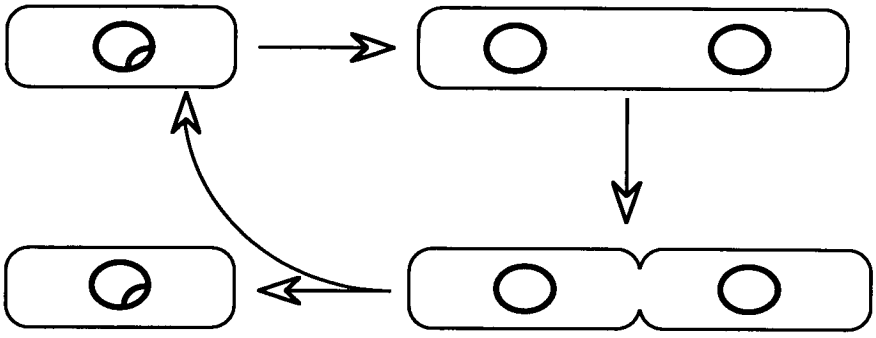

Figure 1.1. A representation of the *E. coli* cell cycle. A newborn cell elongates and replicates its chromosome. The newly replicated chromosomes (open circles) are then partitioned to opposite poles and then the cell divides.

The third chapter of this thesis will describe the cloning and overexpression of *ftsK*, a cell division gene first identified by Begg *et al.*, (1995). The fourth chapter gives an account of efforts to express and visualize FtsK by SDS-PAGE. The fifth chapter deals with the construction and phenotypes of a number of *ftsK* deletion alleles. The role of *ftsK* in cell division and possible future directions are also discussed.

1.1 Cell division

Cell division in *E. coli* takes place after the newly replicated chromosomes have segregated. Ingrowth of the cytoplasmic membrane,

peptidoglycan and outer membrane leads to the formation of a septum at the midpoint of the dividing cell (Donachie, 1993). The newly synthesized peptidoglycan layer is a covalently linked double layer the hydrolysis of which results in cell separation (Wolf-Watz and Normark, 1976). Little is known about the involvement of the outer membrane in cell division although it is thought to be anchored to the peptidoglycan layer via crosslinks with lipoproteins (de Boer *et al.*, 1990; Braun *et al.*, 1976). Division requires the concerted action of at least nine cell division specific gene products. The finding that L-forms of *E. coli* which lack the peptidoglycan layer divide normally, led to the conclusion that invagination of the peptidoglycan and outer membrane is probably a separate process from cytoplasmic membrane constriction (Onada *et al.*, 1987).

Most of the genes that are involved in cell division were discovered as mutations that gave rise to a filamentous temperature sensitive phenotype (*fts*). *fts* mutant cells continue to elongate and segregate their chromosomes at the non-permissive temperature but cell division is blocked, resulting in the formation of long, filamentous cells. The *fts* phenotype is distinct from that of the *par* class of mutants which form filaments that have large masses of DNA at the filament centre or unevenly distributed throughout the elongated cell. The *par* phenotype has been attributed to mutations in genes involved in chromosome replication and resolution.

1.1.1 Cell division genes

Six of the nine cell division genes described so far are clustered in the *m*urein *r*egion *a* or *mra* region at 2.2 minutes on the *E. coli* chromosome (Matsuhashi *et al.*, 1990; Miyakawa *et al.*, 1972). The structure of the *mra* region and the transcriptional and translational regulation of the expression of the genes within it will be discussed in section 1.2.2. The location of cell division genes and other genes involved in the cell cycle or peptidoglycan production is shown in Figure 1.1.2.

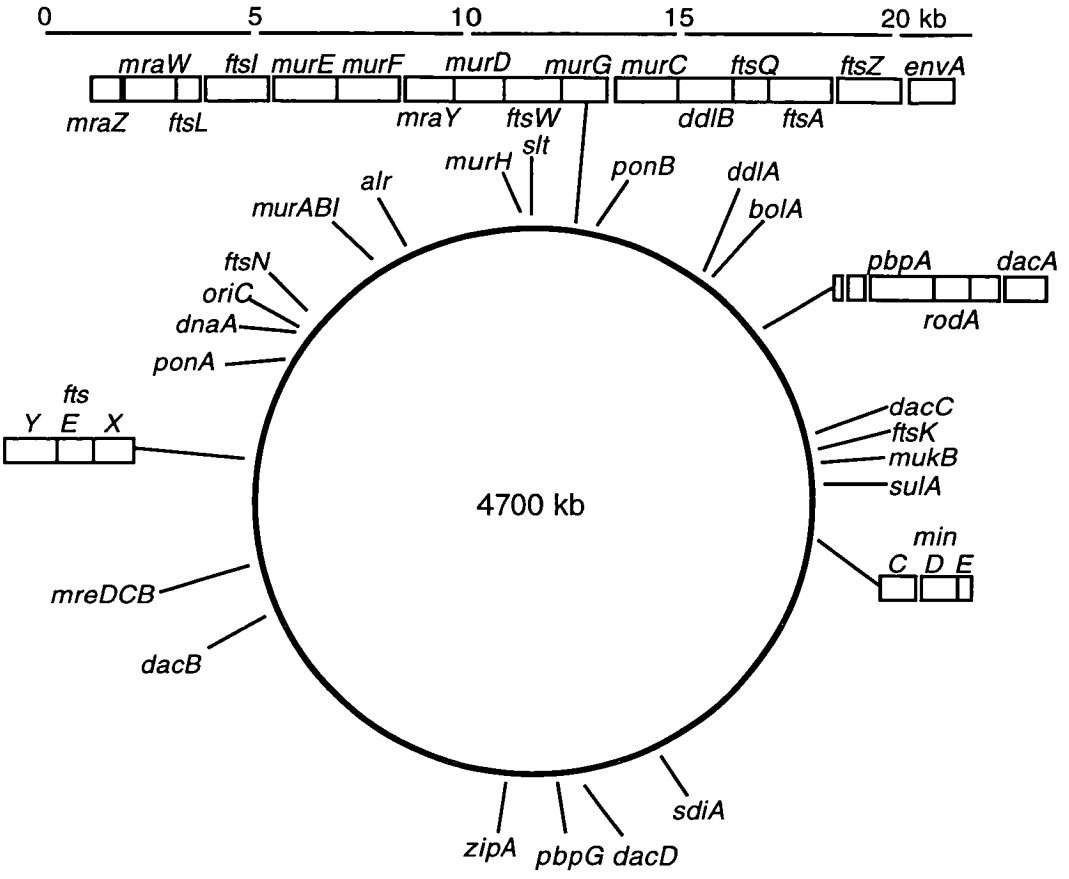

Figure 1.1.2. The location of the genes involved in the cell cycle and peptidoglycan metabolism of *E. coli*. The *E. coli* chromosome is approximately 4700 kb.

1.1.2 *ftsZ*

ftsZ is the penultimate gene in the *mra* region and encodes the 40 kDa cytoplasmic protein FtsZ (Lutkenhaus *et al.*, 1980; Plá *et al.*, 1991). FtsZ is the most abundant of the cell division proteins, numbering between 5000–20,000 molecules per cell (Bi and Lutkenhaus, 1991).

Genetic and molecular evidence suggests that FtsZ is required at an early stage of cell division and that its presence is essential for the completion of the division process. (Addinall *et al.*, 1996; Begg and Donachie, 1985). Temperature sensitive mutants of FtsZ form smooth sided filaments at the non-permissive temperature. Depletion of FtsZ from the cell leads to a block to cell division at all temperatures, indicating that FtsZ is an essential cell division protein (Dai and

Lutkenhaus, 1991). Mutations in other cell division genes such as *ftsA* and *ftsI* produce filaments with slight constrictions. The products of these genes are thought to act at a later stage in cell division than FtsZ (Begg and Donachie, 1985). Combining the *ftsZ* (Ts) mutation with a *rodA* (Ts) (or *pbpA* (Ts)) allele that leads to a loss of shape results in the formation of lemon shaped cells devoid of any constrictions, indicating that cell division is blocked at an early stage and the cell shape is lost. A combination of the *rodA* (Ts) allele with temperature sensitive mutants of *ftsA*, *ftsQ*, *ftsI* or *ftsK*, all later acting cell division genes, leads to swollen chains of cells with partial constrictions. This suggests that division has initiated but cannot be completed (Begg and Donachie, 1985; Begg *et al.*, 1995).

FtsZ has been localised to the cytoplasmic edge of the mid-point of the cell prior to septation and at the leading edge of the invaginating septum during division in a circumferential ring structure (Bi and Lutkenhaus., 1991., Addinall *et al.*, 1996). In the early ground-breaking work of Bi and Lutkenhaus (1991) thin sections of *E. coli* were probed with anti-FtsZ antibody and then gold-labeled secondary antibody. The electron dense regions of FtsZ accumulation were visualized by electron microscopy. Later work employed immunofluorescence microscopy (IFM) and FtsZ fused to the green fluorescent protein (GFP) of *Aequoria victoria* to localize FtsZ in fixed and living cells respectively (Addinall *et al.*, 1996; Ma *et al.*, 1996).

FtsZ rings are present at the mid-cell in 90% of cells in a wild-type population (Addinall *et al.*, 1996). It is inferred, therefore, that the FtsZ ring forms at the potential division site soon after the last round of division has been completed (Bi and Lutkenhaus, 1991; Addinall *et al.*, 1996). The location of the FtsZ ring is dictated by an as yet uncharacterized hypothetical factor and the *min* system. The *min* system functions to restrict division to the mid-cell (see below; reviewed in Lutkenhaus and Addinall, 1997). The hypothetical FtsZ nucleation factor, if it indeed exists, is present at least at all unused division sites (Addinall *et al.*, 1997b). This was demonstrated by shifting a strain bearing the FtsZ84 (Ts) mutant protein to the non-permissive temperature. High temperature renders FtsZ84 non-functional and cell division is blocked, resulting in the formation of smooth sided filaments. Shifting the *ftsZ84* mutant cells back to the permissive temperature results in the rapid formation of FtsZ

rings at the unused division sites, indicating that the positional information locating all unused division sites was already in place (Addinall *et al.*, 1997). The identity of the FtsZ nucleation factor is currently being sought by a number of laboratories.

Properties of FtsZ

Purified FtsZ can bind and hydrolyse GTP (de Boer *et al.*, 1992; Mukherjee *et al.*, 1993; RayChaudhuri and Park, 1992). FtsZ shows limited sequence homology with eukaryotic tubulins and contains the sequence GGGTGTG that is similar to the conserved tubulin GTP binding signature motif GGGTGS/TG (de Boer *et al.*, 1992; Mukherjee *et al.*, 1993, Mukherjee and Lutkenhaus, 1994). The mutants FtsZ3 (GGGAGTG) and FtsZ84 (AGGTGTG) have mutations in this motif and show reduced GTP binding and GTPase activities (de Boer *et al.*, 1992; Mukherjee *et al.*, 1993; RayChaudhuri and Park., 1992). The FtsZ84 mutant protein has an altered substrate preference and binds and hydrolyses ATP *in vitro* (RayChaudhuri and Park, 1994). The method of purification of the FtsZ dictates the kinetics of the GTPase activity. The study of RayChaudhuri and Park (1992) showed that FtsZ purified with GDP bound showed no lag in GTP hydrolysis. Two other groups isolated FtsZ without a bound nucleotide and this form of FtsZ showed a lag in GTPase activity that was inversely proportional to the FtsZ concentration. The lag could be prevented by preincubation of FtsZ with GDP, increasing the temperature or increasing the K^+ concentration. It is proposed that the GTPase activity of FtsZ is dependent upon interactions between FtsZ molecules which then leads to ring formation. GTP binds to the amino-terminal 320 amino acids of *Bacillus subtilis* FtsZ and can be cross linked to a region containing amino acids 67 to 250 (Wang *et al.*, 1997).

The visualization of the FtsZ ring and the similarity of FtsZ to tubulin led to the proposal that FtsZ could aggregate into a cytoskeletal structure *in vivo*. It was found that FtsZ could polymerise *in vitro* in the presence of guanine nucleotides. Mukherjee and Lutkenhaus (1994) found that FtsZ could form a linear polymer when supplied with GTP *in vitro*. In the presence of DEAE dextran, a substance found to enhance tubulin polymerization, FtsZ formed microtubule like structures, possibly bundles of linear polymers, in the presence of either GTP or GDP but did not hydrolyse GTP. In another study purified FtsZ formed

microtubules in the absence of DEAE dextran (Bramhill and Thompson, 1994). The polymerization was dependent on high GTP concentrations. The mutant FtsZ84 protein which has reduced GTPase activity was shown to have a reduced tendency to polymerise. This work has now been shown to have been carried out at a pH dramatically lower than intended, where FtsZ has an increased likelihood of polymerization (Lutkenhaus and Mukherjee, 1996). FtsZ microtubules assemble into two dimensional sheets which are structurally similar to tubulin polymers. (Erickson *et al.*, 1996). A *Rhizobium meliloti* FtsZ-GFP fusion protein was incorporated into the FtsZ ring at the septum in *E. coli* (Ma *et al.*, 1996). Interestingly, cytoskeleton like fibrils could also be seen in the cytoplasm of a proportion of these cells, probably the result of non-productive polymerization.

The crystal structure of a recombinant *Methanococcus jannaschii* FtsZ ($FtsZ_{Mj}$) has recently been reported (Löwe and Amos, 1998). The N-terminal GTP binding domain of $FtsZ_{Mj}$ has a fold similar to that found in $p21^{ras}$ (Tong *et al.*, 1991). The GTP binding by $FtsZ_{Mj}$ is different to that displayed by other members of the GTPase superfamily. The C-terminus of $FtsZ_{Mj}$ shows limited sequence similarity to any other protein presently in the databases but does have structural similarity to chorismate mutase of *B. subtilis* (Löwe and Amos, 1998). $FtsZ_{Mj}$ has an overall structure similar to α- and β- tubulin.

Interactions with FtsZ

Further evidence that FtsZ interacts with itself has come from yeast two-hybrid system studies using *Bacillus subtilis* FtsZ (Wang *et al.*, 1997). Amino acids 100–326 of FtsZ were found to be the minimum required for FtsZ:FtsZ interaction to take place, perhaps defining an FtsZ polymerization domain. It was also shown that *B. subtilis* FtsZ interacted with *B. subtilis* FtsA. An interaction between *B. subtilis* FtsZ and *E. coli* FtsA was also observed, the interaction requiring the non-conserved region of the carboxy-terminus of FtsZ. The FtsZ–FtsA interaction is possibly a mechanism by which FtsA is located to the septum (Addinall and Lutkenhaus, 1996; Ma *et al.*, 1996; Wang *et al.*, 1997).

FtsZ also interacts with the essential cell division protein ZipA (Hale and de Boer, 1997; see section 1.1.10). ZipA was identified by virtue of its binding to radiolabelled FtsZ *in vitro*. The endogenous cell division

inhibitors SulA and MinC (in the presence of MinD) also show interaction with FtsZ in a two-hybrid screen (Huang *et al.*, 1996). FtsZ also interacts with the cytoplasmic region of PBP3 (Bramhill *et al.*, 1995). PBP3 is the product of the *ftsI* gene that is responsible for the production of septal peptidoglycan (Ishino and Matsuhashi, 1981; Spratt, 1975). This possible interaction might link cytoplasmic membrane invagination and the ingrowth of the peptidoglycan layer during cell division (Bramhill *et al.*, 1995).

With the exception of *ftsK*, the effect of depletion of the products of each of the cell division genes has been characterised via the construction of conditional null alleles. In this way it has been shown that each cell division protein is essential for cell division and viability. The importance of FtsZ in the division system is, however, critical. The amount of FtsZ limits cell division (Bi and Lutkenhaus, 1990a). A slight increase in the level of FtsZ leads to the formation of minicells by suppression of the division inhibition by MinCD at the cell poles (Ward and Lutkenhaus, 1985; see 'The *min* system' below). Increasing FtsZ to three times the normal levels induces filamentation, presumably because of unproductive aggregation of FtsZ (Dai and Lutkenhaus, 1992; Ward and Lutkenhaus, 1995). The ratio of FtsZ:FtsA is also important for correct cell division (Dai and Lutkenhaus, 1992; Dewar *et al.*, 1992). Overproduction of FtsA leads also to filamentation but simultaneous overproduction of FtsZ and FtsA results in normal cell division.

FtsZ has been identified in a range of eubacteria and in archaebacteria (Bauman and Jackson, 1996; Beall *et al.*, 1988; Brun and Shapiro, 1993; Doherty and Adams, 1995; Holden *et al.*, 1993; Kobayashi *et al.*, 1997; Margolin *et al.*, 1991; Margolin and Long, 1994; Margolin *et al.*, 1996; Osteryoung and Vierling, 1995; Wang and Lutkenhaus, 1996; Yi and Lutkenhaus, 1985). One of these organisms, *Mycoplasma pulmonis*, lacks a cell wall, indicating that FtsZ does not require the peptidoglycan layer for function (Wang and Lutkenhaus, 1996).

Regulation of FtsZ activity

FtsZ activity is subject to control by several endogenous division inhibitors (see below). That FtsZ is the target for regulation highlights its critical role in the division process.

The *min* system.

The *min* system of *E. coli* dictates the position of the septum. The *minB* operon consists of three genes, *minC*, *minD* and *minE* (de Boer *et al.*, 1988, 1989; Rothfield and Zhao, 1996). In the classical *min* mutant, division can take place at any of the three potential division sites in the cell, that is, the midpoint (new division site) and either of the two poles (old division sites). Polar division leads to the production of small anucleate cells known as minicells. The use of division potential in polar division results in an increase in the average nucleate cell length as in Figure 1.1.3. (Adler *et al.*, 1976; Teather *et al.*, 1972; Donachie and Begg, 1996).

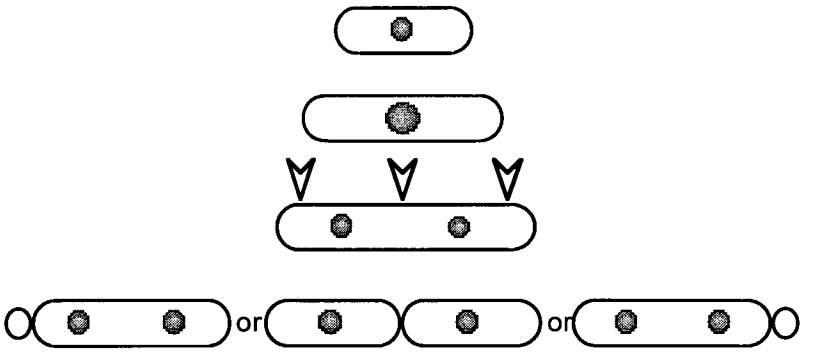

Figure 1.1.3. The *min* phenotype. Any of the potential division sites (indicated by arrows) can be used. The result is an increase in average nucleate cell length. From Donachie, 1993.

The *minC* gene product inhibits division and requires MinD for activity. MinD is a cytoplasmic membrane-associated ATPase (de Boer *et al.*, 1991). Although it is thought that the MinCD complex is required for inhibition of division, overproduction of MinC alone blocks division (de Boer *et al.*, 1992). The target for MinCD is FtsZ; MinCD blocks the formation of the FtsZ ring (Bi and Lutkenhaus, 1990c; Bi and Lutkenhaus, 1993). MinC can also be activated by the DicB protein, which is encoded by a defective prophage, to function as a division inhibitor (de Boer *et al.*, 1990; Labie *et al.*, 1990; Mulder *et al.*, 1992). The MinC/DicB system is not subject to control by MinE (de Boer *et al.*, 1990; see below).

MinE is apparently able to differentiate between the mid-cell and the cell pole. The presence of MinE allows division at the mid-cell but not at the poles. There are two potential mechanisms by which MinE might function. The first suggests that MinE suppresses the inhibitory effect of MinCD at the mid-cell but not at the cell poles (de Boer *et al.*, 1989; Zhao *et al.*, 1995; Huang *et al.*, 1996; Rothfield and Zhao, 1996), the second proposes that MinE is a factor that targets MinCD to the poles (de Boer *et al.*, 1989; Pichoff *et al.*, 1995). MinE can neither confer topological specificity on the MinC/DicB division inhibitor nor can it prevent the filamentation caused by the overexpression of MinC leading to the suggestion that MinE acts through MinD (de Boer *et al.*, 1992). Interaction between MinC and MinD was detected using the yeast two hybrid system (Huang *et al.*, 1996). There was no interaction observed between MinC and MinE but a weak interaction was recorded between MinD and MinE. MinC interacted with FtsZ only when MinD was present, indicating that MinCD is the active division inhibitor. It was proposed that by interacting with FtsZ, MinCD prevented FtsZ ring formation (Huang *et al.*, 1996).

Overproduction of MinE suppresses the division inhibition caused by MinCD not only at the mid-cell but also at the cell poles resulting in the formation of minicells. Deletion of *minC* and/or *minD* leads to a loss of division inhibition at all potential division sites which also leads to minicell formation (de Boer *et al.*, 1989). In the absence of MinE long aseptate filaments form because MinCD blocks division at all potential division sites (de Boer *et al.*, 1989).

MinE forms a ring structure at the mid-cell (Raskin and de Boer, 1997). The MinE ring requires MinD but not MinC or the FtsZ ring for formation. The study proposes that the MinE ring inhibits the action of MinCD at the mid-cell and contradicts the hypothesis that MinE localises MinCD to the cell pole (de Boer *et al.*, 1989; Pichoff *et al.*, 1995). The model put forward by Zhao *et al.*, (1995) and Huang *et al.*, (1996) and developed by Raskin and de Boer (1997) is shown in Figure 1.1.4. Factors X and Y are the hypothetical elements that respectively influence the sites for FtsZ and MinE ring location. The FtsZ ring is prevented from assembling at the cell poles by MinCD. MinCD is prevented from acting at the mid-cell by the MinE ring (see figure legend).

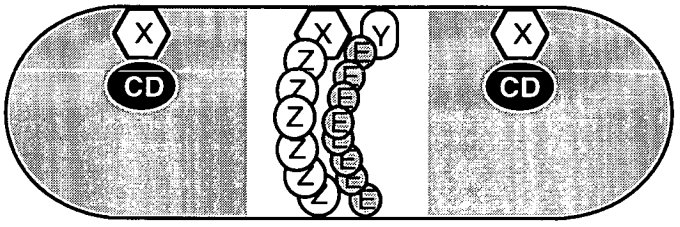

Figure 1.1.4. The proposed model for MinCDE action (from Raskin and de Boer, 1997). MinE recognizes the mid-cell via a hypothetical topological factor Y which results in a zone where MinCD activity is suppressed (unshaded section). Factor X is a hypothetical element present at both the mid-cell and poles that defines potential division sites (see FtsZ section). At the cell poles Factor X is proposed to inhibited by the presence of active MinCD.

The SOS Response

sulA (*sfiA*) is a component of the SOS regulon (Huisman and D'Ari, 1981). Damage to DNA results in a block to chromosome replication and the formation of regions of single stranded DNA (Rupp and Howard-Flanders, 1968). The binding of RecA to these lesions causes RecA to become activated (Salles and Defais, 1984; Sassanfar and Roberts, 1990). Activated RecA associates with LexA, the repressor of the SOS system, resulting in the autocatalytic breakdown of LexA. Two LexA monomers bind to the consensus sequence TA**CTGT**ATATATATA**CAG**TA (known as SOS boxes) and form a dimer (Friedberg *et al.*, 1995; Kim and Little, 1992; Schnarr *et al.*, 1985; Thliveris *et al.*, 1991). Binding of LexA to the SOS box results in the repression of transcription of the downstream genes (Brent, 1982; Brent and Ptashne, 1981; Friedberg *et al.*, 1995; Little and Mount, 1982; Sancar *et al.*, 1982a; Sancar *et al.*, 1982b; Schnarr *et al.*, 1991), transcription of these genes is derepressed under SOS-inducing conditions. Gene products induced during SOS include enzymes active in the repair of damaged DNA. Once the SOS gene products have repaired the single stranded regions of DNA, RecA returns to its non-activated state, LexA no longer breaks down by autocatalysis and the system is repressed once again by LexA binding at SOS boxes. In this manner the set of genes described as the SOS regulon can be induced and repressed as and when required by the cell (Walker, 1996).

Induction of *sulA* results in the inhibition of cell division (Gottesman, 1989; Huisman *et al.*, 1984; Schoemaker *et al.*, 1984). FtsZ is the target for SulA activity (Bi and Lutkenhaus, 1990; Jones and Holland, 1985; Lutkenhaus, 1983). FtsZ mutants resistant to the inhibitory effects of SulA have been shown to have reduced GTP binding capabilities (Bi and Lutkenhaus, 1990b; Dai and Lutkenhaus, 1994). SulA prevents the formation of the FtsZ ring and two-hybrid analysis showed an interaction between FtsZ and SulA (Bi and Lutkenhaus, 1993; Huang *et al.*, 1996). The interaction between FtsZ and SulA involves the hydrolysis of GTP (Higashitani *et al.*, 1995). Upon repair of DNA damage, *sulA* induction is repressed and the cytoplasmic pool of SulA is rapidly degraded by the Lon protease (Gottesman, 1989; Maguin *et al.*, 1986). Khattar (1997) showed that the lethal effects of *sulA* induction in a *lon* mutant strain can be suppressed by overproducing the heat shock protease complex HslVU. It is not known whether HslVU is involved in the degradation of SulA in wild-type cells.

It is possible, therefore, that during SOS induction FtsZ ring formation is blocked to stall division whilst DNA damage is repaired. This block is mediated by the reversible inhibition of FtsZ polymerization by SulA.

The bacteriophage relic e14, present in some strains of *E. coli*, contains the SOS inducible *sfiC* gene (D'Ari and Huisman, 1983; Greener and Hill, 1980; Maguin *et al.*, 1986). SfiC also inhibits division during SOS induction.

There is a third SOS inducible system that blocks cell division upon interference with chromosome replication (Hill *et al.*, 1997). Even in cells devoid of SulA and SfiC activity filamentation is induced upon interference with DNA replication. This *sfi*-independent pathway is LexA dependent but the remaining components remain obscure. It is not known whether the *sfi*-independent filamentation functions by interfering with FtsZ function.

1.1.3 *ftsA*

ftsA is located in the *mra* region immediately upstream of *ftsZ* and encodes the 45 kDa protein FtsA (Lutkenhaus and Donachie, 1979; van de Putte *et al.*, 1964; Robinson *et al.*, 1984). There are approximately 150

molecules of FtsA per cell (Wang and Gayda, 1992). FtsA is found in the cytoplasm, associated with the cytoplasmic membrane and at inner-outer membrane junctions (Chon and Gayda, 1988; Plá *et al.*, 1990). FtsA is an essential cell division protein (Lutkenhaus and Donachie, 1979). Temperature-sensitive *ftsA* mutants form filaments with evenly spaced constrictions at the non-permissive temperature (Donachie *et al.*, 1979). The *ftsA12* (Ts) mutation can be suppressed by overproduction of FtsN (Dai *et al.*, 1993), as can temperature sensitive mutations in *ftsI* and *ftsQ*. As mentioned previously, FtsA is described as a late acting cell division protein (Begg and Donachie 1985). A combination of an *ftsA* (Ts) and *rodA* (Ts) alleles leads to the formation of chains of bloated cells with invaginations at the non-permissive temperature (Begg and Donachie, 1985). The morphology of the cells suggest that the FtsZ ring has formed but cell division has been blocked after the initiation of septation due to the inability of the FtsZ ring to fully constrict. Overproduction of FtsA blocks cell division due to the resultant imbalance in the ratio of FtsA:FtsZ (Dai and Lutkenhaus, 1992; Dewar *et al.*, 1992; Wang and Gayda, 1990). This relationship has led to speculation that FtsA might be involved in preventing unproductive aggregation of FtsZ (Lutkenhaus and Mukherjee, 1996).

It is proposed that FtsA interacts with another component of the septum, PBP3, the *ftsI* gene product (Tormo *et al.*, 1986). PBP3 is a septum specific transpeptidase that synthesizes the ingrowing layer of peptidoglycan during cell division (Botta and Park, 1981; Ishino and Matsuhashi, 1981; Spratt, 1975; Spratt, 1977). FtsA could act as a line of communication involved in co-ordinating invagination of the cell membrane and septal peptidoglycan biosynthesis.

FtsA is a component of the septum (Addinall and Lutkenhaus, 1996b; Ma *et al.*, 1996). The presence of FtsA in the septum is dependent on the formation of the FtsZ ring. In strains where FtsZ ring formation is prevented there is no evidence of FtsA localization. Cells carrying the mutant *ftsZ26* form spiral FtsZ structures as well as rings at the midcell at the permissive temperature (Addinall and Lutkenhaus, 1996a; Bi and Lutkenhaus, 1992). FtsA colocalises with FtsZ in these spiral shaped septa. These data led Addinall and Lutkenhaus (1996b) to suggest that FtsZ recruits FtsA to the septum. As mentioned in section 1.1.2, FtsA from *E. coli* interacts with FtsZ from *B. subtilis* (Wang *et al.*, 1997). The

sequestration of FtsA to the septum could be a directly attributed to the FtsZ ring.

FtsA has an ATP binding domain and limited similarity with sugar kinases, actin and DnaK (Bork *et al.*, 1994; Sanchez *et al.*, 1994). FtsA binds to an ATP affinity column suggesting that FtsA may have ATPase activity (Sanchez *et al.*, 1994). FtsA has both a phosphorylated and non-phosphorylated form (Sanchez *et al.*, 1994). The phosphorylation of FtsA takes place at a threonine residue corresponding to the phosphorylated residue in DnaK. Phosphorylated FtsA is located in the cytoplasm whereas the non-phosphorylated form is associated with the cytoplasmic membrane. Purified phosphorylated FtsA can bind ATP *in vitro* whereas the non-phosphorylated form cannot (Sanchez *et al.*, 1994). The role of the phosphorylation of FtsA is unclear as a mutant FtsA that cannot be phosphorylated still supports division (Sanchez *et al.*, 1995)

1.1.4 *ftsQ*

ftsQ lies in the *mra* region upstream of *ftsA* and encodes the 31 kDa FtsQ protein (Begg *et al.*, 1980; Carson *et al.*, 1991; Robinson *et al.*, 1984; Storts *et al.*, 1989). FtsQ is membrane bound, consisting of a small cytoplasmic N-teminus, a single membrane spanning α-helix and a larger periplasmic domain and is present at approximately 50 molecules per cell (Carson *et al.*, 1991). Proteins with such a structure are said to have a bitopic topology. FtsQ is essential for cell division (Carson *et al.*, 1991). The *ftsQ1* (Ts) mutant forms smooth filaments at 42°C but also forms filaments with evidence of septation at 37°C suggesting that FtsQ is required throughout the division process (Carson *et al.*, 1991).

Overexpression of *ftsQ* has no effect in rich media but blocks division in minimal medium (Carson *et al.*, 1991, Storts *et al.*, 1989). The overproduction of FtsQ is also detrimental to *ftsA* (Ts) and *ftsZ* (Ts) mutants and lethal to the *ftsI23* (Ts) strain at the permissive temperature (Dai and Lutkenhaus, 1992). FtsQ is thought to act at a later stage in cell division than FtsZ because of the constricted morphology of the *ftsQ1/rodA* (Ts) double mutant at the non-permissive temperature (Begg and Donachie, 1985) and also because functional FtsQ is not required for FtsZ ring formation (Addinall *et al.*, 1996). FtsQ is also thought to act at a later stage in cell division than FtsA as the immunolocalization of FtsA to

the potential division site does not require functional FtsQ (Addinall and Lutkenhaus, 1996b).

Guzman *et al.* (1997) reported that the specific sequences of the membrane spanning domain (MSD) of FtsQ was not essential for FtsQ function. FtsQ with the MSDs of FtsI, FtsL or MalF retained the ability to complement a *ftsQ*-null mutant. Swapping the cytoplasmic or periplasmic domains relative to the MSD of FtsQ rendered the protein unable to complement the *ftsQ*-null allele. These results agree with Dopazo *et al.*, (1992) who state that the amino terminus of FtsQ is required for FtsQ function.

1.1.5 *ftsW*

ftsW is located in the *mra* region and possibly encodes two proteins; a 43 kDa protein and a longer, in frame 46 kDa protein which would be initiated at a translation initiation codon 90 bases upstream of the first (Ikeda *et al.*, 1989; Ishino *et al.*, 1989; Khattar *et al.*, 1994; Khattar *et al.*, 1997). Both the 46 kDa protein (FtsWL) and the smaller peptide of 43 kDa (FtsWS) have been identified by SDS–PAGE but the two gene products have never been detected in the same extract (Khattar *et al.*, 1994; Khattar *et al.*, 1997; D.S. Boyle and M.M. Khattar, pers. comm.).

ftsW is an essential cell division gene (Boyle *et al.*, 1997). Depletion of FtsW from the cell results in the formation of aseptate filaments (Boyle *et al.*, 1997). Genetic and molecular evidence suggests that FtsW is required throughout division and that FtsW functions to stabilize the FtsZ ring (Boyle *et al.*, 1997; Khattar *et al.*, 1994; Khattar *et al.*, 1997). A reduced number of FtsZ rings are seen in filaments produced by the depletion of FtsW (Boyle *et al.*, 1997). The *ftsW* null strain required the upstream *mraY–murD* gene junction for complementation, confirming the findings of Ikeda *et al.* (1989) that this region contained a promoter for *ftsW* and downstream genes.

Certain *ftsW* (Ts) alleles can be suppressed by altering physiological levels of ppGpp (Khattar *et al.*, 1997). This finding can be compared to the suppression of the *pbpA* deletion by increased levels of ppGpp (Vinella *et al.*, 1992). These results suggest that the proposed PBP2/RodA and PBP3/FtsW murein synthesising elements are both sensitive to regulation by ppGpp levels.

1.1.6 *ftsI*

The *ftsI* (*pbpB*) gene is located in the proximal end of the *mra* region. The gene encodes the 60 kDa protein PBP3 (FtsI) (Nakamura *et al.*, 1983). PBP1a, PBP1b, PBP2 and PBP3 make up the four high molecular weight penicillin binding proteins with transpeptidase activity so far discovered in *E. coli* (Engelbert *et al.*, 1993). It is estimated that there are 100 molecules of PBP3 per cell (Dougherty *et al.*, 1996; Spratt, 1977; Weiss *et al.*, 1997).

PBP3 is the septum specific peptidoglycan synthesising enzyme and is not required for the elongation mode of peptidoglycan synthesis (Botta and Park, 1981; van Heijenoort, 1996; Schmidt *et al.*, 1981; Spratt, 1977). Inactivation of PBP3 with antibiotics such as cephalexin or furazlocillin results in filaments with slight indentations (Botta and Park, 1981; Spratt and Pardee, 1975). The same phenotype results when *ftsI* (Ts) mutants are shifted to the non-permissive temperature (Begg and Donachie, 1985; Fletcher *et al.*, 1979; Spratt, 1977). A conditional null allele of *ftsI* proved the essential nature of the gene (Hara *et al.*, 1997). PBP3 is not required for initiation of septation or the very early stage of septal peptidoglycan synthesis as there is a period of penicillin-insensitive peptidoglycan synthesis (PIPS) during which PBP3 is not required (Nanninga, 1991).

PBP3 has a bitopic structure similar to FtsQ, FtsN and FtsL with a small cytoplasmic region, an MSD and a catalytically active periplasmic carboxy-terminus (Bowler and Spratt, 1989; Nagasawa *et al.*, 1989). The periplasmic domain of PBP3 exhibits transpeptidase and disputed transglycosylase activities (Ishino and Matsuhashi, 1981; Ghuysen, 1994). PBP3 has a lipoprotein modification sequence but since PBP3 is known not to be a lipoprotein the importance of this is not yet fully understood (Hayashi *et al.*, 1988). An 11 amino acid polypeptide is cleaved from the carboxy-terminus of the mature PBP3 protein (Nagasawa *et al.*, 1989). This modification was proposed to be a potential method of regulation of PBP3 activity. It was later determined that a PBP3 protein translated without these 11 amino acids functioned well and that a mutant resistant to the cleavage was also fully functional (Hara *et al.*, 1989; Hara *et al.*, 1991). The importance of this post-translational modification has still to be determined.

The level of tripeptide acceptor in the peptidoglycan layer appears to exert a controlling effect on PBP3 activity (Begg *et al.*, 1990). The *ftsI23* (Ts) gene product can be suppressed at the non permissive temperature by increasing the proportion of tri- to pentapeptide side chains in the peptidoglycan layer. This can be achieved by overproducing the D-ala:D-ala carboxypeptidase PBP5 (Begg *et al.*, 1990). Similarly, overproducing the MurF, a D-ala:D-ala adding enzyme in a wild-type strain results in a block to division, possibly due to the reduced levels of tripeptide acceptor (Mengin-Lecreulx *et al.*, 1989). Thus the amount of tripeptide acceptor present in the peptidoglycan layer may dictate whether the cell will divide or elongate.

Inhibition of PBP3 with cephalexin causes FtsZ rings to disassemble. (Pogliano *et al.*, 1997). This is followed by the reconstitution of a few FtsZ rings at unused potential division sites. This suggests a role for PBP3 in the localization of future division sites (Pogliano *et al.*, 1997).

Interactions with PBP3

PBP3 may interact with several of the cell division proteins (Donachie, 1993). FtsZ was found to bind to the cytoplasmic domain of PBP3 *in vitro* (Bramhill *et al.*, 1995). Overproduction of FtsQ is tolerated in rich media but is lethal in strains bearing the *ftsI23* (Ts) allele at the permissive temperature (Dai and Lutkenhaus, 1992). An interaction between PBP3 and FtsA was suggested by Tormo *et al.*, (1986) because a mutant FtsA protein can alter the ampicillin-binding characteristics of PBP3.

Subcellular localization of PBP3.

PBP3 localises to the septum during division (Weiss *et al.*, 1997). This localization was seen in 50% of the cells in the population. Interestingly it was also found that in 10–20% of the cells PBP3 could be located at one cell pole. The localization of PBP3 to the mid-cell is expected from the accumulated genetic and biochemical evidence that PBP3 a septal peptidoglycan synthesising enzyme. The importance of the polar localization is less clear. Weiss *et al.*, (1997) suggest that polar PBP3 could be a relic from a previous division, a result of polar insertion of PBP3 into the membrane or an artifact. A model for the dynamic localization of PBP3 was offered: PBP3 locates to the septum early in the

division process and remains at the division site during invagination. Upon completion of division it is postulated that PBP3 redistributes from the pole to the mid cell prior to the next round of division.

1.1.7 *ftsL*

ftsL (*mraR*) is located immediately upstream of *ftsI* in the *mra* region at 2.2 minutes of the *E. coli* chromosome (Guzman *et al.*, 1992; Ueki *et al.*, 1992). *ftsL* encodes the 13 kDa protein FtsL of which there are approximately 50 molecules per cell (Guzman *et al.*, 1992). The *ftsL* locus was first located by Ishino *et al.*, (1989) who described two temperature sensitive mutations in the region that gave different phenotypes at the non-permissive temperature. *fts36* had a filamentous phenotype at 42°C and *lts33* lysed at 42°C. Guzman *et al.* (1992) showed that *ftsL* is an essential cell division gene by constructing a conditional null allele of the gene. Depletion of FtsL from the cell causes lethal filamentation. Overproduction of FtsL causes filamentation only in minimal medium (Guzman *et al.*, 1992). FtsL is proposed to have a bitopic membrane topology similar to FtsQ, FtsN and PBP3, consisting of a 37 aa amino-terminal cytoplasmic region, a 20 aa hydrophobic MSD and a 64 aa periplasmic carboxy-terminus region (Guzman *et al.*, 1992). FtsL has a proposed leucine zipper domain which may result in FtsL forming a dimer (Guzman *et al.*, 1992). Guzman *et al.* (1992) suggested that FtsL may act in a complex with the other bitopic proteins as a signal transducing network during cell division.

1.1.8 *ftsN*

ftsN was initially identified as a multicopy suppressor of the temperature-sensitive *ftsA12* cell division mutation and subsequently the *ftsI23* and *ftsQ1* temperature sensitive alleles (Dai *et al.*, 1993). *ftsN* maps to 88.5 minutes of the *E. coli* chromosome and encodes the 36 kDa protein FtsN. FtsN is essential for cell division, depletion of which leads to the formation of smooth sided filaments suggesting that FtsN is an early acting cell division protein (Dai *et al.*, 1993). FtsN has a bitopic topology similar to FtsQ, PBP3 and FtsL, consisting of a short cytoplasmic region, a single membrane spanning hydrophobic region and a large periplasmic

domain (Dai *et al.*, 1996). It appears that the functional domain of FtsN is located in the periplasm (Dai *et al.*, 1996). Substitution of the cytoplasmic and the MSDs with the first MSD of the MalE protein allows for normal division. Similarly, fusing the periplasmic domain of FtsN to the cleavable signal sequence of MalE has no effect on cell division, indicating that FtsN does not have to be membrane bound to function. It is proposed that the periplasmic domain of FtsN interacts with a periplasmic or cytoplasmic membrane bound component of the cell division apparatus (Dai *et al.*, 1996).

Subcellular location of FtsN

FtsN locates to the potential division site and to the septum during division (Addinall *et al.*, 1997a). The localization of FtsN is dependent on the formation of the FtsZ ring and the subsequent recruitment of FtsA to the mid-cell (Addinall *et al.*, 1997a). Functional PBP3 and FtsQ was also found to be required for the localization of FtsN as filaments caused by temperature sensitive mutations in these genes had no FtsN rings (Addinall *et al.*, 1997a). These findings point towards FtsN acting at a later stage in cell division than FtsZ, FtsA, FtsQ and PBP3, which contradicts both the earlier proposal that FtsN could be an early cell division gene and that the smooth morphology of some division mutants indicates an early function for the protein (Addinall *et al.*, 1997a; Dai *et al.*, 1993). The localization of FtsN to the septum is mediated through the periplasmic domain (Addinall *et al.*, 1997a). The MalG-FtsN fusion protein also localised to the septum, proving that the cytoplasmic and membrane spanning regions do not influence the positioning of FtsN (Addinall *et al.*, 1997a).

1.1.9 *ftsK*

ftsK is located at 20 minutes on the *E. coli* genetic map and encodes the FtsK protein with a predicted size of 147 kDa (Begg *et al.*, 1995). TOE44 was one of a number of strains produced by a mutagenesis and enrichment procedure designed to select mutants temperature sensitive for only a short period of the cell cycle (Begg *et al.*, 1980). At high temperature TOE44 formed filaments with no obvious evidence of invagination and with normally segregated chromosomes. Mapping the

temperature sensitive mutation in TOE44 led to the discovery of a previously unidentified gene, *ftsK*, situated downstream of *lrp* (Begg *et al*, 1995). Combining the *ftsK44* (Ts) mutation with the *rodA* (Ts) allele indicated that *ftsK* might act at a very late stage in division. Interestingly, the temperature sensitive phenotype of the *ftsK44* mutation was found to be suppressed by deletion of *dacA*, which encodes the D-ala:D-ala carboxypeptidase PBP5 (Spratt, 1975; Spratt, 1980; see section 1.3.7). It was suggested that this suppression might indicate a role for FtsK in peptidoglycan synthesis or modification.

Complementation of *ftsK44* at elevated temperatures by cloned chromosomal DNA fragments yielded some surprising observations. In all cases it was found that a region 5′ to the upstream *lrp* gene was required for complementation by cloned *ftsK* fragments. This region does not include the assigned promoter for *lrp* (Begg *et al.*, 1995; Wang *et al.*, 1994). The 134 bp gap between *lrp* and *ftsK* contains a previously characterised SOS inducible promoter named *dinH* (Lewis *et al.*, 1992). It was also shown that the entire *ftsK* ORF was not required for complementation of *ftsK44*. A 2 kb cloned fragment containing DNA upstream of *lrp* and 1170 bp of the 5′ end of *ftsK* complemented *ftsK44* (the *ftsK* ORF is 3998 bp). The *ftsK44* mutation was sequenced and found to be a GC to CG transversion at bp 239 of the *ftsK* ORF. This mutation resulted in the substitution of glycine-80 by an alanine residue, located in the predicted membrane spanning amino terminus of FtsK (Begg *et al.*, 1995).

The carboxy-terminus of FtsK shows sequence similarity to the SpoIIIE family of DNA translocases (Flannagan *et al.*, 1994; Hagege *et al.*, 1993; Kataoka *et al.*, (unpub.); Kendall and Cohen, 1988; Oswald *et al.*, 1993; Tomura *et al.*, 1993; Wu and Errington, 1994; Wu *et al.*, 1995; Wu and Errington, 1997). The SpoIIIE of *Bacillus subtilis* translocates DNA not already situated at the pole into the prespore and is located at the septum during asymmetrical division (Wu and Errington, 1997). The carboxy-terminus of FtsK contains the ATP/GTP binding motifs (Walker *et al.*, 1982) found in common with other members of the SpoIIIE family. Figure 1.1.9 overleaf shows a line up of the C-terminus of FtsK with other representatives from the FtsK/SpoIIIE family of proteins.

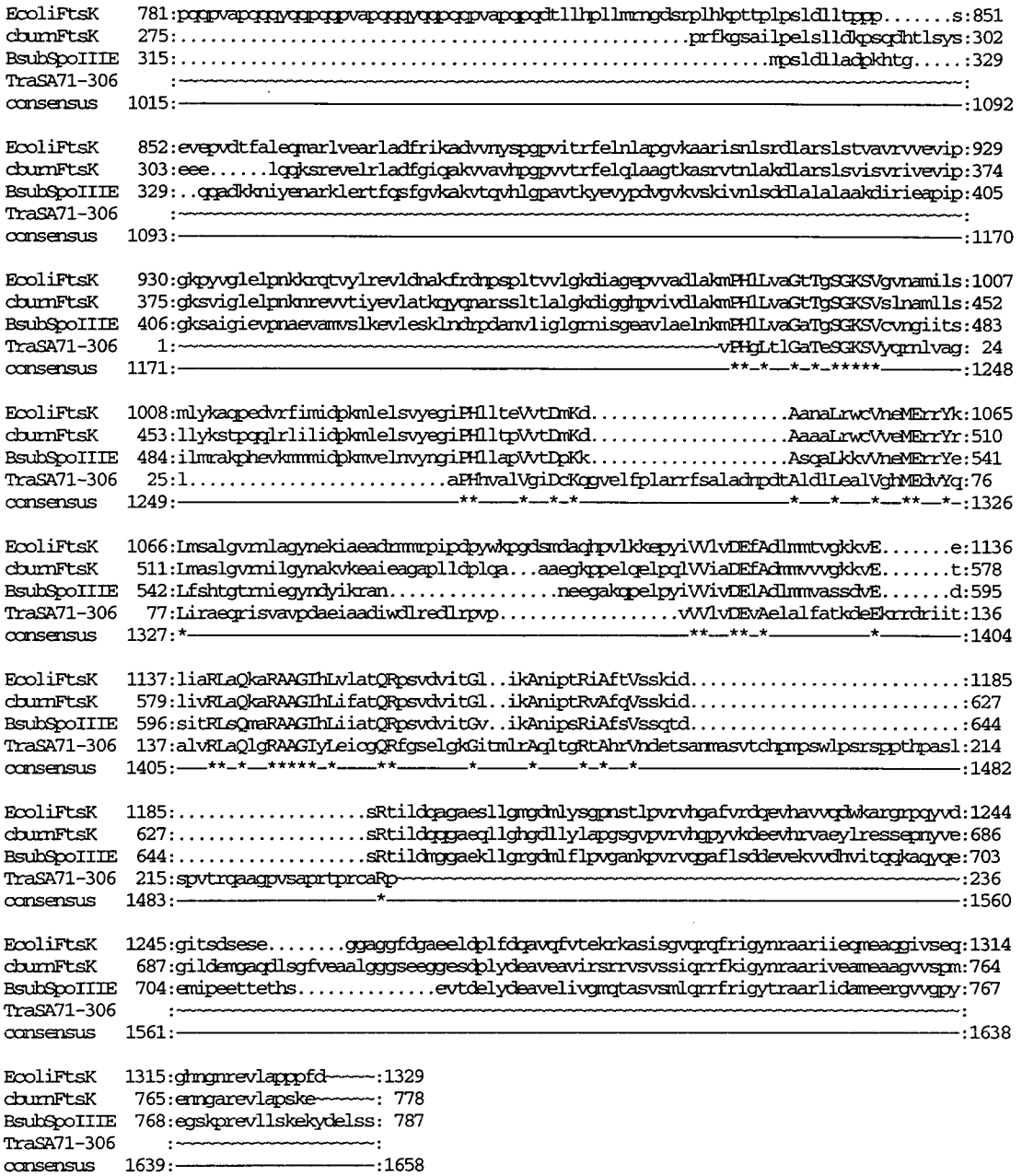

Figure 1.1.9. An alignment of C-terminal region of *E. coli* FtsK (EcoliFtsK, aa 781–1329), *C. burnetii* FtsK (cburnFtsK, aa 275–778), SpoIIIE from *Bacillus subtilis* (BsubSpoIIIE, aa 315-787) and aa 71–306 of *S. ambofaciens* TraSA (TraSA71-306). Identical residues are in uppercase and are indicated by asterisks (*) in the consensus line. The alignment was created by the PileUp program from the Wisconsin GCG Package.

The amino-terminus of FtsK shows a satisfactory degree of similarity only with the SpoIIIE from *Coxiella burnetii* (Oswald *et al.*, 1993). A number of initiation codons exist within the *ftsK* ORF but a TTG initiation codon upstream of the first ATG was proposed because this extended region shows similarity with the N-terminus of the *C. burnetii* SpoIIIE and possesses a potentially strong ribosome binding site. Begg *et al.* (1995) predicted that the amino-terminus of FtsK consists of 4-5 transmembrane bound domains and that the carboxy-terminus of the protein is located in the cytoplasm.

The central region of FtsK has no clear similarity to any other proteins of the family. This region does, however, contain three proline/glutamine rich repeat regions similar to those found in γ-gliadins and C-hordeins from cereal seeds (Okita *et al.*, 1985). The γ–gliadins act as storage proteins. The γ–gliadin proline/glutamine rich regions are thought to adopt extended or compact helical structures depending on temperature and solvent (Tatham *et al.*, 1985; 1989; Tatham *et al.*, 1984). The importance of this region is unknown, although it was proposed by Begg *et al.* (1995) that it might be functionally similarly to the contractile elastin proteins, conferring mechanical elasticity to the cytoplasmic C-terminus on FtsK (Urry, 1995; Urry *et al.*, 1983).

The involvement of the membrane spanning amino terminus in cell division and peptidoglycan synthesis is implied by the filamentous phenotype of the *ftsK44* mutant and by the fact that it can be suppressed by a deletion of *dacA* (see section 1.3.7). No direct evidence exists for the involvement of FtsK in chromosome transfer other than its similarity to the SpoIIIE family of proteins.

The insertion of a Tn*10*d-*cat* sequence at bp 2000 of *ftsK* was found to induce *uspA* expression, encoding the universal stress protein A (UspA), during stationary phase (Diez *et al.*, 1997; T. Nyström, pers. comm.). This insertion also caused a proportion of the cells to form chains with no obvious evidence of chromosomal abnormalities. The *ftsK1*::*cat* strain survived stationary phase poorly and did not tolerate media with elevated salt levels. A protein of between 41 and 67 kDa was also detected by *in vitro* transcription/translation of a cloned region of the 3′ end of the *ftsK* ORF. The full involvement of FtsK in stress adaptation has yet to be elucidated.

1.1.10 *zipA*

The 36 kDa FtsZ interacting protein A or ZipA was identified as a protein that bound radiolabelled FtsZ *in vitro* (Hale and de Boer, 1997). The *zipA* gene was identified from an expression library and found to map to 52 minutes of the *E. coli* genetic map. *zipA* is an essential gene, depletion of ZipA in a *zipA* conditional null strain resulted in lethal filamentation (Hale and de Boer, 1997). It is estimated that there are between 100–1000 molecules of ZipA per cell. Overproduction of ZipA is toxic to the cells resulting in the formation of filaments. Interestingly, the lethal filamentation induced by overexpression of *zipA* can be alleviated by simultaneously increasing the levels of FtsZ (Hale and de Boer, 1997). This indicates that there is possibly a required ratio of ZipA:FtsZ as with FtsA:FtsZ.

ZipA was found to be an integral cytoplasmic membrane protein. The predicted amino acid sequence of ZipA led Hale and de Boer (1997) to suggest the ZipA has a bitopic topology of type 1b. Type 1b bitopic proteins have a hydrophobic amino-terminus that acts as a membrane anchor, with the remainder of the protein located in the periplasm (Pugsley, 1993).

A ZipA::GFP chimera localised to the mid-cell prior to division and also to the septum during division (Hale and de Boer, 1997). This finding, along with the evidence of direct ZipA:FtsZ interaction, the location of ZipA and that cells depleted of ZipA form smooth sided filaments led to the suggestion that ZipA could be the hypothetical Factor X that dictates the position of the potential division site. However it has since been found that there are no ZipA rings in filaments formed by the inactivation of FtsZ (P.A.J. de Boer, pers. comm.). It is likely, therefore, that ZipA requires the prior formation of the FtsZ ring for localization in a manner similar to FtsA localization (Addinall and Lutkenhaus, 1996).

1.1.11 *ftsY, ftsE* and *ftsX*

ftsY, *ftsE* and *ftsX* form an operon at 76 minutes on the *E. coli* genetic map (Gill *et al.*, 1986). Temperature-sensitive mutations in *ftsE* lead to filamentation (Gill *et al.*, 1986; Gibbs *et al.*, 1992). Taschner *et al.* (1988) questioned whether *ftsE* is a true cell division gene as the filamentation elicited by the mutant is medium dependent. The function

of *ftsX* is not known. All three proteins are associated with the cytoplasmic membrane (Gill and Salmond, 1987). FtsY is a prokaryotic homologue of the eukaryotic signal recognition particle (SRP) receptor protein, SRα. The SRP is involved in the binding and export of periplasmic and other non-cytoplasmic proteins (Luirink *et al.*, 1994; Miller *et al.*, 1994).

1.1.12 *envA* (*lpxC*)

envA (*lpxC*) is the last gene in the *mra* region and it encodes the 34 kDa protein EnvA (LpxC) (Beall and Lutkenhaus, 1987; Sullivan and Donachie, 1984; Young *et al.*, 1995). *envA* is an essential gene and the *envA22* temperature sensitive mutant forms chains of cells at the restrictive temperature, implicating EnvA in cell separation (Beall and Lutkenhaus, 1987; Wolf-Watz and Nomark, 1976). The *envA22* mutation also renders the cell hyperpermeable to antibiotics (Grunstrom *et al.*, 1980; Normark, 1970). It has since been shown that *envA* encodes a UDP-3-*O*-acyl-N-acetylglucosamine deacetylase and is involved in LipidA biosynthesis, thus the inhibition of cell separation in the mutant strain could be indirect (Young *et al.*, 1993; Young *et al.*, 1995). *envA* has also been shown to be a multicopy suppressor of a mutant defective in OmpF assembly (Kloser *et al.*, 1996). It is proposed that multicopy *envA* suppresses the OmpF mutant by reducing lipopolysaccahride levels in the outer membrane thereby increasing its fluidity (Kloser *et al.*, 1996).

Table 1.1 overleaf summarises the information in the above sections.

Gene	Protein	MW of Protein(kDa)	Phenotype of Mutation (s)	Cellular Location	Function(s)	Interaction(s)
ftsZ	FtsZ	40	filamentation, altered septal morphology	Cytoplasm / DS	Cytoskeletal component	FtsZ, FtsA, ZipA, SulA
ftsA	FtsA	45	filamentation, increased resistance to penicillins	Cytoplasm, associated with cytoplasmic membrane/ DS	ATP-binding	FtsZ
ftsQ	FtsQ	31	filamentation	ICMP / DS	Unknown	Unknown
ftsW	FtsW	46/43	filamentation	ICMP /DS	PBP3 activator? FtsZ ring stability?	FtsZ ? PBP3 ?
ftsI	PBP3	60	filamentation	ICMP /DS	Division specific transpeptidase	FtsZ ? FtsA
ftsL	FtsL	13	filamentation	ICMP /DS	Unknown	Unknown
ftsN	FtsN	36	filamentation	ICMP /DS	Unknown	FtsA?,FtsQ,PBP3?
ftsK	FtsK	147	filamentation	ICMP /DS	Chromosome segregation ?	Unknown
zipA	ZipA	36	filamentation	ICMP /DS	Unknown	FtsZ
ftsY	FtsY	54.5	filamentation	Cytoplasm, associated with cytoplasmic membrane	SRα homologue	Unknown
ftsE	FtsE	24.4	filamentation	ICMP	Unknown	Unknown
ftsX	FtsX	38.5	filamentation	ICMP (?)	Unknown	Unknown
envA (lpxC)	EnvA (LpxC)	34	chain formation	Cytoplasm, associated with cytoplasmic membrane	UDP-3-*O*-acyl-*N*-acetylglucosamine deacetylase	Unknown

Table 1.1. Summary of the properties of the cell division genes noted in this chapter. ICMP – Integral cytoplasmic membrane protein, DS – Localised to the division site during septum formation.

1.2 The *mra* region.

As mentioned in section 1.1.2 the *mra* region is situated at 2.2 minutes on the *E. coli* genetic map and contains 16 genes. Figure 1.2.1 shows a diagram of the *mra* region. The *mra* region was first identified by Miyakawa *et al.* (1972) as one of two regions that contained genes involved in peptidoglycan biosynthesis. Subsequent work by many groups has led to the construction of the map of the entire *mra* region (Begg *et al.*, 1980; Fletcher *et al.*, 1978; Ikeda *et al.*, 1990a; 1990b; Ishino *et al.*, 1989; Lugtenberg and van Schijndel-van Dam, 1972a; 1972b; 1973; Lutkenhaus *et al.*, 1980; Maruyama *et al.*, 1988; Mengin-Lecreulx *et al.*, 1989; Robinson *et al.*, 1984; Robinson *et al.*, 1986; Salmond *et al.*, 1980; Tao and Ishiguro, 1989; Yi and Lutkenhaus, 1985; Yi *et al.*, 1985).

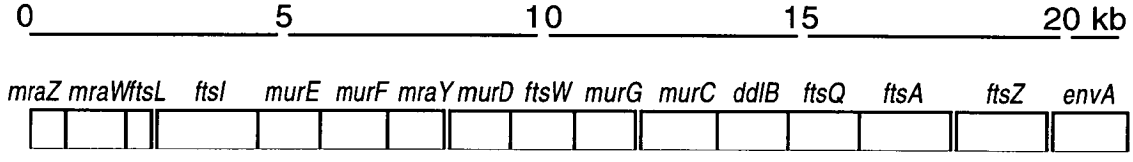

Figure 1.2.1. The *mra* region of the *E. coli* chromosome (adapted from Lutkenhaus and Mukherjee, 1996). Transcription is from left to right. A summary of the gene products, their molecular weights and proposed functions is presented in Table 1.2.1.

The first two genes in the region *mraZ* and *mraW* are as yet uncharacterized. Six of the ORFs have been identified as essential cell division genes, seven are involved in peptidoglycan precursor biosynthesis or transport and one, *envA* is involved in lipopolysaccharide synthesis (Boyle *et al.*, 1997; Lutkenhaus and Mukherjee, 1996; Young *et al.*, 1995). The function and molecular weight of each protein encoded by the *mra* region is summarized in Table 1.2.1 below. These genes are transcribed in the same direction and could be co-transcribed as the only terminator in the region is downstream of the last gene of the cluster, *envA* (Beall and Lutkenhaus, 1987).

Gene	Protein	MW of Protein (kDa)	Function	Phenotype of Mutation(s)
mraZ	MraZ	17	unknown	unknown
mraW	MraW	35	unknown	unknown
ftsL (*mraR*)	FtsL (MraR)	13	unknown	filamentation / lysis
ftsI (*pbpB*)	PBP3 (FtsI)	60	transpeptidase (transglycosylase?)	filamentation
murE	MurE	53	*meso*-DAP adding enzyme	lysis
murF	MurF	47	D-alanyl-D-alanine adding enzyme	unknown
mraY	MraY	40	linking UDP-MurNAC-pentapeptide to undecaprenol phosphate	unknown
murD	MurD	47	D-glutamate adding enzyme	unknown
ftsW	FtsW	46/43	unknown	filamentation
murG	MurG	38	*N*-acetylglucosaminyl transferase	aberrant morphology / filamentation
murC	MurC	54	L-alanine adding enzyme	lysis
ddlB	DdlB	33	D-alanine:D-alanine ligase	25% reduction in D-ala:D-ala ligase activity
ftsQ	FtsQ	31	unknown	filamentation
ftsA	FtsA	45	suspected ATPase	filamentation / increased resistance to penicillin
ftsZ	FtsZ	40	GTPase, possible polymerization into septal ring	filamentation / aberrant septal morphology / UV resistance in a *lon* strain
envA	EnvA	34	UDP-3-*O*-acyl-N-acetylglucosamine deacetylase	chain formation

Table 1.2.1. The genes and gene products of the *mra* region. The data was collated from van Heijenoort, (1996) and Lutkenhaus and Mukherjee, (1996).

Many promoters have been identified in the region, although their individual contributions to total transcription is the source of much debate. It is possible to consider the region as one large operon, as transcripts initiated from any one of the promoters could contribute to expression of the downstream genes, with transcription terminating only downstream of *envA* (Beall and Lutkenhaus, 1987). Several of the genes within the *mra* region overlap, that is, the translation initiation codon for one gene is located within the coding sequence for the upstream gene (Boyle, 1996; Ikeda *et al.*, 1990a; 1990b; Mengin-Lecreulx *et al.*, 1989; Robinson *et al.*, 1984; Robinson *et al.*, 1986).

1.2.2 Transcriptional organization of the *mra* region

A recent report by Hara *et al.* (1997) has shown the presence of a σ70 promoter upstream of *mraZ*, named P_{mra}, which contributes to the transcription of the first nine genes of the *mra* region from *mraZ* through to *ftsW*. By replacing P_{mra} with P_{lac} it was shown that it was necessary to supply the genes from *mraZ* to *ftsW* on a plasmid when P_{mra}:: P_{lac} was uninduced for the cells to remain viable. It was not required to provide extra copies of genes downstream of *ftsW* when the P_{mra}:: P_{lac} was uninduced indicating that the P_{mra} probably does not significantly contribute to the expression of genes downstream of *ftsW*. Hara *et al.* (1997) also describe a promoter in the 5′ end of *ftsW* that could be responsible for contributing to the expression of *ftsZ*.

A potential promoter with similarity to the σ70 consensus was discovered just upstream of *ftsL* (Ishino *et al.*, 1989). This promoter also has an overlapping sequence with homology to the SOS box consensus, perhaps indicating that this promoter functions during SOS induction although this has not been shown experimentally. *ftsI* expression is negatively controlled by *mreB* (Wachi and Matsuhashi, 1989). A mutation in *mreB* results in the overproduction of PBP3 and the cells have a spherical morphology. It is not known which promoter or controlling sequences are negatively controlled by *mreB* (Wachi and Matsuhashi, 1989). Several potential promoters in the *ftsI–murD* region have been described (Mengin-Lecreulx *et al.*, 1989). Complementation of both an *ftsW* (Ts) and a null-allele requires the *murD* promoter in the

mraY-murD junction (Boyle *et al.*, 1997; Ikeda *et al.*, 1989; Mengin-Lecreulx *et al.*, 1989).

1.2.2.1 Transcription within the *ddlB-envA* region

Of various promoters so far described within the *ddlB–envA* region (shown in Figure 1.2.2) some are subject to regulation. It is proposed that the regulation of some promoters is responsible for maintaining the ratio of FtsA:FtsZ which is critical for correct division to take place during different growth conditions (Dai and Lutkenhaus, 1992; Dewar *et al.*, 1992).

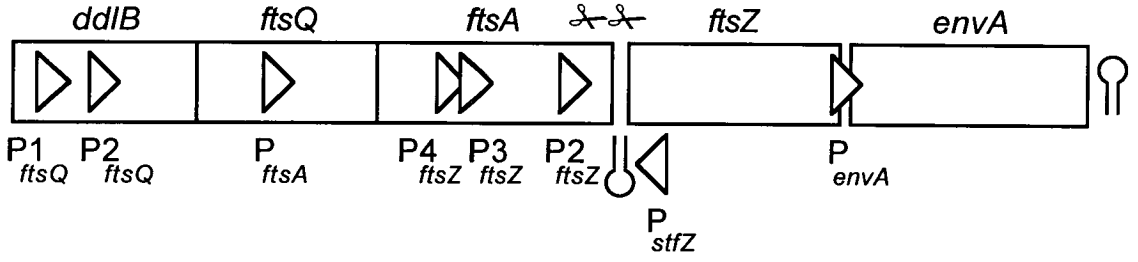

Figure 1.2.2. The *ddlB-envA* genes of the *mra* region and the identified promoters. The two RNaseE cleavage sites identified by Cam *et al.* (1996) are indicated(✂). Promoters, ▷, and terminators, ⚲ are indicated. Adapted from Lutkenhaus and Mukherjee (1996) and Flärdh *et al.* (1997).

ddlB contains two promoters, $P1_{ftsQ}$ and $P2_{ftsQ}$ (Robinson *et al.*, 1984; Aldea *et al.*, 1990; Figure 1.2.2.1) Transcription from these promoters produces 46% of the expression in the *ddlB–ftsZ* region (Flärdh *et al.*, 1997). $P1_{ftsQ}$ is a so-called gearbox promoter. Transcription from gearbox promoters increase as growth rate slows. Slow growing cells are smaller than fast growing cells; the increase in the level of FtsZ as growth rate decreases could be responsible for the cells dividing when smaller than fast growing cells. $P1_{ftsQ}$ transcription is dependent on the stationary phase sigma factor σ^S which could result in the extra expression from $P1_{ftsQ}$ as growth rate slows. $P2_{ftsQ}$ transcription is regulated by the transcription factor SdiA (Sitnikov *et al.*, 1996; Wang *et al.*, 1991). Cloning *sdiA* on a multicopy plasmid results in increased expression form $P2_{ftsQ}$ and

minicells form due to the overproduction of FtsZ (Wang *et al.*, 1991). The actual role of *sdiA* in the cell cycle is unclear as deletion of the gene has no apparent effect on the cell (Wang *et al.*, 1991).

A promoter within the *ftsQ* gene, named P_{ftsA} has been identified (Dewar and Donachie, 1990; Robinson *et al.*, 1984; Yi *et al.*, 1985). This promoter is estimated to be responsible for 12% of the total transcription from the *ddlB–ftsZ* region (Flärdh *et al.*, 1997).

There are three promoters located in *ftsA*, $P2_{ftsZ}$, $P3_{ftsZ}$ and $P4_{ftsZ}$. (Aldea *et al.*, 1990; Dewar *et al.*, 1989; Lutkenhaus and Wu, 1980; Robinson *et al.*, 1984; Sullivan and Donachie, 1984). A promoter named $P1_{ftsZ}$ was described by Aldea *et al.* (1990) but this was later discovered to be a site where *ftsZ* mRNAs were cleaved by RNaseE (Cam *et al.*, 1996). $P3_{ftsZ}$ and $P4_{ftsZ}$ produce 46% of the transcription from the *ddlB–ftsZ* region (Flärdh *et al.*, 1997). $P3_{ftsZ}$ and $P4_{ftsZ}$ are gearbox promoters and their activity is inversely proportional to growth rate (Dewar *et al.*, 1989; Flärdh *et al.*, 1997; Smith *et al.*, 1993). $P2_{ftsZ}$ is a weak promoter responsible for 5% of the transcription from the *ddlB–ftsZ* region (Flärdh *et al.*, 1997). At least one of the promoters located in *ftsA* is positively regulated by RcsB, an activator of colonic acid biosynthesis (Gervais *et al.*, 1992). In a screen for suppressors of the *ftsZ84* (Ts) allele, multicopy *rcsB* was discovered to increase the levels of colanic acid produced by the cell which could be correlated to increased *ftsZ* expression (Gervais *et al.*, 1992) The method by which increased colanic acid levels might influence *ftsZ* transcription is not known. The three DnaA boxes located in *ftsQ* and *ftsA* do not influence transcription from the *ftsQ–ftsA* region (Masters *et al.*, 1989; Schaper and Messer, 1995; Smith *et al.*, 1996). mRNAs which have their 5′ ends from the $P2_{ftsZ}$ region show periodic fluctuations (Garrido *et al.*, 1993; Zhou and Helmstetter, 1994). Garrido *et al.*, (1993) attribute this periodicity to promoter activity but Zhou and Helmstetter (1994) suggest that replication forks passing through the *mra* region could inhibit transcription resulting in periodic promoter activity. There is an *envA* specific promoter situated in the junction between the *ftsZ* and *envA* genes (Beall and Lutkenhaus, 1987).

Transcription from the *ddlB–ftsZ* region is not solely responsible for the expression of *ftsZ*. Due to the operon like structure of the *mra* region it is possible that even the distal *mra* promoter might contribute towards *ftsZ* expression (Hara *et al.*, 1997). In support of this theory, a

fragment of DNA extending more than 6 kb upstream of *ftsZ* was required for complementation of an *ftsZ* null allele (Dai and Lutkenhaus; 1991).

1.2.3 Translational regulation of *ftsQ*, *ftsA* and *ftsZ*

Mukherjee and Donachie (1990) showed that *ftsQ*, *ftsA* and *ftsZ* have different translational efficiencies. The synthesis of each protein is not only regulated by the relative abundance of the mRNA for the gene but also by the effectiveness of its ribosome binding site.

1.2.4 Regulation of *ftsZ* expression by antisense RNAs

There are two antisense RNAs that affect the translation of FtsZ mRNA. The first, DicF is encoded by a defective prophage present in some strains of *E. coli* (Béjar and Bouché, 1985; Bouché and Bouché, 1989). The active DicF RNA is produced by RNaseE and RNaseIII cleavage of untranslated RNA from the prophage region (Faubladier *et al.*, 1990). The effect of DicF during normal conditions is unknown but at high copy number and at high temperature DicF inhibits cell division by binding to and sequestering the ribosome binding site of *ftsZ* mRNA (Tétart and Bouché, 1992).

There is one report of an antisense RNA produced from the opposite strand at the *ftsA–ftsZ* gene junction named *stfZ* (Dewar and Donachie, 1993). This antisense RNA could bind to *ftsZ* mRNA thus preventing the translation of the mRNA and might be a mechanism for regulating *ftsZ* expression. Like DicF, the division inhibitory effect of *stfZ* has only been seen when the *stfZ* region is cloned in high copy number and the cells are grown at high temperature (Dewar and Donachie, 1993). It appears that the filamentation caused by overproduction of *stfZ* is strain specific (G.C. Draper, unpublished observations).

The requirement of the cell for the correct ratio of FtsA:FtsZ is therefore maintained by a combination of promoter strength, promoter activation/repression, mRNA processing, translation efficiencies and regulation by antisense RNAs.

1.3 Peptidoglycan structure and synthesis

Peptidoglycan (murein) is the polymer responsible for maintaining the shape and bearing the stresses exerted on the *E. coli* cell. Pressures of up to five atmospheres are exerted by the components of the cytoplasm and without a functional peptidoglycan layer cell shape would be lost and the cell would be prone to lysis (Mitchell and Moyle, 1954). The Gram-negative envelope consists of an inner cytoplasmic membrane surrounded by the peptidoglycan layer which is in turn encompassed by an outer membrane. The peptidoglycan sacculus is a flexible non-crystalline polymer that is linked to the inner membrane at regions of peptidoglycan synthesis and to the outer membrane by cross links to lipoproteins (Braun *et al.*, 1976; Höltje and Glauner, 1990). The peptidoglycan layer has been shown to be a maximum of 7 nm ± 0.5 n m thick (Glauner *et al.*, 1988; Hobot *et al.*, 1984). 75–80% of the sacculus is a single layer of peptidoglycan. The remaining 20–25% of the peptidoglycan is present as a triple layer which can be identified in both septal and cylindrical cell wall peptidoglycan (Labischinski *et al.*, 1991; 1993).

Peptidoglycan is a homopolymer of glycan chains which are linked together via peptide bridges. The repeating unit of the peptidoglycan polymer is a disaccharide pentapeptide. The disaccharide pentapeptide consists of two amino sugars, *N*-acetylglucosamine (GlcNAc) and *N*-acetyl-muramic acid (MurNAc) (Figure 1.3.1). The two amino sugars are covalently linked by a β 1-4 glycosidic bond. The pentapeptide that is linked to the carboxyl group of each MurNAc residue via an amide bond is L-alanyl-D-glutamyl-D-*meso*-diaminopimelyl-D-alanyl-D-alanine (Höltje and Glauner, 1990).

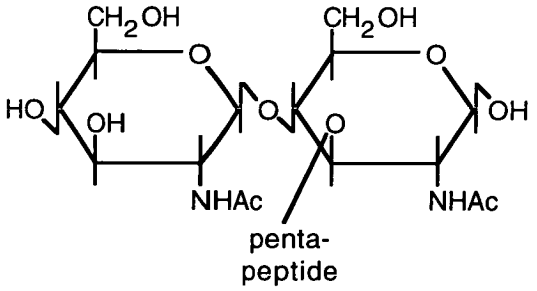

Figure 1.3.1. The structure of the unprocessed repeating unit of peptidoglycan. From van Heijenoort, 1996.

The disaccharide pentapeptide is synthesized in the cytoplasm and is presented ready for incorporation into the existing peptidoglycan in a bactoprenol phosphate (LipidII) bound form (Bupp and van Heijenoort, 1993; Umbreit and Strominger, 1972) The disaccharide pentapeptide is attached to the pre-existing peptidoglycan by a transglycosylation reaction. The pentapeptide side chains of the newly formed glycan strand are then modified to create substrates for cross linking of peptide side chains. Cross linking of modified side chains creates a peptidoglycan sheet that surrounds the cytoplasm and its membrane (van Heijenoort, 1996).

Glauner *et al.* (1988) demonstrated that the average length of a glycan strand is 30 disaccharide subunits. The glycan chains are thought to traverse the circumference of the cell in an orientation perpendicular to the long axis of the cell. It has been estimated that 100 average glycan chains are needed to equal the circumference of the cell (Schwartz, 1993). X-ray analysis showed that the glycan chains have a helical structure with 4.5 disaccharide subunits per revolution (Labischinski *et al.*, 1985). Thus, two out of the four side chains per revolution extend in parallel to the long axis of the cell and can be used as substrates for cross linking with the remaining two protruding towards the cytoplasmic and outer membranes. The degree of cross linking varies according to the age of the culture (Glauner *et al.*, 1988). Mature peptidoglycan from stationary phase cultures has a higher degree of cross linking than newly synthesized peptidoglycan found in exponentially growing cells. de Jonge *et al.* (1989) discovered that after 4 minutes 96% of newly inserted glycan chains had their peptide side chains modified by the removal of the terminal D-alanine group, forming tetrapeptide side chains.

1.3.1 Synthesis of disaccharide pentapeptide

The disaccharide pentapeptide subunit is assembled in the cytoplasm whilst bound to the inner side of the cytoplasmic membrane and then presented to the periplasm as part of a molecule named LipidII.

The initial stage of peptidoglycan precursor production takes place in the cytoplasm and involves the synthesis of UDP-*N*-acetylglucosamine (UDP-GlcNAc) from fructose-6-phosphate in a four step process. The biosynthesis of the second UDP-linked amino sugar, UDP-*N*-acetylmuramic acid (UDP-N-MurNAc) requires UDP-GlcNAc and phosphoenol pyruvate (PEP). The MurA protein catalyses the linking of UDP-GlcNAc with PEP to form UDP-GlucNAc-enolpyruvate (Venkateswaran and Wu, 1972). The MurZ protein can also function as a UDP-GlcNAc PEP transferase (Marquardt *et al.*, 1992). UDP-GlcNAc enolpyruvate is then reduced to UDP-MurNAc by the UDP-GlcNAc enolpyruvate reductase MurB (Anwar and Vlaovic, 1979; Pucci *et al.*, 1992).

MurC adds an L-alanine group to UDP-MurNAc, followed by the sequential addition of D-glutamate and *meso*-diaminopimelic acid, catalysed by MurD and MurE respectively (Maruyama *et al.*, 1988; Mengin-Lecreulx *et al.*, 1989; Tao and Ishiguro, 1989). D-glutamate is synthesised from L-glutamate by the L-glutamate isomerase MurI (Doublet *et al.*, 1993; Doublet *et al.*, 1992). The terminal D-alanyl:D-alanine group is added to the existing tripeptide side chain as a dipeptide (Lugtenberg and van Schinjdel von Dam, 1972; Marayuma *et al.*, 1988). There are two alanine racemases, encoded by *alrR* and *dadX*, which convert L-alanine to the D-alanine required (de Roubin *et al.*, 1992; Wisjman, 1972; Wild *et al.*, 1985). Two D-alanine residues are linked together by one of two D-alanine:D-alanine ligases, DdlA and DdlB (Lugtenberg and van Schinjdel von Dam, 1973; Zawadzke *et al.*, 1991). The D-alanyl:D-alanine peptide is linked to the UDP-MurNAc-L-alanyl-D-glutamyl-*meso*-DAP molecule by the action of the MurF protein to yield UDP-MurNAc-pentapeptide (Lugtenberg and van Schinjdel von Dam, 1972; Marayuma *et al.*, 1988).

The MurNAc-pentapeptide moiety of UDP-MurNAc-pentapeptide is transferred to undecaprenol (bactoprenol) phosphate, a C_{55} isoprenoid lipid situated in the cytoplasmic membrane and UMP is released (van Heijenoort *et al.*, 1992; Ikeda *et al.*, 1991; Wright *et al.*, 1967). The

production of bactoprenol pyrophosphate-MurNAc-pentapeptide, named LipidI, is catalysed by MraY (Ikeda *et al.*, 1991). The transfer of the GlucNAc moiety of UDP-GlucNAc to LipidI produces bactoprenol pyrophosphate-MurNAc(-pentapeptide)-GlucNAc (LipidII) and UMP. This step is catalysed by MurG (Mengin-Lecreulx *et al.*, 1991). LipidII carries the completed disaccharide pentapeptide which is situated on the inner face of the cytoplasmic membrane. In order for LipidII to present the completed subunit into the periplasm for donation to peptidoglycan biosynthesis, LipidII is thought to flip in the membrane, possibly facilitated by the MurH protein (Dai and Ishiguro, 1988). A summary of the biosynthesis of the disaccharide pentapeptide precursor of peptidoglycan is presented in Figure 1.3.2.

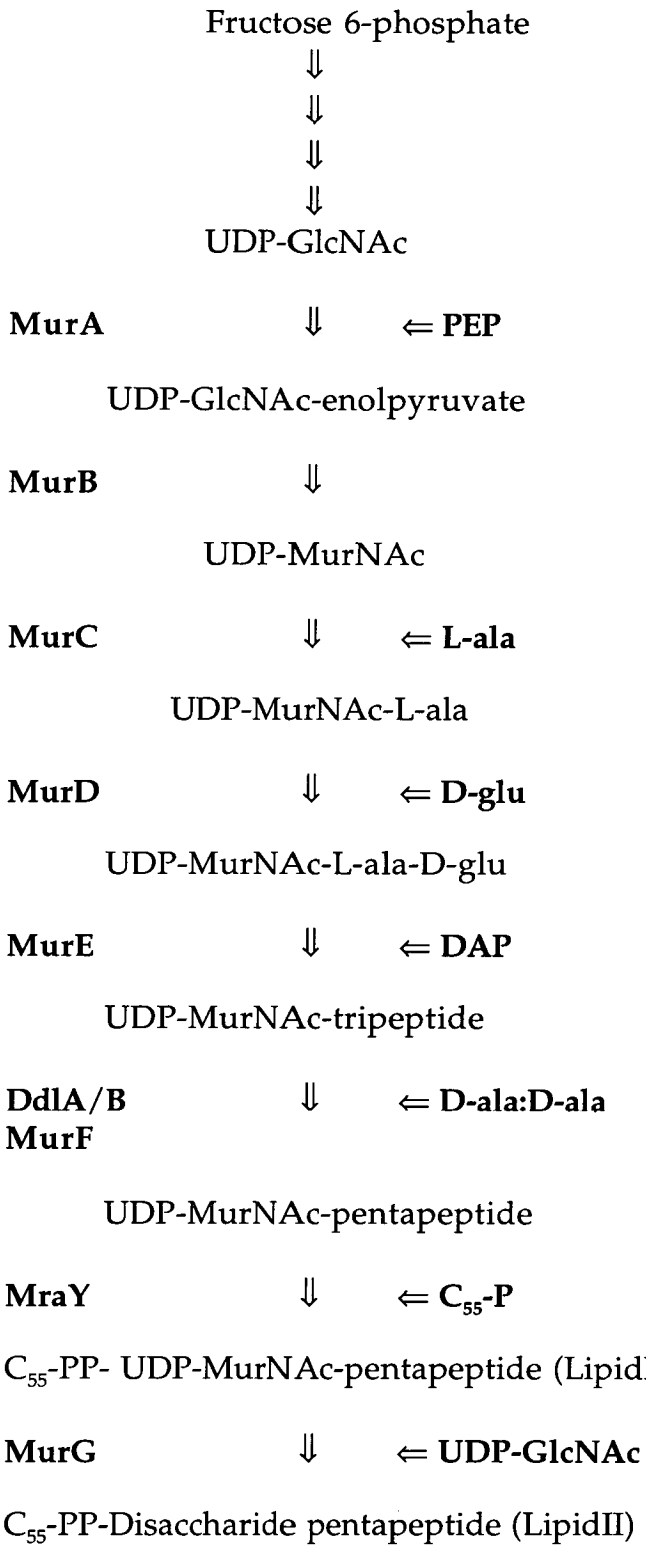

Figure 1.3.2. Assembly of disaccharide pentapeptide. From van Heijenoort, 1998.

The extension of the glycan chain occurs by transglycosylation (see The penicillin binding proteins, below) whilst in the LipidII state. It is not known whether new disaccharide pentapeptide monomers are added at the MurNAc reducing end or the non-reducing GlcNAc end, although the latter occurs in *Bacillus licheniformis* and *Micrococcus luteus* (van Heijenoort, 1996; Ward and Perkins, 1973; Weston *et al.*, 1977). The mechanism of release of the completed glycan strand from the LipidII state is not known.

1.3.2 The penicillin binding proteins

The bacterial cell wall is the target for the action of penicillin (Park and Strominger, 1957). Penicillin is an analogue of the D-alanyl:D-alanine component of the peptidoglycan precursor, disaccharide pentapeptide (Tipper and Strominger, 1965). Penicillin competes with disaccharide pentapeptide for binding to penicillin binding proteins (PBPs). Penicillin inhibits PBP function by covalently binding to a serine residue located in the active site of all PBPs (Ghuysen, 1991). A number of PBPs were identified by Spratt and Pardee (1975) as proteins that bound ^{14}C labelled penicillin. Now ten distinct PBPs have been identified, named, in descending molecular weight, PBP1a, 1b, 1c, 2, 3, 4, 5, 6, 6b and 7/8 (Spratt, 1977).

PBPs 1a, 1b, 2, and 3, collectively known as the high molecular weight PBPs, are bifunctional peptidoglycan synthesising enzymes. These enzymes exhibit transpeptidase activity that catalyses the formation of cross links between the peptide side chains of glycan strands. The high molecular weight PBPs also possess transglycosylase activity. Transglycosylation involves the addition of disaccharide pentapeptide monomers to pre-existing glycan strands (Tamaki *et al.*, 1977; Ishino *et al.*, 1980; Ishino and Matsuhashi, 1981; Ishino *et al.*, 1986). One report, however, disagrees with the observation that PBP3 has transglycosylase activity (Ghuysen, 1994). These enzymes have an N-terminal cytoplasmic membrane anchor and an active site in the periplasmic C-terminus (Adachi *et al.*, 1987; Broome-Smith *et al.*, 1985; Edelman *et al.*, 1987; Spratt and Bowler, 1987).

PBPs 4, 5, and 6 are D-alanyl:D-alanine carboxypeptidases. These enzymes catalyse the removal of the terminal D-alanine group from the

pentapeptide side chains of peptidoglycan (Spratt *et al.*, 1977; Matsuhashi *et al.*, 1979).

1.3.3 PBP1a, 1b and 1c

PBP1a and PBP1b are 94 and 89 kDa proteins encoded by the *ponA* (*mrcA*) and *ponB* (*mrcB*) genes respectively (Broome-Smith *et al.*, 1985; Tamaki *et al.*, 1980). There is an estimated 100 and 120 molecules of PBP1a and PBP1b per cell respectively (Spratt, 1975). Inactivation of either PBP1a or PBP1b individually is not lethal, but the double mutant is inviable (Kato *et al.*, 1985; Tamaki *et al.*, 1977). The two genes are therefore thought to be functionally redundant (Suzuki *et al.*, 1978; Kato *et al.*, 1987). Despite the compensatory nature of PBP1a and PBP1b it is thought that they do have subtly different roles (del Portillo and dePedro, 1991; Schmidt *et al.*, 1981). PBP1b prevents the cell from lysing when PBP2 or PBP3 are inhibited whereas PBP1a activity alone does not. PBP1b is the major transpeptidase whereas PBP1a and PBP1b exhibit similar transglycosylase activities (van Heijenoort *et al.*, 1993; Spratt, 1977). PBP1b has been shown to bind the soluble lytic glycosylase Slt *in vitro*. Slt also binds PBP3 and PBP7 *in vitro* possibly defining a septal peptidoglycan synthesising complex (Höltje , 1993; Höltje *et al.*, 1995; Höltje, 1996).
Little is known about PBP1c. PBP1c was only detected when PBPs were incubated in the presence of an ^{125}I-derivative of ampicillin (Rojo *et al.*, 1984).

1.3.4 PBP2

PBP2 has a molecular weight of 66 kDa and is encoded by the *pbpA* gene located in the *mrd* region at 15 minutes of the *E. coli* genetic map (Asoh *et al.*, 1986; Matsuhashi *et al.*, 1990). *In vitro* PBP2 is a transpeptidase but requires the RodA protein for transglycosylase activity (Ishino *et al.*, 1986). RodA is encoded by the *rodA* gene located immediately downstream of *pbpA* and the genes are probably cotranscribed (Asoh *et al.*, 1986; Matsuzawa *et al.*, 1989). An estimated 50–70% of peptidoglycan made by growing cells can be attributed to the activity of PBP2 (Park and Burman, 1973). Mecillinam, a β-lactam antibiotic of the penicillin family, binds and inhibits the transpeptidation

but not the transglycosylation activity of PBP2 (Lund and Tybring, 1972; Matsuhashi *et al.*, 1979). Inactivation of PBP2 with mecillinam causes the cells to grow as spheres (James *et al.*, 1975). The shape loss as a result of PBP2 inhibition led to the conclusion that PBP2 is essential for *E. coli* to maintain its rod shape. Inactivation of either PBP2 or RodA results in loss of shape (Matsuzawa *et al.*, 1973; Spratt, 1975). Ishino *et al.* (1989) proposed that the PBP2 and RodA proteins dictate the elongation mode of peptidoglycan synthesis and the PBP3/FtsW system proteins function together to co-ordinate the growth of septal peptidoglycan.

Conditional mutants and null mutations of *pbpA* are lethal at optimal growth conditions but can be suppressed by reducing the growth rate, increasing FtsZ production or overproducing ppGpp (Addinall *et al.*, 1995; Begg and Donachie, 1985; Vinella *et al.*, 1992; Vinella *et al.*, 1993).

1.3.5 PBP3

PBP3 (FtsI) is dealt with in detail in section 1.1.6. PBP3 is essential for the production of septal peptidoglycan (Botta and Park ,1981; Schmidt *et al.*, 1981; Spratt, 1977). As mentioned previously PBP3 has been shown to have transpeptidase and transglycosylase activities *in vitro*, but the transglycosylase activity is disputed (Ishino and Matsuhashi, 1981; Ghuysen, 1994). The proportion of tripeptide side chains in the peptidoglycan layer modulates PBP3 activity (Begg *et al.*, 1990). The activity of the proteins responsible for the production of tripeptide side chains could be a regulator of PBP3 activity.

It is proposed that PBP3 and the essential cell division protein FtsW might function in concert during septation (Ikeda *et al.*, 1989; see section 1.3.4). Khattar *et al.* (1994) disagreed with this proposal because the available *ftsW* mutants appeared to exhibit an early block to division and thus FtsW was unlikely to be required at the same time at the late acting PBP3. The isolation of additional *ftsW* mutants (Boyle *et al.*, 1997; Khattar *et al.*, 1997) indicated that FtsW is probably required throughout the division process, reaffirming the proposal of Ikeda *et al.* (1989).

1.3.6 PBP4

PBP4 is a 49 kDa protein encoded by *dacB* which is located at 69 minutes of the *E. coli* chromosome (Iwaya and Strominger, 1977; Korat *et al.*, 1991). It is estimated that there are 100 PBP4 molecules per cell (Spratt, 1975). PBP4 has both DD-endopeptidase and DD-carboxypeptidase *in vivo* (Korat *et al.*, 1990). The DD-carboxypeptidase activity is responsible for cleaving the terminal D-alanine from newly synthesised glycan chain. The DD-endopeptidase activity hydrolyses peptidoglycan cross links in a manner similar to the penicillin-insensitive autolysin MepA (Keck *et al.*, 1990). This hydrolysis of existing cross links has been proposed to enable the insertion of new glycan strands enabling the peptidoglycan sacculus to grow (Höltje, 1993). PBP4 has been implicated in division as DD-endopeptidase activity increases immediately before division initiates (Hackenback and Messer, 1977). Endopeptidase activity prior to division could result in an increased proportion of tripeptide side chains in the peptidoglycan layer. Tripeptide side chains have been suggested to be the preferred substrate for the transpeptidation reaction carried out by the septal peptidoglycan synthesising enzyme PBP3 (Begg *et al.*, 1990).

PBP4 is a soluble periplasmic protein loosely associated with the cytoplasmic membrane (Mottl, 1992). Boronic acid has been shown to compete with penicillin for binding to PBP4, a phenomenon particular to this PBP (Mottl, 1992). PBP4 is dispensible and strains deficient in DD-endopeptidase activity are penicillin tolerant (Iwaya and Strominger, 1977; Kitano *et al.*, 1980). Cells with reduced growth rates show increased penicillin tolerance and have reduced DD-endopeptidase activity suggesting that PBP4 is involved in penicillin induced lysis (Cozens *et al.*, 1989).

1.3.7 PBP5

PBP5 is a 42 kDa protein encoded by the *dacA* gene of the *mrd* cluster located at 15 minutes on the *E. coli* genetic map (Broome-Smith *et al.*, 1988; Matsuhashi *et al.*, 1990; Spratt 1975). It is estimated that there are 1800 PBP5 molecules per cell (Spratt, 1975). PBP5 is produced as a preprotein that is processed before being inserted into the cytoplasmic membrane (Pratt *et al.*, 1986). PBP5 is anchored to the cytoplasmic

membrane by atypical amphipathic helices located at the carboxy-terminus (Jackson and Pratt, 1987). PBP5 has DD-carboxypeptidase activity *in vitro* and shows approximately 10 times more activity than the DD-carboxypeptidase, PBP6 (Amanuma and Strominger, 1984). For this reason PBP5 is considered to be the major DD-carboxypeptidase *in vivo*. Inactivation of PBP5 results an increase in the amount of pentapeptide side chains, the substrate for PBP5 (Glauner, 1986). Overproduction of PBP5 results in the formation of spherical cells that lyse (Markiewitz *et al.*, 1985). The overproduction of PBP5 suppresses the *ftsI23* (Ts) allele (Begg *et al.*, 1990). Deletion of *dacA* suppresses the *ftsK44* (Ts) allele at the non-permissive temperature which led to speculation that FtsK has a function in peptidoglycan metabolism (Begg *et al.*, 1990).

1.3.8 PBP6 and 6b

PBP6 is a 40 kDa protein encoded by the *dacC* gene (Ananmura and Strominger, 1984; van der Linden *et al.*, 1992). PBP6b has an apparent molecular mass of between 41–43 kDa and is encoded by the *dacD* gene (Baquero *et al.*, 1996). PBP6 and 6b are DD-carboxypeptidases, although this property has been questioned for PBP6 (Ananmura and Strominger, 1984; van der Linden *et al.*, 1992). There are 600 PBP6 molecules per cell (Spratt, 1975). PBP6 shows one tenth the DD-carboxypeptidase activity of the major DD-carboxypeptidase, PBP5 (Ananmura and Strominger, 1984). PBP6 is inserted into the cytoplasmic membrane by the carboxy-terminus of the protein, in a manner similar to PBP5 (Jackson and Pratt, 1987). PBP6 activity increases during stationary phase (Buchanan and Sowell, 1982; van der Linden *et al.*, 1992). The PBP6 structural gene, *dacC*, is regulated by the BolA DNA-binding protein, which in turn is regulated by the stationary phase σ-factor, σ^S (Aldea *et al.*, 1989; Lange and Hengge-Aronis, 1991). Neither PBP6 nor PBP6b are essential for cell growth (Baquero *et al.*, 1996; Broome-Smith and Spratt, 1982)

Edwards and Donachie (1995) describe a triple deletion mutant of PBPs 4, 5 and 6. The mutant strain was viable and analysis of purified peptidoglycan showed the presence of tripeptide side chains indicating the possibility that there may be further DD-carboxypeptidases.

1.3.9 PBP7/8

pbpG encodes the 34 kDa PBP7 protein (Henderson *et al.*, 1995; Spratt, 1977). PBP8 is a DD-endopeptidase formed by the cleavage of PBP7 by the OmpT protease (Henderson *et al.*, 1994; Romeis and Höltje, 1994). PBP7 and 8 have only been detected in exponentially growing cells and are both dispensible (Henderson *et al.*, 1995; Spratt, 1977). PBP7 and 8 are soluble periplasmic proteins (Henderson *et al.*, 1995; Romeis and Höltje, 1994). PBP7 binds to Slt *in vitro*, indicating that PBP7 and/or PBP8 might be components of a septal peptidoglycan synthesising complex (Romeis and Höltje, 1994a; Höltje *et al.*, 1995).

1.3.10 Penicillin insensitive peptidoglycan hydrolases

There are a number of proteins involved in the metabolism of peptidoglycan that do not bind penicillin (Höltje, 1993). MepA, like the other DD-endopeptidases PBP4 and PBP7/8, exhibits DD-endopeptidase activity but differs from these two in its penicillin insensitivity (Tamioka and Matsuhashi, 1978; Keck and Schwartz, 1979).

Three LD-carboxypeptidases which cleave the terminal D-alanine moiety from tetrapeptide side chains have also been characterised (Beck and Park, 1976; Metz *et al.*, 1986; Ursinus *et al.*, 1992). It is these proteins that produce the tripeptide peptidoglycan side chains proposed to regulate septation (Begg *et al.*, 1990).

There are four lytic transglycosylases, two of which are soluble, (Slt35 and Slt70) (Betzner and Keck, 1989; Höltje *et al.*, 1995) Slt35 is a enzymatically active soluble form of the outer membrane lipoprotein MltB, which also shows transglycosylase activity (Dijkstra *et al.*, 1995; Ehlert *et al*, 1995). MltA and MltC are also membrane bound (Dijkstra and Keck, 1996; Lommatzch *et al.*, 1997; Ursinus and Höltje, 1994). Triple deletion mutants lacking MltA, MltB (and thus Slt35) and Slt70 have no obvious phenotype other than altered sensitivity to mecillinam and aztreonam as was earlier reported for a Slt70 defective strain (Templin *et al.*, 1992). These results do not support the hypothesis that lytic transglycosylases are essential for growth (Shockman and Höltje, 1994) although it is possible that remaining transglycosylase(s) in the triple deletion mutant could be acting as a suppressor. In addition, the existence

of other, as yet unidentified, peptidoglycan hydrolases cannot be ruled out.

1.4 Chromosome replication

Meselson and Stahl (1958) discovered that replication of *E. coli* chromosomes occurs in a semi-conservative manner. It was slso observed that one generation after the removal of the pulse isotope (^{15}N) all the DNA had a hybrid density (equal proportions of ^{14}N and ^{15}N). This implied that chromosome replication is a regular process. It was later shown that the replication forks proceed bi-directionally from a fixed point (Bird *et al.*, 1972; Masters and Broda, 1971; Masters *et al.*, 1970; von Meyenburg *et al.*, 1977; Prescott and Kuempel, 1972) at a constant 960 bp min^{-1}(Cooper and Helmstetter, 1968). The amount of DNA per cell is proportional to the growth rate (Schaechter *et al.*, 1958). Since the progression of the replication fork is constant, the modulation of the amount of DNA in the cell at different growth rates must be dictated by another mechanism. It was subsequently found that the rate of initiation of chromosome replication changes with growth rate (Cooper and Helmstetter, 1968). Donachie (1968) refined this idea, stating that replication initiates with every doubling of a fixed cell mass.

1.4.1 Initiation of chromosome replication

Replication of chromosomal DNA requires an initiation step that melts the origin of replication to allow entry of the DNA polymerase III holoenzyme. The origin of replication, *oriC*, is located at 84.3 minutes of the *E.coli* genetic map (von Meyenburg *et al.*, 1977; Hiraga, 1976). Initiation requires DnaA and a number of other proteins. Binding of DnaA to *oriC* results in the unwinding of an AT rich region, providing a point of entry for DNA polymerase (Messer and Weigel, 1996).

oriC contains a number of DnaA binding regions, known as DnaA boxes, as well as binding sites for accessory proteins. There are five DnaA boxes located within *oriC* as well as binding sites for the FIS and IHF DNA bending proteins (Filutowitz and Roll, 1990; Gille *et al.*, 1991; Kano *et al.*, 1991; Roth *et al.*, 1994). The binding of these proteins to *oriC* is thought to assist DnaA with the unwinding of the region (Messer and Weigel, 1996).

The IciA protein binds to the AT rich region *in vitro* and prevents its unwinding if supplied before DnaA (Hwang and Kornberg, 1990; Thöny *et al.*, 1991). The significance of IciA is unknown since neither overexpression nor insertional inactivation of the gene has much phenotypic effect (Thöny *et al.*, 1991). The Rob and H-NS proteins also bind *oriC* at specific sites (Skarstad *et al.*, 1993; Messer and Weigel, 1996).

DnaA binds ATP and ADP (Sekimitzu *et al.*, 1987). DnaA–ATP and DnaA–ADP both bind DNA but only DnaA–ATP is active for initiation. In the absence of *oriC*, phospholipids cause DnaA to release bound ATP *in vitro*, whereas when *oriC* is provided the phospholipids promote ATP binding by DnaA (Crooke *et al.*, 1992).

There are 11 GATC *dam* methylation sites within *oriC* (Oka *et al.*, 1980). The methylation status of these sites is critical for the initiation of replication (Messer *et al.*, 1985). Newly replicated origins are hemimethylated and only fully methylated copies of *oriC* can initiate replication (Russel and Zinder, 1987). Methylation of newly replicated hemimethylated DNA is rapid at all sites with the exception of *oriC*, which takes approximately 1/3 of the generation time to be methylated by Dam (Campbell and Kleckner, 1990; Ogden *et al.*, 1988). Hemimethylated *oriC* interacts with the cytoplasmic membrane, possibly delaying the methylation of the GATC sequences (Ogden *et al.*, 1988). The recently identified SeqA protein is thought to sequester *oriC* DNA, delaying its methylation (Garwood and Kohiyama, 1996; von Freiesleben *et al.*, 1994; Lu *et al.*, 1994).

1.4.2 Termination of chromosome replication

The finding that the *E. coli* chromosome replication initiates at *oriC*, proceeds a bi-directional manner and terminates at a region diametrically opposed from *oriC* led to the proposal that the region between *trp* and *his* could contain a replication terminus (Bird *et al.*, 1972; Masters and Broda, 1972). The teminus region contains a locus for the resolution of dimeric chromosomes formed by an odd number of cross-overs, known as *dif* (Blakely *et al.*, 1991; Clerget, 1991; Kuempel *et al.*, 1991). The XerC and XerD recombinases are required for *dif* to function (Colloms *et al.*, 1990; Blakely *et al.*, 1993) XerC and XerD exhibit site-specific topoisomearse activity (Cornet *et al.*, 1997). Cell division has to

take place for the resolution of chromosome dimers at *dif* (Steiner and Kuempel, 1998). The terminus region has a number of regions prone to hyperrecombination (Nishitani *et al.*, 1993). These regions are known as Hot DNA. It is proposed that replication arrest at a *Ter* site (see below) can cause the formation of double strand breaks which are repaired by the RecBCD enzyme, resulting in recombination (Horiuchi *et al.*, 1994). Hyperrecombination also takes place at the terminus recognition zone (TRZ) (Louarn *et al.*, 1994).

Polar traps in the terminus prevent the replication forks from proceeding through the region. There are seven *Ter* sites to which the Tus protein binds. *TerA, D* and *E* prevent the passage anticlockwise forks wheras *TerC, B, F* and *G* prevent clockwise replication forks from passing through the terminus (François *et al.*, 1989; Hidaka *et al.*, 1988; Hidaka *et al.*, 1991; Hill, 1996; Sharma and Hill, 1992). Tus binds to the *Ter* site as a monomer and the lack of dyad symmetry within Tus is thought to be responsible for the polar effect of *Ter*/Tus mediated replication arrest (Coskun-Ari *et al.*, 1994; Sista *et al.*, 1991). Strains which have 360 kb of the 450 kb terminus region deleted retain viability but filament, display evidence of abnormal DNA segregation, produce anucleate cells and show induction of the SOS response (Henson and Kuempel, 1986).

1.4.3 Chromosome partition

Jacob *et al.* (1963) proposed that newly replicated plasmids and chromosomes could be partitioned through attachment to the cell membrane. Growth at the midcell could then result in the passive partition of the DNA molecules. The finding that extension of peptidoglycan is achieved by the apparently random insertion of glycan chains into the sacculus (except during division) and the lack of any attachment machinery appears to have disproved the hypothesis (Woldringh *et al.*, 1987). Despite this, the theory encouraged the search for partition apparatus.

Many mutations result in the abnormal partition of chromosomes (Donachie, 1993). These *par* mutants which have incorrectly placed chromosomes have now been mapped to genes involved in DNA synthesis and to DNA topoisomerases (Hussain *et al.*, 1987; Kato *et al.*,

1990; Kato *et al.*, 1989; Norris *et al.*, 1986; Stewart and D'Ari, 1992). A *par* phenotype is also seen in mutants defective in the *dif* locus or the XerC protein (Blakely *et al.*, 1991; Clerget, 1991; Kuempel *et al.*, 1991). Hiraga and co-workers described a new gene, *mukB* whose product appeared to be involved in chromosome segregation. A temperature-sensitive *mukB* mutant showed evidence of isolated nucleoids and clumps of DNA as well as anulceate cells (Hiraga, 1993; Hiraga *et al.*, 1989; Niki *et al.*, 1991; Niki *et al.*, 1992). A strain in which *mukB* is deleted is viable, but not at elevated temperatures. MukB has no influence on plasmid partition as F plasmids were shown to segregate equally well into both nucleate and anucleate cells (Ezaki *et al.*, 1991). MukB encodes a protein with a similar secondary structure to eukaryotic myosin and kinesin heavy chains (Niki *et al.*, 1992; Hiraga, 1992). MukB also binds ATP and GTP and interacts in a non-specific manner with DNA (Niki *et al.*, 1992). It is suggested that MukB acts as a potential cytoskeletal structure that could act either as a scaffold for DNA translocation or a motor that actively partitions DNA (Niki *et al.*, 1991; Niki *et al.*, 1992).

Important advances in the study of chromosome segregation where made during 1997 by a number of groups. A mitotic like apparatus was discovered in *Bacillus subtilis* (Lin *et al.*, 1997; Sharpe and Errington, 1996). The Spo0J protein binds to the *soj–spo0J* locus (Mysliwiec *et al.*, 1997) on the chromosome and is located toward the cell poles (Lin *et al.*, 1997). This finding was in agreement with the observations of Webb *et al.* (1997) who localised the origin region of the *B. subtilis* chromosome to the cell poles. The origin and the *soj–spo0J* region are both located in the same third of the chromosome of *B. subtilis*. The terminus region is not sequestered at the cell poles.

The soj–*spo0J* locus of *B. subtilis* is a homologue of the *parA parB parS* region of the *E. coli* bacteriophage P1 and the *sopA sopB* genes of the F plasmid (Austin and Abeles. 1983a; 1983b; Mori *et al.*, 1989). *parA* and *parB* are required for partition of P1 during the prophage stage of the bacteriophage life cycle (Gerdes and Molin, 1986). ParA is an ATPase which interacts with the DNA binding protein ParB (Davis *et al.*, 1992; Watanabe *et al.*, 1992). The ParB protein binds to the *parS* region (Martin *et al.*, 1987; Davis and Austin, 1988).

The developmental bacterium *Caulobacter crescentus* has a region homologous to the *parA parB parS* region of P1 (Mohl and Gober, 1997). Both ParA and ParB localise to the cell poles after chromosome replication and prior to cell division. ParB binds to a region functionally homologous to the P1 *parS* region. Overexpression of either *parA* or *parB* resulted in aberrant chromosome segregation and loss of ParA and ParB localization (Mohl and Gober, 1997). The authors suggest that ParA and ParB might be components of a bacterial mitotic apparatus.

The origin of the *E. coli* chromosome was shown to be located more towards the cell poles. The F plasmid and P1 prophage genomes migrate to the cell quarters, indicating different mechanisms for chromosome and episome segregation (Gordon *et al.*, 1997). *E. coli* does not have a region homologous to the *parA parB parS* region of other organisms and plasmids.

CHAPTER 2
MATERIALS AND METHODS

Chapter 2. Materials and Methods.

2.1 Bacterial and Phage Strains, Plasmids and General Materials

2.1.1 Bacterial Strains

Strain	Genotype	Source/Reference
BL21 (DE3)	*E. coli* B strain. F^- *ompT* *hsdS*$_B$ (r_B^- m_B^-) λDE3	Studier and Moffat (1986)
C600	F^- e14$^-$ (McrA$^-$) *thr-1 leuB6 thi-1 lacY1 supE44 rfbD1 fhuA21*	Laboratory stock
C6SA5	C600 *sulA*::Tn*5* [from GC2481] Kan^R	This work
CDK1	MG1655 *aroA*::Tn*10* *ftsK*$_{677}$::*cat* Cmp^R Tet^R	This work
CDK2	MG1655 *aroA*::Tn*10* *ftsK*$_{\Delta 54-2201}$::*cat* Cmp^R Tet^R	This work
CDK3	CDK2 *dacA*::*kan* Cmp^R Tet^R Kan^R	This work
CDK5	MG1655 *aroA*::Tn*10* *ftsK*$_{\Delta 54-3669}$::*cat* Cmp^R Tet^R	This work
CDK44	TOE44 *aroA*::Tn*10* [from ME8436]	This work
CSR603	*thr–1 ara–14 leuB6* Δ(*gpt-proA*)62 *lacY1 tsx-33 glnV44 phrB1 galK2* λ$^-$ *rac-0 gyrA98 recA1 rpsL31 kdgK51 xylA5 mtl-1 argE3 thi-1 uvrA6* Nal^R Str^R	Sancar and Rupert (1978)
DH5α	ϕ80*dlac*ΔM15 Δ(*lacZYA–argF*)U169 *recA1 endA1 hsdR17* (r_K^- m_K^+) *supE44,* λ$^-$ *thi-1 gyrA96 relA1*	Laboratory stock
GC2481	*sulA*::Tn*5* Kan^R	R. D'Ari
JC10-240	Hfr:P045; *lysA>serA ilv-318 thr-300 srlC300*::Tn*10 thi-1 recA56 relA rpsE300* Tet^R	A. J. Clark
K146	MG1655 *aroA*::Tn*10* *ftsK*$_{667}$::*cat* Cmp^R Tet^R	This work
K2:1	MG1655 *aroA*::Tn*10* *ftsK*$_{\Delta 54-2201}$::*cat* Cmp^R Tet^R	This work

Strain	Genotype	Source/Reference
ME8436	F' 106–*poxB–aroA*::Tn*10*–*putPA galK35* λ^- *pyrD34 his-68 recA1 rpsL118 malA1* λ^R *xyl-7 mtl-2 thi-1* Tet^R	National Institute of Genetics, Japan
MG1655	prototroph	Laboratory stock
MG1655 *pcnB*::*kan*	MG1655 *pcnB*::*kan* Kan^R	This work
MGAT	MG1655 *aroA*::Tn*10* [from ME8436] Tet^R	This work
MGdak	MG1655 *dacA*::*kan* [from SP1070)	This work
MG*recA*	MG1655 *recA56 srlC300*::Tn*10* [from JC10–240] Tet^R	This work
MGSA5	MG1655 *sulA*::Tn*5* Kan^R	This work
MM38K24	*argG6 asnA31* or *asnB32 his-1 leuB6 metB1 pyrE gal-6 lacY1 pcnB*::*kan xyl-7 supE44 bgl fhuA2 gyrA rpsL104 tsx-1 uhp* Kan^R	Masters *et al.*, 1993
NACK6	W3110 *aroA*::Tn*10* $ftsK_{\Delta 54-3669}$::*cat* Cmp^R Tet^R	This work
SP1070	*dacA*::*kan* Kan^R	B. Spratt
TOE44	*thr-1 ara-14 leuB6* Δ(*gpt-proA*)62 *lacY1 tsx-33 qsr'-0 glnV44 galK2* λ^- rac-0 *hisG4 rfbD1 mgl-51 thyA12 rpsL31 kdgK51 xylA5 mtl-1 argE3 thi-1 deoB16 ftsK44*	Begg *et al.* (1995)
TP8503	*thi-1 leu supE42* Δ(*lac–proB*) *fhuA* Tmp^R	Masters *et al.* (1989)
TPλ	TP8503 λ p(*sfiA*::*lac*)	This work
W3110	Inv: *rrnD–rrnE*	Laboratory stock

Table 2.1.1. *E. coli* strains used in this study.

2.1.2 Bacteriophage strains

Bacteriophage P1 and λ lysates used in this study are listed in Table 2.1.2. Phage lysates were stored at 4°C as LB-broth suspensions containing a few drops of chloroform to prevent microbial growth.

Bacterio-phage	Description	Source/Reference
P1	Wild–type bacteriophage	Laboratory stock
λvir	Virulent	Laboratory stock
λ p(*sfiA*::*lac*)	λ phage containing the *sulA* promoter (P_{sfiA}) transcriptionally fused to *lacZ*.	Huisman and D'Ari, 1983
λ214 (λ1F10)	λ215 containing stuffer fragment from 20.07–20.87 minutes	Kohara *et al.*, 1987

Table 2.1.2. Bacteriophages used in this study.

2.1.3 Plasmids

The plasmids used in this study are shown in Table 2.1.3. Plasmids were stored as dried pellets at –70°C until required.

Plasmid	Description	Source/Reference
pBADK	*Eco*RI-*Xba*I insert from pUCK cloned into *Eco*RI/*Xba*I digested pBAD18. 9.0 kb. Amp^R.	This work
pBADK'	pBADK with 2.6 kb *Bsu*36I–*Xba*I fragment removed, end-filled and religated. 6.4 kb. Amp^R.	This work
pBADK'3	pBADK with 3.7 kb *Bsa*BI–*Xba*I fragment removed, end filled and religated. 5.3 kb. Amp^R.	This work
pBR322	General purpose cloning vector. Tet^R Amp^R	Bolivar *et al.* 1977
pBR325	General purpose cloning vector. Tet^R Amp^R Cmp^R.	Bolivar, 1978

Plasmid	Description	Source/Reference
pBS58	*ftsQ*, *ftsA* and *ftsZ* cloned into pGB2. $Spec^R$.	Bi and Lutkenhaus, (1990a)
pCD99	*Eco*RI–*Xba*I insert from pUCK cloned into *Eco*RI/*Xba*I digested pUC18. 7.1 kb. Amp^R.	This work
pCD101	*Eco*RI–*Kpn*I fragment from λ214 cloned into *Eco*RI/*Kpn*I digested pUC19. 9.9 kb. Amp^R.	This work
pCD101 -ΔRV	*Eco*RV fragment from pCD101 removed and the plasmid religated. 7.8 kb. Amp^R.	This work
pCD109	*Sal*I-*Sph*I fragment from pGEMT-K cloned into *Sal*I/*Sph*I digested pUC18. 4 kb. Amp^R.	This work
pCDCAT	*cat* gene PCR product (with promoter) from pBR325 using *Not*I and *Mfe*I mutagenic primers digested with *Not*I and *Mfe*I and ligated into *Not*I-*Mfe*I digested pCD101. 9.2 kb. Amp^R Cmp^R.	This work
pCDCAT2	pCDCAT with *Bsu*36I–*Nru*I fragment removed, end-filled and religated. 7.7 kb. Amp^R Cmp^R.	This work
pGB2	pSC101-based cloning vector. 4.9 kb. $Spec^R$.	Churchward *et al.*, 1984.
pGB101	*Eco*RI–*Sal*I fragment from pCD101 cloned into *Eco*RI/*Sal*I digested pGB2. 11.2 kb. $Spec^R$.	This work
pGEM-T	T-overhang PCR product cloning vector. 3 kb. Amp^R.	Promega
pGEM-TK	1.3 bp PCR product of the 5' end of *ftsK* with an introduced *Nde*I site and an ATG codon replacing the *ftsK* TTG initiation codon cloned into pGEM-T. 4.3 kb. Amp^R.	This work

Plasmid	Description	Source/Reference
pHL1	*Hin*dIII chromosomal fragment from the 88.5 minute region cloned into *Hin*dIII digested pBR322. Amp^R.	M. Khattar
pKATG	*Not*I–*Sal*I fragment from pCD99 cloned into *Not*I/*Sal*I digested pCD109. 7.1 kb. Amp^R.	This work
PKBCAT	*Ecl*136II–*Hin*dII fragment from pUCAT18 containing the *cat* gene cloned into the *Bsa*BI of pCD101. *cat* gene is in the same orientation as *ftsK*. 11.4 kb. Amp^R Cmp^R.	This work
pKC1	*Eco*RI–*Mfe*I fragment from pCD99 removed, the linear plasmid religated. 4.8 kb. Amp^R.	This work
pKD140	3' region of *cytR* and *ftsN* cloned into the *tet* gene of pBR322. Amp^R.	Dai *et al.* 1993
pKHS3	pJF118HE clone containing *ftsWS* transcribed by P_{tac} and T7 Ø10 promoters. Amp^R.	Khattar *et al.* 1997
pKT1	*Xba*I–*Nhe*I fragment removed from pCD99, the linear plasmid end-filled and religated. 3.5 kb. Amp^R.	This work
pKT2	*Xba*I–*Bsu*36I fragment removed from pCD99, the linear plasmid end-filled and religated. 4.5 kb. Amp^R.	This work
pKT3	*Xba*I–*Cla*I fragment removed from pCD99, the linear plasmid end-filled and religated. 4.7 kb. Amp^R.	This work
pKT4	*Xba*I–*Nco*I fragment removed from pCD99, the linear plasmid end-filled and religated. 6.4 kb. Amp^R.	This work
pLysS	Phage T7 *gene1* cloned in pACYC184 *tet* gene. 5.5 kb. Cmp^R.	Laboratory stocks

Plasmid	Description	Source/Reference
pSU44	*trxB*, *lrp* and *ftsK'* fragment from λ215 cloned into pUC19.	Begg *et al.*, 1995
pT7-3	ColEI origin. Polylinker downstream of the the 'phage T7 Ø10 promoter. *bla* gene transcribed in the same direction as Ø10 promoter. 2.4 kb. Amp^R.	Tabor and Richardson, 1988
pT7-3K	*Eco*RI–*Xba*I fragment from pUCK cloned into *Eco*RI/*Xba*I pT7-3. 6.8 kb. Amp^R.	This work
pT7-5	Similar to pT7-3 but orientation of *bla* gene reversed. 2.4 kb. Amp^R.	Tabor and Richardson, 1988
pT7-5K	*Eco*RI–*Xba*I fragment from pUCK cloned into *Eco*RI/*Xba*I pT7-3. 6.8 kb. Amp^R.	This work
pT7-7	Similar to pT7-3 but allows construction of translational fusions to N-terminus of Ø10. Also carries an *Nde*I site for fusion of in frame genes to Ø10 rbs and translation initiation codon. 2.5 kb. Amp^R.	Tabor and Richardson, 1988
pT7-7KATG	*Nde*I–*Sal*I fragment from pKATG cloned into *Nde*I/*Sal*I digested pT7-7. 6.9 kb. Amp^R.	This work
pTLK'	*Bsa*BI–*Sma*I fragment removed from pCD101, the plasmid religated. 5.5 kb. Amp^R.	This work
pUCK	4.4 kb *Sca*I*Nde*I fragment from λ214 containing the promoterless *ftsK* ORF end-filled and cloned into *Sma*I digested pUC19. 6.1 kb. Amp^R	This work
pUC18	General purpose cloning vector. 2.7 kb. Amp^R.	Yannisch-Perron *et al.* (1985)
pUC19	General purpose cloning vector. 2.7 kb. Amp^R	Yannisch-Perron *et al.* (1985)

Table 2.1.3. Plasmids used in this study.

2.1.4 Growth media and buffers

Growth media are listed in section 2.1.2. Phage, bacterial and other commonly used buffers are listed in section 2.1.3. LB-broth and LB-agar were the media of choice in most manipulations except where otherwise stated. Where bacteriophage λ was used the media was supplemented with 10 mM $MgSO_4$ and 0.2% maltose; for bacteriophage P1, the media was adjusted to 2.5 mM $CaCl_2$. For selection/testing of auxotrophies V B minimal medium containing appropriate supplements was used. Arabinose and glucose were added to media at a concentration of 0.2% (w/v) unless stated otherwise in the text.

2.1.5 Growth media

Luria Broth (LB-broth)	Difco Bacto tryptone	10 g
	Difco Bacto yeast extract	5 g
	NaCl	5 g
	Distilled water to 1 litre	
	pH to 7.2 using NaOH	
LB-agar	LB-broth + 15 g Difco agar per litre	
LBΔNaCl	as above but without NaCl	
LB top agar	LB-broth + 6.5 g Difco agar per litre	
Nutrient broth	Oxoid No. 2 nutient broth	
	Distilled water to 1 litre	
Nutrient agar	Nutrient broth + 12.5 g Davis NZ agar	
MacConkey agar	Peptone	20 g
	Bile salts No.3	1.5 g
	NaCl	5 g
	Neutral red	0.03 g
	Crystal violet	0.001 g
	Difco agar	15 g
	Distilled water to 1 litre	

VB minimal medium	20x VB salts	50 ml
	20% carbon source	10 ml
	Thiamine HCl (1 mg ml^{-1})	2 ml
	Supplements as required.	
	Distilled water to 1 litre	
VB minimal agar	As VB minimal broth + 15 g Difco agar per litre	
20x VB salts	$MgSO_4$	4 g
	Citric acid	40 g
	KH_2PO_4	400 g
	$NaNH_4.HPO_4.H_2O$	70 g
	Distilled water to 1 litre	
	Store over 1 ml of chloroform	
Spizizen's minimal medium	$(NH_4)_2SO_4$	10 g
	K_2HPO_4	70 g
	KH_4PO_4	30 g
	Sodium citrate.$2H_2O$	5 g
	$MgSO_4.7H_2O$	1 g
	Distilled water to 1 litre	
SOC broth	Bactotryptone	4 g
	Bacto yeast extract	1 g
	5 M NaCl	0.4 ml
	1 M $MgCl_2$	2 ml
	1 M KCl	0.5 ml
	1 M $MgSO_4$	2 ml
	glucose	0.72 g
	Distilled water to 200 ml	

2.1.6 Commonly used buffers

Bacterial buffer	$MgSO_4.7H_20$	2 g
	Na_2HPO_4	7 g
	KH_2PO_4	3 g
	NaCl	4 g

	Distilled water to 1 litre	
Phage Buffer	Na_2HPO_4	7 g
	KH_2PO_4	3 g
	NaCl	5 g
	1 M $MgSO_4$	1 ml
	0.1 M $CaCl_2$	10 ml
	1% gelatin solution	1 ml
	Distilled water to 1 litre	
TE buffer	10 mM Tris-HCl (pH 8.0)	
	1 mM EDTA (pH 8.0)	
TAE buffer	40 mM Tris-acetate	
(working solution)	2 mM EDTA	
50x stock TAE buffer	Tris base	242 g
	Glacial acetic acid	57.1 ml
	Distilled water to 1 litre	

2.1.7 Minimal medium supplements

Amino acid supplements were stored in stock solutions of pure amino acids at a concentration of between 2–10 mg ml^{-1} depending on the solubility of the particular amino acid. Sparingly soluble amino acids such as tyrosine were dissolved in 0.01 M NaOH. The final concentration of the amino acids in the media was normally in the order of 20–100 mg ml^{-1}. If a rich minimal media was required, vitamin free casamino acids (CAA) was used. The stock concentration of CAA was 100 mg ml^{-1} and the final concentration in the medium was 2 mg ml^{-1}. CAA lacks tryptophan and this was added to CAA media if the bacterial strain to being used had an auxotrophy for this amino acid.

2.1.8 Selection for antibiotic resistance

The routine concentration of antibiotics used in this study are shown in Table 2.1.1. Those antibiotics dissolved in water were filter sterilised using a 0.45 µm filter (Gelman).

Antibiotic	Abbreviation	Solvent	conc. of stock solution (mg ml^{-1})	final conc. in media ($\mu g\ ml^{-1}$)
Ampicillin	Amp	H_2O	100	50–100
Chloramphenicol	Chl	ethanol	20	25
D–cycloserine	Cyc	H_2O		
Kanamycin	Kan	H_2O	25	25
Naladixic acid	Nal		150	150
Nitrofurantoin	Nit	dimethyl-formamide	2	0.1–5
Spectinomycin dihydrochloride	Spec	H_2O	50	50
Tetracycline hydrochloride	Tet	50% ethanol	10	10

Table 2.1.1 Antibiotic solutions used in this study.

2.2 DNA Techniques

2.2.1 Large scale preparation of plasmid DNA

For preparation of large amounts of plasmid DNA an *endA*$^-$ strain such as DH5α was used as the host. A single colony was used to inoculate 5 ml of LB-broth (with appropriate antibiotics and supplements) which was incubated at a suitable temperature with shaking overnight. 0.5 ml of the overnight was used to inoculate 500 ml of LB-broth containing appropriate antibiotics and supplements and the culture was incubated at a suitable temperature overnight with vigorous shaking. The culture was chilled on ice for 15 minutes and transferred to two 250 ml centrifuge bottles. The cells were pelleted by centrifugation at 5000 rpm in a pre–cooled GSA rotor for 15 minutes at 4°C. The supernatant was discarded and the pellets resuspended in a total of 200 ml of chilled TE buffer. The

concentrated cells were centrifuged as above and the supernatant removed and discarded. Care was be taken to remove all the supernatant and any excess was removed with a Pasteur pipette. The pellet was resuspended in 5 ml of Tris–sucrose buffer and transferred to a 50 ml centrifuge tube. 1 ml of lysozyme (20 mg ml^{-1}) was added and mixed in gently by swirling. The tube was placed on ice for five minutes with frequent swirling. 1 ml 0.5 M EDTA (pH 8.0) was added and mixed in by gentle swirling followed by 0.8 ml RNAseA (10 mg ml^{-1}) which was also mixed in by gentle swirling. The tube was placed on ice for five minutes with frequent swirling. 6 ml of Triton lysis solution was added, the mouth of the tube covered with Parafilm and mixed by gentle inversion. The tube was placed on ice for 10 minutes until the cells lysed and the mixture became viscous and partially cleared. If clearing was not apparent then up to 1.5 ml of a 10% Triton X-100 solution was added to aid lysis of the cells.

The cellular debris and bulk chromosomal DNA were pelleted by centrifuging the lysate in a pre-cooled SS-34 rotor at 15,000 rpm for 30 minutes at 4°C. To avoid sedimentation of larger plasmids (>15 kb) a shorter run was used, typically 15 minutes. The supernatant was decanted into a sterile glass measuring cylinder and 17.1 g CsCl added and mixed by inversion until dissolved. 342 µl of ethidium bromide (10 mg ml^{-1}) was added and the volume made up to 23 ml with TE and mixed thoroughly. The solution was transferred to two Beckman Ti50 Sorval crimp seal tubes and the tubes, stoppers and caps balanced to within 0.05 g.

The tubes were placed in a Ti50 fixed angle rotor and centrifuged at 38,000 rpm for 60 hours at 18°C or 45,000 rpm for at 24 hours at 18°C. The tubes were removed from the rotor avoiding unnecessary handling. Under UV illumination two ethidium bromide stained bands could be seen, the lower band contained the supercoiled plasmid DNA. This band was extracted by the insertion a 0.9 x 40 mm needle into the top of the tube which acted as a vent and another needle, attached to a 5 ml syringe, inserted just below the band ensuring that the bevel of the needle pointed up. 1–2 ml was withdrawn to ensure that the entire plasmid band was drawn into the syringe. The plasmid/CsCl solution was transferred to a 10 ml syringe. To extract the ethidium bromide an equal volume of isopropanol saturated with CsCl and H_2O was drawn into the syringe. This was shaken gently to mix the two phases and allowed to settle. The

isopropanol/ethidium layer (uppermost) was expelled and care was taken not to lose any of the plasmid containing (bottom) layer. This extraction procedure was repeated four times and then the plasmid/CsCl solution transferred to 8/32 inch dialysis tubing. The ends of the tubing was sealed with clips and the plasmid/CsCl solution was dialysed against 5 litres of TE at 4°C for 1 hour. The TE was replaced with fresh, chilled TE buffer and the dialysis repeated for a further 4 hours. The TE was changed once more and the dialysis allowed to continue overnight at 4°C. 0.5 ml aliquots of plasmid solution were transferred to microcentrifuge tubes and 50 µl 3 M sodium acetate (pH 5.0) and 1 ml ice cold ethanol added, mixed gently and placed on ice for 30 minutes. The plasmid DNA was precipitated by centrifugation at 14,000 rpm for 30 minutes at 4°C. The ethanol was removed and the pellets washed with 1 ml of ice cold 70% ethanol. The tubes were vortexed briefly and centrifuged for a further 10 minutes at 14,000 rpm at 4°C. The 70% ethanol was removed and the pellet dried in a Savant Speed-Vac. The DNA was stored as these dried pellets at –70°C until required whereupon 110 µl TE was added and 1 hour allowed for the pellet to dissolve. The DNA concentration was determined as described in section 2.2.7.

solutions used

Tris-sucrose buffer	50 mM Tris-HCl (pH 8.0) 25% w/v sucrose
Triton lysis solution	50 mM Tris-HCl (pH 8.0) 62.5 mM EDTA 0.1% v/v Triton X-100

2.2.2 Plasmid minipreparation by alkaline lysis

This routine plasmid 'miniprep' procedure is a slight variation on that first described by Birnboim and Doly (1979). 5 ml LB-broth containing the appropriate selective antibiotics was inoculated with a single colony. The culture was incubated at a suitable temperature overnight with shaking. The cells were pelleted by centrifuging the culture in a bench top

centrifuge at 4500 rpm for 5 minutes. The supernatant was removed and discarded and the pellet resuspended in 100 µl TGE buffer. The concentrated cell suspension was transferred to a microcentrifuge tube and 200 µl lysis solution was added. This was mixed by inverting the tube 5 times and allowed to stand at room temperature for 3 minutes whereupon 150 µl of ice cold 3 M sodium acetate (pH 5.0) was added. The tubes were vortexed briefly and placed on ice for 5 minutes. The cell debris was pelleted by centrifugation at 14,000 rpm for 10 minutes. The supernatant was decanted into a fresh microcentrifuge tube and an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1) added. The tubes were vortexed for 1 minute and then centrifuged for 5 minutes at 14,000 rpm. The aqueous layer was transferred to a fresh microcentrifuge tube, 2 volumes of ice cold ethanol added and placed on ice for 5 minutes. The DNA was precipitated by centrifugation at 14,000 rpm for 5 minutes. The ethanol was removed and the pellet washed in 700 µl 70% ethanol. The tubes were centrifuged for 2 minutes at 14,000 rpm, the 70% ethanol removed and the pellet was dried in a Savant Speed-Vac. The pellet was dissolved in 50 µl TE containing 0.2 mg ml^{-1} RNAse Cocktail (HT Biotechnology, Cambridge UK).

solutions used

TGE	10 mM EDTA 25 mM Tris-HCl (pH 8.0) 1% w/v glucose
Lysis solution	0.2 M NaOH 1% w/v SDS

2.2.3 Large scale preparation of bacteriophage λ DNA

5 ml of LB-broth was inoculated with a single colony of DL307 and incubated at 37°C overnight with shaking. 200 ml of LB-broth supplemented with 0.2 M $MgSO_4$ was inoculated with 0.5 ml of the DL307 overnight culture and incubated at 37°C with vigorous shaking. When the OD_{600} = 0.5 the required λ phage in lysate form was added to give a multiplicity of infection (m.o.i.) of 0.1 for a *c*I⁻ phage and an m.o.i. of 1 for

a cI^+ phage. The OD_{600} was followed. The OD_{600} increased as the cells continued to grow and divide and then fell as the cells lysed and released the phage. This took up to 8 hours. When lysis was well established 0.2 ml chloroform was added and the culture incubated for a further 10 minutes at 37°C with shaking. 8 g of solid NaCl was added to the lysed culture dissolved by swirling and placed on ice for 1 hour. RNAse A and DNAse were added to a concentration of 1 mg ml^{-1} each and mixed by swirling. The mixture was incubated at room temperature for 1 hour. The lysed culture was transferred to a 250 ml centrifuge bottle and centrifuged in a GSA rotor at 10,000 rpm for 10 minutes at 4°C. The supernatant was transferred to a sterile 1 l flask and 20 g of PEG 8000 added, dissolved by swirling and placed on ice for at least 1 hour but preferably overnight. The lysate/PEG 8000 solution was transferred to a 250 ml centrifuge bottle and centrifuged in a GSA rotor at 10,000 rpm for 10 minutes at 4°C. The supernatant was removed and discarded and the PEG/phage pellet was resuspended in 5 ml of phage buffer and transferred to a Universal bottle. An equal volume of chloroform was added and vortexed gently for 30 seconds to wash the PEG 8000 from the phage. The chloroform/phage/PEG 8000 mixture was centrifuged at 4500 rpm for 10 minutes to layer the PEG 8000 at the chloroform (lower) : phage buffer (upper) interface. The phage buffer layer, which contained the phage was carefully removed. CsCl in phage buffer solutions were prepared to densities of 1.3, 1.5 and 1.7 g/cc. A step gradient was set up by placing 2 ml if the 1.3 g/cc solution in a 35 ml polypropylene ultracentrifuge tube and then successively underlaying the 1.5 g/cc solution then the 1.7 g/cc solution. The bacteriophage solution was carefully layered on top of the step gradient and balanced to within 0.05 g with another tube. The step gradients were centrifuged at 35,000 rpm in a MSE 16x4 swinging bucket rotor for 2 hours at 18°C. The tubes were carefully removed from the rotor and clamped firmly. A piece of black card was placed behind the tube to aid the visualization of the opaque grey/blue phage band at the top of the 1.5 g/cc step. The band was extracted using a syringe with a 0.9 x 40 mm needle. The phage in phage buffer/CsCl was transferred to 8/32 inch dialysis tubing and dialysed against 5 l TE buffer overnight at 4°C. 0.5 ml aliquots were transferred to microcentrifuge tubes and 0.5 ml phenol (Tris equilibrated, pH 8.0) added. The tubes were placed on a blood mixer for 5 minutes then centrifuged for 5 minutes at 14,000 rpm. The aqueous layer

was transferred to a fresh microcentrifuge tube and an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1) added. The tubes were placed on a blood mixer for 5 minutes and then centrifuged for 5 minutes at 14,000 rpm. The aqueous layer was removed and an equal volume of chloroform added. The tubes were placed on a blood mixer for 5 minutes then centrifuged for 5 minutes at 14,000 rpm. The aqueous layer was transferred to a fresh microcentrifuge tube and 1/10 volume of 3 M sodium acetate (pH 5.0) added. 2 volumes of ice cold ethanol were added and mixed by inversion and the tubes placed on ice for 15 minutes. The phage DNA was precipitated by centrifuging at 14,000 rpm at 4°C for 15 minutes. The ethanol was removed and the pellets washed with 1 ml of ice-cold 70% ethanol and then centrifuged at 14,000 rpm for 5 minutes at 4°C. The pellets were dried in a Savant Speed-Vac. The DNA was stored as these dried pellets at –70°C until required whereupon 110 µl TE was added and 1 hour allowed for the pellet to dissolve. The DNA concentration was determined as described in section 2.2.7.

2.2.4 Large scale preparation of chromosomal DNA

5 ml LB-broth with appropriate supplements was inculated with a single colony of the strain of interest and incubated at a suitable temperature with shaking overnight. 250 µl of this overnight culture was used to inoculate 25 ml LB-broth supplemented with appropriate antibiotics. The culture was incubated with shaking overnight at a suitable temperature. The culture was transferred to a sterile Universal bottle and chilled on ice for five minutes. The culture was centrifuged at 4500 rpm for 10 minutes at room temperature to pellet the cells. The supernatant was discarded and the pellet was resupended in 5 ml STE. 250 µl 10% SDS and 250 µl proteinase K solution (4 mg ml^{-1}) where added, the mixture gently swirled and then incubated at 50°C for 6 hours without shaking. The mixture was transferred to a 25 ml glass beaker and an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1 v/v) added. This was mixed gently by swirling and allowed to stand either at room temperature for 1 hour or at 4°C overnight. The mixture was transferred to a Universal bottle and centrifuged at 4500 rpm at room temperature for 15 minutes. The upper aqueous layer (approximately 5 ml) was transferred to a 25 ml glass beaker and 500 µl of 3 M sodium acetate (pH

5.5) added and mixed by swirling. 11 ml of ice cold ethanol was added and a glass rod was used to spool the precipitated DNA out of the mixture. The DNA was transferred to a microcentrifuge tube and washed with 1 ml of 70% ethanol. The DNA was allowed to air dry for five minutes and then redissolved in 5 ml TE buffer overnight. 25 µl of a 10 mg ml^{-1} RNAseA solution was added, mixed by swirling and incubated at 37°C for 1 hour. 125 µl 10% SDS and 63 µl proteinase K (4 mg ml^{-1}) were added and the mixture incubated without shaking at 50°C for 1 hour. The mixture was transferred to a 25 ml glass beaker and an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1 v/v) added. This was mixed gently by swirling and allowed to stand either at room temperature for 1 hour or at 4°C overnight. The mixture was transferred to a Universal bottle and centrifuged at 4500 rpm at room temperature for 15 minutes. The upper aqueous layer (approximately 5 ml) was transferred to a 25 ml glass beaker and adjusted to 500 µl 3 M sodium acetate (pH 5.5) added and mixed by swirling. 11 ml ice cold ethanol was added and a glass rod used to spool the precipitated DNA from the mixture. The DNA was transferred to a microcentrifuge tube and washed with 1 ml 70% ethanol. The DNA was allowed to air dry for ten minutes before being redissolved in 500 µl TE buffer. This method yields approximately 500 mg of chromosomal DNA.

solutions used

STE TE buffer, 10 mM NaCl.

2.2.5 Small–scale preparation of chromosomal DNA

5 ml of LB-broth containing appropriate selective antibiotics was inoculated with a single bacterial colony and incubated overnight at the permissive temperature. 1.5 ml of the culture was transferred to a microcentrifuge tube and centrifuged for 2 minutes at 14,000 rpm. The supernatant was discarded. The pellet was resupended in 567 µl of TE buffer, 3 µl of proteinase K (20 mg ml^{-1}) and 30 µl 10% SDS. The mixture was vortexed thoroughly and incubated at 37°C for 1 hour. 100 µl of 5M NaCl was added and mixed thoroughly. 80 µl of hexadecyltrimethyl ammonium bromide (CTAB)/NaCl solution was added, mixed

thoroughly and incubated for 10 minutes at 60°C. An equal volume of chloroform/isoamyl alcohol (1:1 v/v) was added, mixed thoroughly and the tubes were centrifuged at 14,000 rpm for 5 minutes. The aqueous upper layer was transferred to a fresh microcentrifuge tube and an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1 v/v) added, mixed thoroughly and centrifuged for 5 minutes at 14,000 rpm. The supernatant was transferred to a fresh microcentrifuge tube and 2 volumes of ice-cold ethanol was added. The contents were mixed and centrifuged for 5 minutes at 14,000 rpm. The ethanol was removed and the pellet washed in 1 ml of ice-cold 70% ethanol then centrifuged for 5 minutes at 14,000 rpm. The 70% ethanol was removed and the pellet dried in a Savant Speed-Vac. The DNA was dissolved in 100 µl of TE buffer and 15 µl used per restriction digest.

solutions used

CTAB/NaCl solution 4.1 g NaCl was dissolved in 80 ml H_2O and 10 g CTAB (hexadecyltrimethyl ammonium bromide) was added slowly while heating and stirring.

2.2.6 Precipitation of DNA

1/10 volume 3 M sodium acetate (pH 5.0) was added to the DNA solution and mixed by vortexing. 2 volumes of ice cold ethanol were added and the tubes placed on ice for 10 minutes. The mixture was centrifuged at 14,000 rpm in a microcentrifuge for 10 minutes. The ethanol was removed and 1 ml of ice cold 70% ethanol added. The tubes were vortexed for 10 seconds and centrifuged at 14,000 rpm for five minutes. The 70% ethanol was removed and the pellet dried in a Savant Speed-Vac. The pellet was dissolved in a suitable volume of TE or dH_2O.

2.2.7 Determination of DNA concentration

10 µl of the DNA solution was added to 990 ml of TE buffer in a quartz cuvette. The OD_{260} and the OD_{280} were taken. An OD_{260} of 1.0 corresponds to 50 mg ml^{-1}. An OD_{260}/OD_{280} ratio of 1.8 indicates relatively

pure DNA. contaminants such as proteins and carbohydrates increase or decrease this ratio.

2.2.8 Restriction of DNA

The digestion of DNA using restriction endonucleases was performed in 20–50 µl volumes containing 0.1–1 µg DNA, 1x appropriate restriction enzyme buffer and restriction enzyme in 2–5 fold excess. The final volume was made up using distilled water. Some restriction endonucleases require the presence of BSA in the reaction mixture so, when required, BSA was added according to manufacturers instructions.

Partial digestion of DNA.

For the partial digestion of DNA six two–fold serial dilutions of restriction enzyme were added to DNA of a fixed concentration. The greatest concentration of enzyme used in such reactions was 0.5 Units of enzyme per µg of DNA. The digestion reactions were incubated for 1 hour. The reactions were terminated using TAE loading buffer and the samples analysed by agarose gel electrophoresis.

Digestion of DNA using two restriction enzymes.

For digestion of DNA using two restriction enzymes one of two approaches was taken. If the enzymes had a compatible buffer then both enzymes were added in equal concentration to the reaction mixture. If the enzymes had different buffer requirements the digestion conditions would be made to suit the restriction enzyme which had a requirement for a low salt buffer. The digest would be incubated for 1–2 hours and then the digest volume would be doubled using the appropriate amount of the second buffer (high salt), distilled water and the second restriction enzyme. The reaction would be incubated at the optimum temperature for a further 1–2 hours.

Alternatively, after the DNA had been digested by one enzyme, the DNA was purified from the reaction using Promega Wizard DNA Clean–Up Columns according to the manufacturers instructions. The eluted DNA in solution was then subjected to restriction by the second restriction enzyme.

2.2.9 'Filling in' of recessed 3' termini

The Klenow fragment of DNA polymerase I was employed to 'end fill' recessed 3' termini created by certain restriction endonucleases, to create blunt ended DNA molecules. The Klenow reactions were performed two ways:

I) The Klenow enzyme is at least partially functional in a wide variety of restriction enzyme buffers. Restriction digests, typically 20 µl, were allowed to proceed to completion (1–2 hours). The volume of the reaction was made up to 30 µl with dNTPs (such that each dNTP was present at a final concentration of 20 mM), 2 units of Klenow enzyme, the appropriate amount of 10x restriction enzyme buffer and distilled water. The samples were incubated at 37°C for 20 minutes then the salts, protein and unincorporated dNTP's were removed by using the Promega DNA Clean-Up Columns according to the manufacturers instructions.

II) 0.5 µg DNA, 1x Nick Translation Buffer (Boehringer Mannheim), each dNTP at a concentration of 20 mM and 2 units of Klenow enzyme were mixed and made up to 20 µl with dH_2O. The samples were incubated at 37°C for 20 minutes then the salts, protein and unincorporated dNTP's were removed by using the Promega DNA Clean-Up Columns according to the manufacturers instructions.

2.2.10 Agarose gel electrophoresis of DNA

Agarose gel electrophoresis was used to separate DNA fragments after digestion with restriction endonucleases and identify PCR products. The concentration of agarose used depended upon the sizes of DNA fragments being separated. For fragments of 300 bp to 1.5 kb 1.5% agarose was used, between 1.5 kb to 4 kb 1% agarose and above 4 kb 0.8% agarose. The agarose was dissolved in TAE buffer by brief boiling, cooled to 60°C and then poured into the gel tray, the comb(s) inserted and left to solidify for 30 minutes. Two types of electrophoresis equipment were used both made by BRL. The minigel (5 x 7.5 cm) used for rapid (1–2 hours) separation of DNA fragments usually for ligation reactions or preparing probes for hybridization. The midigel (11 x 15 cm) was used to analyse

When larger DNA fragments of 1 kb or greater were extracted the Qiagen Gel Extraction Kit was used according to the manufacturer's instructions. The reason for employing the two different systems is that the Geneclean system has a tendency to shear larger DNA molecules when two or more silica particles bind to the DNA fragment. Repeated pelleting and resuspension of the Glassmilk with bound DNA can exert forces on the DNA that can lead to degradation. The Qiagen system employs a fixed bed of silica so these forces are not encountered.

2.2.12 Ligation of DNA fragments

Ligation reactions were performed in 20 µl volumes. The total amount of DNA used per reaction was 0.5–1.0 µg with 1x ligation buffer (Boehringer Mannheim), T4 DNA ligase and TE buffer to make up to 20 µl when necessary. For sticky ended ligations a threefold molar excess of fragment to vector was used whilst for blunt ended ligations, a six to one molar ratio was used. Ligase concentration was 1 Unit per reaction for blunt ended DNA ligations and 0.2 Units per reaction for sticky-ended DNA ligations. Sticky-ended ligations were incubated at 16°C for 3 hours and blunt-ended ligations incubated overnight also at 16°C.

2.2.13 Preparation and transformation of competent cells (Chung Method)

The method developed by Chung *et al.* (1989) was used for routine transformations of plasmid DNA (not ligations). 5 ml of medium containing the appropriate selective antibiotics was inoculated with a single colony and incubated with shaking at the appropriate temperature overnight. 100 µl of the overnight culture was used to inoculate 20 ml of LB-broth, with appropriate selection, and incubated at a suitable temperature until the OD_{600} reached 0.4. The culture was transferred to a sterile Universal bottle and placed on ice for 10 minutes. The chilled culture was centrifuged in a bench centrifuge at 4500 rpm for 5 minutes. The supernatant was discarded and the Universal bottle was inverted to drain off excess media. The cells were resuspended in 1 ml of TSS and placed on ice. The cells and could be used immediately or stored at –70°C for up to 3 months. Plasmid DNA (typically 10–200 ng) was added to 100

potential clones and run chromosomal digests prior to Southern blotting onto nylon filters. The wells of the gels were always loaded dry and then the TAE buffer was carefully added to each reservoir until it overflowed across the surface of the gel joining both together. The minigel was run at 60–70 mA and midigels were typically run overnight at 25 mA. Once the gel had run sufficiently (this could be approximately gauged by observing the marker dye migration) the power supply was sitched off and the DNA in the gel stained with ethidium bromide/TAE solution (0.5 $\mu g\ ml^{-1}$) for 30 minutes and then destained in TAE for a further 30 minutes. After staining the DNA fragments could be visualised by exposing the gel to UV illumination.

2.2.11 Extraction of DNA from agarose gel slices

Two approaches were employed to extract DNA fragments from agarose gels after electrophoresis. The Geneclean kit manufactured by BIO 101 was used to isolate small fragments of 1 kb or less. The principle behind the system is a silica matrix to which DNA binds in high salt solution but not in low salt solution. Agarose gels were stained with ethidium bromide so that the DNA bands could be visualised in the gel when placed on a UV transilluminator. The desired bands were excised using a flamed scalpel blade and each gel fragment placed in a sterile microcentrifuge tube, weighed and three volumes of 6 M sodium iodide added to each. These were incubated at 50°C for 5 minutes or until the gel slice dissolved. 5 µl of Glassmilk silica suspension was added to each of the samples, these were vortexed and placed on ice for five minutes with frequent vortexing. The tubes were centrifuged at 14,000 rpm for 30 seconds to pellet the Glassmilk and the supernatant discarded. The pellet was resuspended in 0.5 ml of New Wash (an ethanol based buffer to remove the sodium iodide) and centrifuged as above. This process was repeated a further two times and then the pellet resuspended in 10 µl of TE buffer. This was incubated at 50°C for 10 minutes and then centrifuged as above to pellet the Glassmilk. The aqueous solution contained the DNA previously bound to the Glassmilk. This is transferred to a fresh microcentrifuge tube and centrifuged once more to remove residual Glassmilk. The DNA fragment in solution was then available for further manipulation.

µl of competent cells, gently mixed and left on ice for 30 minutes. After this time 0.9 ml of LBG (LB-broth supplemented with 0.2 mM glucose) was added to the transformation mixture. This was then incubated at the permissive temperature for 1–2 hours whilst being mixed on a rotating blood mixer. This is to allow the expression of plasmid encoded antibiotic resistance markers. 100 µl aliquots were plated onto selective agar and incubated overnight at an appropriate temperature. As a control, an aliquot of cells only were plated on the selective medium.

TSS	Difco bactotryptone	10 g
	Difco yeast extract	5 g
	NaCl	10 g
	PEG 3350	100 g
	$MgSO_4$	20 mM
	PIPES (pH 6.5)	20 mM
	Distilled water to 1 litre.	

2.2.14 Preparation and transformation of competent cells ($CaCl_2$ method)

For transformation of ligation mixtures the following procedure was followed. 5 ml of medium containing the appropriate selective antibiotics was inoculated with a single colony and incubated with shaking at the appropriate temperature overnight. 100 µl of the overnight culture was used to inoculate 20 ml of LB-broth, with appropriate selection, and incubated at a suitable temperature until the OD_{600} reached 0.6. The culture was transferred to a sterile Universal bottle and placed on ice for 10 minutes. The chilled culture was centrifuged in a bench centrifuge at 4500 rpm for 5 minutes. The supernatant was discarded and the universal was inverted to drain off excess media. The cells were resuspended in 10 ml ice-cold 0.1 M $MgCl_2$/0.1 M $RbCl_2$ (9:1 v/v). The cell suspension was centrifuged in a bench centrifuge at 4500 rpm for 5 minutes. The supernatant was removed and the pellet of bacterial cells resuspended in 1 ml ice-cold 0.1 M $CaCl_2$/0.1 M $RbCl_2$ (9:1 v/v). The cells were stored on ice for at least 30 minutes (the competence of the cells has been shown to increase after overnight storage on ice). Immediately before use 1.5 µl dimethylsulphoxide (DMSO) was added per 100 µl of the competent cells. 5 µl of the ligation mix was added to 100 µl

of competent cells, mixed gently and placed on ice for 30 minutes. The mixture was transferred to a 42°C waterbath for 90 seconds and then placed on ice for 5 minutes after which 0.9 ml LB-broth 0.2 mM glucose was added. The culture was then incubated at a suitable temperature for 1 hour to allow to expression of the antibiotic selection marker after which 200 µl aliquots plated on selective media.
note: If the selection is for tetracycline resistance alone the 1 hour expression step can be omitted.

2.2.15 Preparation of cells for high efficiency electro-transformation

1 litre of LB-broth (2 x 500 ml in 2 litre conical flasks), containing selective agents if required was inoculated with 1 ml of a fresh overnight culture and incubated with vigorous shaking at a suitable temperature until the OD_{600} reached 0.7–1.0. The culture was transferred to 4 250 ml GSA centrifuge pots and placed on ice for 20 minutes. The culture was centrifuged in a chilled GSA rotor at 8000 rpm for 5 minutes at 4°C. The supernatant was discarded without disturbing the pellet. The pellets were resuspended in 250 ml of chilled sterile dH_2O and pelleted as above. This procedure was repeated using decreasing volumes of chilled sterile dH_2O (i.e. 125 and 50 ml). The pellets were pooled by resuspending in a total of 20 ml of chilled 50% glycerol in dH_2O, (v/v), transferred to a 36 ml Corex tube and centrifuged at 10,000 rpm for 5 minutes at 4°C in a pre–chilled Sorval SS–34 rotor. The supernatant was discarded and the pellet resuspended in 2 ml of sterile chilled 50% glycerol to give a cell concentration of ~2–3 x 10^{10} ml^{-1}. Aliquots of 130 µl were placed in sterile microcentrifuge tubes and placed on ice. The cells were snap frozen in a dry ice/ethanol bath and stored at –70°C until needed. Cells could be stored in this manner for up to 6 months.

2.2.16 Preparation of DNA for electroporation

DNA ligation mixtures used for transforming cells by electroporation have to be in salt free solution to prevent arcing as the DNA solution/cell suspension is exposed to a large electrical charge. The presence of salts result in a premature release of the charge and a greatly reduced transformation efficiency.

The ligation mixture was briefly centrifuged in a microcentrifuge and dH_2O added to increase the total volume to 50 µl. 0.5 ml of N–isobutanol was added and the mixture vortexed and centrifuged for 30 minutes at 14,000 rpm at 4°C. The supernatant was removed, the pellet dried in a Savant Speed-Vac and resuspended in 10 µl dH_2O. For each transformation 1–2 µl of the ligation mixture was used.

2.2.17 Electro-transformation of ligation mixtures

The electro–transformation competent cells were thawed slowly at room temperature and placed on ice. The equipment used was a Biorad Genepulser and Pulse Controller. The Genepulser was set at 25 mF and 2.5 kV and the Pulse Controller at 200 Ohms. The pulse at these settings has a time constant of 4.5–5.0 milliseconds giving a field strength of 12.5 kV/cm. 40 µl of the competent cells was mixed with 2 µl of the ligated DNA. The mixture of cells and DNA was transferred to a chilled electroporation cuvette (1 mm electrode width, Biorad) and the side of the cuvette gently tapped to ensure that the mixture was distributed evenly on the bottom. The cuvette was placed in the safety chamber slide and inserted into the chamber. The cells were pulsed and immediately resuspended the cells in 1 ml of SOC broth (see Table 2.1.2) and transferred to a microcentrifuge tube. The tubes containing the transformed cells were placed on a blood mixer for 1–2 hours to allow the transformants to express for antibiotic selection at a suitable incubation temperature. 100 µl aliquots of the cell suspension were spread on appropriate selective plates and incubated overnight at a suitable temperature.

2.2.18 DNA sequencing

Introduction.

DNA sequencing was performed using the Pharmacia T7 Sequencing Kit. The kit is based upon the chain–terminating dideoxynucleotide sequencing method developed by Sanger *et al.*, (1977). In the original procedure, primer extension was catalysed by the Klenow fragment of DNA polymerase I. The kit replaces the Klenow enzyme with the T7 DNA polymerase, which has the advantage of creating longer chain terminated fragments with a more even distribution of label

between fragments. The major practical difference in using T7 polymerase over Klenow is that primer extension reactions are performed in two stages, a labelling reaction and a termination reaction. The two stages are necessary because the enzyme uses dideoxynucleotides very readily, and therefore in order to allow the synthesis of long chain-terminated fragments, dideoxynucleotides are excluded from the first stage of the reaction, being added for the second. Even so, the time required for the reactions using the T7 enzyme is considerably less than those using the Klenow enzyme.

Annealing of primer to single stranded template.

The DNA templates used in the sequencing reactions were double stranded plasmid DNAs purified either by CsCl density centrifugation of by the Promega SV Miniprep Kit. The concentration of the template was adjusted to 1 mg ml^{-1} with distilled water. The oligonucleotide primers used where obtained from Genosys or Perkin Elmer and tended to be between 17–24 bases in length

The template was denatured by adding 2 µg DNA to 8 µl 1 M NaOH. This mixture was left at room temperature for five minutes whereupon 1 µl of 3 M sodium acetate (pH 5.0) was added followed by 20 µl of ethanol. The tube was placed on ice for 10 minutes then centrifuged at 14,000 rpm at 4°C for 10 minutes. The ethanol was replaced with 20 µl of 70% ethanol, vortexed briefly and centrifuged at 14,000 rpm at 4°C for 5 minutes. The 70% ethanol was removed and the almost invisible pellet allowed to air dry briefly before being dissolved in 10 µl dH_2O
The following was added to an microcentrifuge tube on ice:

Template DNA (2 µg)	10 µl
Primer (0.80 mM)	2 µl
Annealing buffer	2 µl
Total	14 µl

The contents of the tube were mixed thoroughly and incubated at 60°C for 10 minutes. The tube was then left at room temperature for at least 10 minutes; if the rest of the sequencing reaction was to be performed at a later time then the tube could be stored at –20°C until required.

Sequencing reaction.

For each template to be sequenced, four wells of a microtitre plate were labelled 'A', 'C', 'G' and 'T' respectively and 2.5 µl of the corresponding dideoxynucleotide mix added to each well. To the tube containing the annealed template and primer the labelling mix, (dCTP, dGTP, dTTP and dGTP in solution), T7 DNA polymerase and α–[^{35}S] dATP were added as follows:

Annealed template and primer	14 µl
Labelling mix	3 µl
α–[^{35}S] dATP	1 µl (10 µCi)
T7 DNA polymerase (1.5 U µl^{-1})	2 µl
Total	20 µl

This labelling reaction was incubated at room temperature for 5 minutes. While this was proceeding the previously dispensed sequencing mixes were incubated at 37°C for 1 minute in a water bath. After the 5 minute incubation of the labelling reaction, 4.5 µl was added to each of the prewarmed sequencing mixes and returned to the water bath for a further 5 minutes to allow chain termination to occur. Finally, 5 µl of Stop solution was added to each reaction, these could then be stored at –20°C until required for electrophoresis. When the samples were needed for loading onto the sequencing gel they were heated to 80°C for 2 minutes to denature the DNA. Immediately after this incubation 3 µl of each sample was loaded onto the gel.

DNA sequencing gel electrophoresis.

DNA sequencing was performed using a 30 x 40 cm BRL sequencing apparatus. The glass sequencing plates were thoroughly cleaned with ethanol and chloroform. The shorter of the two plates was desiliconized using dimethylsilane to ease separating the plate from the sequencing gel after running the samples. The plates were assembled using 0.2 m m spacers and taped together to prevent leakage.

The gel was prepared by adding together the following:

Acrylamide (filtered, 40% w/v)	15 ml
Urea	43 g
d.H2O	35 ml
10x TBE	10 ml

The urea was allowed to dissolve with the aid of magnetic stirring. Once dissolved, 1 ml of freshly prepared 10% ammonium persulphate solution was added followed by 35 µl of TEMED. This was then stirred slowly for a few seconds and was then slowly poured between the sequencing plates. The flat edge of a 60 well shark–tooth comb was pushed between the plates to layer the top of the gel. Saran wrap was wrapped around the exposed areas of the plate and the top and each side of the gel was clamped with bulldog clips. The gel was then set aside for at least 30 minutes for the acrylamide to polymerise. Once set, the bulldog clips, Saran wrap, tape and comb were removed and distilled water was squirted along the top of the gel. The shark–toothed comb was then replaced with the teeth pointing downwards until just touching the top of the gel. The gel was then clamped into the sequencing apparatus and TBE buffer poured into the top and bottom reservoirs. The gel was then pre–run at ~66 W (~1500 V) for 1 hour. After this the gel was ready to be loaded with the sequencing reactions. The samples were loaded in the order A, C, G and T immediately after denaturing the DNA (see above). The gel was then electrophoresed at 66 W until the blue dye ran off the end of the gel. Once electrophoresis was complete the glass plates were removed from the apparatus and the shorter glass plate was carefully removed. The plate with the gel attached was placed in a fixing bath containing 10% methanol and 10% acetic acid in water for 20 minutes. The plate and gel were then removed and a sheet of water dampened blotting paper laid over the gel. A dry sheet of blotting paper was then laid over this and gently pressed down. The sheets of blotting paper were then carefully peeled from the glass plate with the gel adhered to the paper. The paper and gel sandwich was then dried in a vacuum gel–drier for 1 hour at 80°C. When dry the gel was placed in an autoradiography cassette and allowed to develop at room temperature. In most instances a good signal was achieved after 24 hours.

2.2.19 Southern blotting of DNA onto nylon filters

Genomic DNAs (7 μg) were digested with appropriate restriction enzymes to produce fragments of calculated size. The digests were run on a midigel at 20 mA for at least 12 hours and then stained with ethidium bromide and photographed under UV illumination. The gel was then washed in 200 ml of 0.25M HCl for 15 minutes to depurinate the DNA. The gel was washed in 200 ml of 0.5M NaOH/1.5M NaCl solution for 15 minutes to denature the DNA. This step was repeated and the gel rinsed in dH_2O. The gel was then washed in 1 M Tris HCl (pH 8.0)/1.5 M NaCl solution for 45 minutes and again rinsed in dH_2O. Finally the gel was soaked in 20x SSC buffer (pH 7.2) for 5 minutes.

Ten sheets of blotting paper, cut to the same size as the gel, were soaked in 6x SSC buffer and laid upon a glass plate in a tray. The gel was then laid upon this with the upper surface laid face down. A sheet of positively charged nylon membrane (Boehringer Mannheim), cut to the size of the gel, was soaked in 6x SSC buffer and laid upon the upper surface of the gel. A further six sheets of blotting paper soaked in 6x SSC buffer were laid on top of this followed by 15 sheets of paper towelling. Finally, a glass plate was placed on top and a 1 kilogram weight placed upon this. 6x SSC buffer was then poured into the tray until half the lower blotting paper was submerged. After 2 hours the weight and glass plate were removed and any damp paper towels were removed and replaced with fresh ones and the glass plate and weight placed back. The blot was then left overnight. The nylon filter was removed and a corner cut out as a marker for both sides of the blot. The DNA was then fixed on the filter by exposing to UV using a UV crosslinker on 'autocrosslink' setting (1800 UV Stratalinker, Stratagene). The blot was now ready to be used for hybridization with the prepared labelled DNA probe.

solutions used

20x SSC buffer	NaCl	525.9 g
	Sodium citrate	264.6 g
	Adjust to pH 7.2	
	Add distilled water to 3 litres	

2.2.20 Preparation of labelled DNA probe

This method is adapted from that described by Feinberg and Vogelstein (1984). The required probe DNAs were always cloned in plasmids. 1 µg of the plasmid DNA was cut with appropriate restriction enzymes and the subjected to agarose gel electrophoresis. The gel was stained in ethidium bromide (0.5 mg ml^{-1})and after destining the gel was observed on a long wave UV transilluminator and the desired band excised cleanly. The DNA was then purified from the agarose gel slice using the Geneclean method. The DNA can now be labelled by adding approximately 50 ng of the fragment DNA to the following mixture:

DNA	~50 ng
OLB	10 µl
BSA (10 mg ml^{-1})	2 µl
α–[^{35}P] dCTP (10 mCi ml^{-1})	5 µl
Klenow enzyme	2 Units
dH_2O to 50 µl	

The reaction was incubate overnight at room temperature in a lead containment vessel.

solutions used

OLB buffer (Solutions A:B:C in the ratios 2:5:3 [v/v])

Solution A: 1.25 M Tris-HCl pH 8.0
0.125 M MgCl2
0.5 mM of each dATP dGTP and dTTP
0.025 mM β–mercaptoethanol

Solution B: 2 M Hepes–NaOH pH 6.6

Solution C: 4.5 mg ml^{-1} pd(N)6 (in TE buffer)

2.2.21 Stripping probes from nylon filters

Once the Southern blot had been probed, it was sometimes necessary to analyse the a blot with a second, different probe. In these cases the initial labelled probe was removed. The blot was incubated at 45°C for 30 minutes in 30 millilitres 0.4 M NaOH and then washed in 40 millilitres 0.1 X SSC, 0.1% SDS, 0.2 M Tris-HCl (pH 7.5) solution for 30 minutes at 45°C. The signal from the blot should was then negligible and the blot ready to be hybridized with the next labelled probe.

2.2.22 The Polymerase Chain Reaction (PCR)

PCR was performed to confirm clonings, confirm gene replacements and to create engineered molecules for cloning. Primers were usually between 17–24 bases in length and were obtained from the Oswel DNA Service, Genosys or Perkin-Elmer. For general purpose PCR reactions such as confirmation of replacements and clonings *Taq* DNA polymerase (Promega) was used. For reactions were the PCR product would be cloned Vent DNA polymerase (NEB) was used because this enzyme has a 3'→5' proof–reading activity. A typical reaction mixture is presented below.

Taq (or Vent) DNA polymerase	1 Unit
10x Taq (or Vent) buffer	10 µl
Primer 1	60 pmol
Primer 2	60 pmol
dNTP's	0.2 mM each
$MgCl_2$	1.5 mM
template	10 ng plasmid or 0.1 µg chromosomal DNA
distilled water to 100 µl	

The melting temperature used was always 94°C for 1 minute. Annealing temperature varied depending on which primers were being used. The annealing temperature (T_m) was gauged by the following formula.

$$4(nA+nT) + 2(nG+nC) = T_m$$

were nA, nT, nG and nC are the number of adenine, thymine, guanine and cytosine residues in the primer. The extension temperature used was always 72°C. As a rule of thumb, 1 minute per Kb of extension was allowed.

In some instances, the template for the PCR reaction was a crude cell extract. 15 µl of an exponentially growing culture was centrifuged at 14,000 rpm for 1 minute. The supernatant was discarded and the cells washed three times in distilled water. The cells were finally resuspended in 100 µl of dH_2O. The cells were then placed in a 95°C heating block for 5 minutes, after which 20 µl was used as a template for PCR.

2.3 Protein Techniques

2.3.1 *In vivo* protein labelling using T7 RNA polymerase

5 ml of LB Amp/Cmp was inoculated with a single colony of the BL21(DE3) (pLysS) host containing the plasmid clone of interest. The culture was incubated overnight at 37°C and then centrifuged at 5000 rpm for 5 minutes to pellet the cells. 4 ml of the supernatant was removed and the pellet resuspended in the remaining 1 ml. 0.5 ml of this concentrated overnight culture was used to inoculate 25 ml of Spizizen's broth with minimal supplements and Amp/Cmp. The culture was incubated at 37°C with shaking until the OD_{600} reached 0.8 and then four 0.5 ml aliquots placed into sterile microcentrifuge tubes which were labelled as follows:

–IPTG/–Rif
+IPTG/–Rif
–IPTG/+Rif
+IPTG/+Rif

The tubes labelled +IPTG had 3 µl of IPTG (20 mg ml^{-1}) added and all tubes were then incubated at 37°C on a blood mixer for 30 minutes. To the tubes marked +Rif 3 µl of freshly prepared rifampicin (100 mg ml^{-1}) was added and the incubation continued as before for 45 minutes. 1 µl of L-[^{35}S] methionine (ICN) at an activity of 5 mCi ml^{-1} was then added to each tube. The tubes were immediately vortexed and incubated for 1 minute at room temperature to allow incorporation of the label. The tubes were centrifuged for 30 seconds at 14,000 rpm to pellet the cells and the supernatant discarded. 200 µl of 1X SDS loading buffer was added to each tube and the tubes vortexed for 2 minutes or until the pellet was resuspended. Samples were then either boiled for 2 minutes or incubated at 37°C for 1 hour. These could be stored at –70°C until required for analysis by SDS-PAGE.

solutions used

SDS-PAGE loading buffer :	100 mM Tris-HCl (pH 6.8)
2X solution	200 mM β-mercaptoethanol
	4% SDS
	0.2% bromophenol blue
	20% glycerol

2.3.2 Visualization of plasmid encoded proteins (Maxicell method)

Plasmid encoded proteins were identified using the method of Sancar *et al.*, (1979). 5 ml LB-broth containing appropriate selective agents was inoculated with a single colony of plasmid containing CSR603. The cultures were incubated overnight with shaking at a suitable temperature. 5 ml of M9 CAA medium with appropriate selection and supplements was inoculated with 50 µl of the overnight culture and incubated with shaking at a suitable temperature until the OD_{600} reached 0.5. 3 ml of this culture was transferred to a glass Petri dish and irradiated for 20 seconds with 254 nm ultraviolet light at an intensity of 10 ergs/mm^2/s. 2.5 ml of the culture was transferred to a test tube, D-cycloserine added to a final concentration of 150 mg ml^{-1} and incubated overnight with shaking at 37°C. 2 ml of the culture was centrifuged at 14,000 rpm for 3 minutes to pellet the cells. The supernatant was removed and the pellet resuspended

in 1 ml of sulphate-free Hershey salts. the cells were pelleted by centrifuging at 14,000 rpm for 3 minutes. The supernatant was removed and the pellet resuspended in 0.8 ml sulphate-free Hershey medium. The culture was incubated with shaking at 37°C for 1 hour. 0.2 ml sulphate-free Hershey medium containing 5 µCi [^{35}S]-methionine (ICN) was added to the culture which was then incubated for 1 hour at 37°C. The cells were pelleted by centrifugation at 14,000 rpm for 5 minutes. The pellet was washed twice with 0.5 ml 100 mM NaCl before being resuspended in 50 ml dH_20. 50 µl of 2 x SDS-PAGE loading buffer was added, the tube briefly vortexed and the samples placed in a boiling water bath for 5 minutes. The samples were then analysed by SDS-PAGE or were stored at –70°C until required.

2.3.3 *In vitro* translation using a linear DNA template

This protocol is based on the bacterial cell-free coupled transcription–translation system first described by De Vries and Zubay (1967) and allows the *in vitro* expression of genes contained on a bacterial plasmid or a bacteriophage genome. The kit was supplied by Promega. *In vitro* transcription is from a linear DNA template from endogenous promoters. The mRNA transcripts produced are then translated *in vitro* where the introduction of a label aids visualization of any proteins produced. The S30 cell extract used by the kit is prepared from *E. coli* B strain SL119 which is deficient in OmpT endoprotease, Lon protease and Exonuclease V (the RecBCD enzyme). Supplied with the kit is a S30 premix without amino acids which is optimised for a given aliquot of S30 with all other requirements. These include the NTPs, tRNAs, appropriate salts and an ATP regenerating system. Also supplied is an amino acid mixture lacking methionine for facilitating the radiolabelling of the translation products.

The reaction for labelling was prepared as follows;

DNA template	4 µg
Amino acid mixture minus methionine	5 µl
S30 premix	20 µl
[^{35}S] methionine (10 mCi ml^{-1})	1.5 µl
S30 extract	15 µl

Sterile distilled water to a final volume of 50 µl.

The mixture was vortexed gently and then centrifuged in a microcentrifuge for 5 seconds to bring the reaction mixture to the bottom of the tube. The reactions were incubate at 37°C for 2 hours. The reaction was stopped by placing the reactions on ice for 5 minutes. To each 50 µl reaction 200 µl acetone was added, the tube vortexed and placed on ice for 5 minutes. This precipitated the protein so as to separate it from the PEG used in the S30 extract. The mixture was centrifuged for 5 minutes at 14,000 rpm and the supernatant removed. The pellet was resupended in 200 µl of 1X SDS-PAGE loading buffer and 20–30 µl used per well for analysis by SDS-PAGE.

2.3.4 Preparation of SDS-PAGE gels

The Hoefer SE600 dual cooled vertical slab unit which is able to run one or two 16 x 18 cm gels was used for most SDS-PAGE experiments. The gel plates were washed in distilled water and then wiped with ethanol. The gel plates were laid together separated by 0.75 mm spacers and then clamped together. The plates were then clamped into the base–plate of the gel pouring apparatus. The resolving gel was poured first. All the ingredients bar the were mixed together in a 50 ml glass beaker, the TEMED and the 10% ammonium persulphate solution were added last. The 10% ammonium persulphate solution was always freshly prepared. The solution was quickly mixed and then drawn up in a 25 ml pipette and poured between the gel plates. The resolving mix was poured into the gap until 4 cm from the top of the plates. This was then layered with isobutanol saturated with 1x stacking gel buffer and allowed to polymerise at room temperature for 15 minutes. The isobutanol was poured off and the air–gel interface was thoroughly rinsed with distilled water. Excess water was removed from the gel space using a strip of blotting paper. The 4% stacking gel was then poured into the remaining area of the gel and the 0.75 mm 10 well comb inserted. The gel apparatus was then left at room temperature for 30 minutes for the stacking gel to polymerise. The comb was then removed and each well rinsed three times with Tris–glycine running buffer to remove any unpolymerized acrylamide. The gel was then ready to be used to run protein samples.

solutions used

Stock acrylamide: The 40% v/v bis-acrylamide was supplied premade by Sigma Chemical Co.

10% resolving gel (40 ml)

bis–acrylamide (40% v/v)	10 ml
4x resolving gel buffer	10 ml
dH_2O	19.2 ml
10% SDS	400 µl
10% ammonium persulphate	400 µl
TEMED	25 µl

4x resolving gel buffer.
45.5 g Tris base dissolved in 200 ml of distilled water adjusted to pH 8.8 with concentrated HCl. Made up to 250 ml with distilled water, filtered and sterilised.

4% stacking gel (10 ml)

bis–acrylamide (40% v/v)	1.3 ml
4x stacking gel buffer	2.5 ml
dH_2O	6.0 ml
10% SDS	100 µl
10% ammonium persulphate	100 µl
TEMED	10 µl

4x stacking gel buffer.
15.25 g of Tris base dissolved in 200 ml of distilled water adjusted to pH 6.8 with concentrated HCl and made up to 250 ml with distilled water, filtered and sterilised.

2.3.5 Running SDS-PAGE gels

The samples were first thawed, if frozen, and then boiled for 2 minutes or incubated at 37°C for 60 minutes as a non–boiled sample. The samples were centrifuged at 14,000 rpm in a microcentrifuge for 5 minutes. Each well was filled half full with running buffer. 30 µl of each

sample and 8 µl of the marker proteins were loaded into separate wells. The remaining space in the wells was topped up with running buffer and the upper reservoir clamped on top of the gel plates. The lower reservoir was filled two thirds full with running buffer. The gel plates/upper reservoir were removed from the pouring stand and placed in the tank. The upper reservoir was filled with running buffer and the lid attached. The gel was run at 30 mA until the marker dyes had migrated from the stacking gel into the resolving gel then amperage was increased to 40–45 mA. The gel was run until the marker dye reached the bottom of the gel.

The clamps were unscrewed and a plastic wedge used to prise open the plates. Each gel was placed in a polythene sandwich box (25 x 25 x 8 cm) and 70 ml of Coomassie stain added to each and incubated at 37°C with gentle shaking for 20 minutes. The stain was poured off and the gel rinsed in 50 ml of destain to remove traces of the stain from the gel and the box. 100 ml of destaining solution was added and foam bungs placed in the box to absorb the Coomassie stain that would leach from the gel. The destaining gel was incubated at 37°C with gentle shaking until the marker bands were clearly visible and the background of the gel was destained thoroughly. The destain was removed and 60 ml of fixing solution added. Fixing the gel prevented the gel from cracking and shrinking during drying. The gel in fixing solution was incubated at 37°C with gentle shaking for 20 minutes. The fixative was removed and a sheet of blotting paper just larger than the gel was soaked with distilled water and laid over the gel. A dry sheet of similarly sized blotting paper was laid on top of the first sheet and the gel/paper sandwich removed carefully from the box. The sandwich was laid on the bed of a vacuum gel dryer gel side up and a sheet of Saran wrap placed over the gel. The gel was dried for 1 hour under vacuum at 80°C. The dried gels were then either taped into an autoradiogram cassette and an X–ray film added or placed in a Phosphorimager cassette. The gels in the autoradiography cassette were usually left overnight to expose the film before developing. The position of the non–labelled marker proteins were marked on the exposed X–ray film by over laying it on the dried gel. The gels in the Phosphorimager cassette were usually left for three hours before scanning.

solutions used

5x Running buffer stock solution:	Tris base	15.1 g
	Glycine	94 g
	10% (w/v) SDS	50 ml

Tris glycine electrophoresis buffer;	Working solution:	
	25 mM Tris·HCl	
	250 mM glycine (pH 8.0)	
	0.1% SDS	

Coomassie blue solution. Coomassie brilliant

	blue (type R250)	0.25 g
	Methanol : H_2O (1:1 v/v)	90 ml
	Glacial acetic acid	10 ml
	Distilled water to 1 litre.	

Once prepared, filter through a Whatman No. 1 filter to remove particulates.

Destain solution:	methanol	500 ml
	Glacial acetic acid	750 ml
	Distilled water to 5 litres	

Fixing solution: Destain solution containing 5% glycerol (v/v)

2.4 Bacteriophage techniques

2.4.1 Production of bacteriophage P1 lysates

5 ml of LB-broth containing 2.5 mM $CaCl_2$ was inoculated with a single colony of the desired strain and incubated overnight at the permissive temperature without shaking. 1 ml of the overnight culture was mixed with 5 x 10^5 P1 in a large sterile test tube and incubated for 30 minutes at 37°C to allow the phage to adsorb into the cells. A cells only control was also prepared. 3 ml of LB-broth and 4 ml of molten LC top agar, cooled to 45°C, containing 2.5 mM $CaCl_2$ were added to the cells/phage mixture and mixed. The mixture was immediately poured onto an LC bottom agar plate and gently swirled until the surface had an

even covering of the top agar. The plates were left at room temperature for 15 minutes to allow the agar to set and then incubated overnight at 37°C in an upside down position. The cells only control had an even lawn of cells whilst the infected culture would be partly or completely lysed, resulting in cleared top agar. The top agar layer was scraped off with a sterile scalpel blade into a 250 ml beaker containing 4 ml of LB-broth and a few drops of chloroform. The mixture was incubated at 30°C with vigorous agitation for 30 minutes. The mixture was transferred to a Universal bottle and centrifuged at 4500 rpm for 15 minutes to pellet the top agar. The supernatant was decanted into a 1/2 ounce Bijou bottle. A few drops of chloroform was added and the lysate stored at 4°C.

2.4.2 Phage P1–mediated transduction

5 ml of LB-broth containing 2.5 mM $CaCl_2$ was inoculated with 0.2 ml of a fresh overnight culture of the recipient strain. The culture was incubated at a permissive temperature with shaking until the OD_{600} reached ~0.8. The culture was centrifuged at 4500 rpm for 5 minutes, the supernatant removed and the cells resuspended in 0.5 ml of LB-broth containing 2.5 mM $CaCl_2$. 130 µl of P1 lysate was placed in a microcentrifuge tube, a drop of chloroform added and the tube vortexed for 10 seconds and then centrifuged at 14,000 rpm for 1 minute to pellet any debris. 50, 10 and 1 µl aliquots were added to 100 µl of cell suspension. A cells only and phage only control was also included. The tubes were incubated at 37°C for 15 minutes. When selecting for relief of auxotrophy, 1 ml of phage buffer was added to the cells which were then plated on minimal selective media without having an incubation period. When antibiotic resistance was being selected 1 ml of phage buffer was added, the mixture vortexed briefly and then centrifuged at 14,000 rpm for 1 minute to pellet the cells. The supernatant was removed and the pellet was resuspended in 1 ml of LB-broth 0.2% glucose. The cells were allowed to express the antibiotic marker for 1 hour before 200 µl aliquots were plated onto selective media. The plates were then incubated overnight and any colonies growing on the selective agar are transductants provided the cells only and lysate only control plates are clear of growth. When selecting on minimal media at 30°C a 2 day incubation period was sometimes required

for the colonies to reach an appreciable size (colonies of 1 millimetre in diameter).

2.4.3 Preparation and selection of λ lysogens

A culture of the desired strain to be lysogenized was grown up in LB-broth supplemented with 0.2 mM $MgSO_4$ and 0.2% maltose to mid–log phase. The λ lysate was diluted so that approximately 200 λ phage particles could be added to the 0.3 ml of the culture. 3 ml of molten LC–top agar cooled to 45°C supplemented with 0.2 mM $MgSO_4$ was added to the cells/phage mixture and the mixture poured onto a fresh LB-agar plate and left to set. This was incubated overnight at 37°C. The aim was to promote the formation of isolated λ phage plaques. A sterile toothpick was used to touch the centre of a plaque and this was then used to streak onto a fresh LB-agar plate which was incubated overnight at 37°C. The resulting single colonies from the plate could now be tested for the presence of λ phage. Lysogenized bacteria are immune to lysis by λ phages with the same immunity as the one used to lysogenize the strain but sensitive to λ phages that are virulent, or carrying a different immunity. An LB-agar plate was streaked with the λ lysate used for the lysogeny and a virulent λ phage (λ*vir*). Sterile toothpicks were used to cross–streak the single colonies over the λ phage and the λ*vir* and incubated overnight at 37°C. The streaks which were immune to the λ phage used for lysogeny and sensitive to λ*vir* were presumed to be λ lysogens.

2.5 Bacterial Techniques

2.5.1 β-galactosidase assays

The method used to determine the β-galactosidase activity was as described by Miller (1972). 0.5 ml of the desired culture was added to 0.5 ml of Z buffer. 1 ml of the same culture was taken and the OD_{600} measured. If the promoter activity was known to be high then 0.1 ml of culture was added to 0.9 ml Z buffer. 50 µl of chloroform was added to the culture/Z buffer mixture which was vortexed for 30 seconds. The samples were stored at 4°C until all sampling had been performed. 200 µl of freshly prepared *o*–nitrophenyl-β-D-galactopyranoside (ONPG)(4 mg ml^{-1})

was added to each sample which were then vortexed. A control of 0.5 ml of the culture medium used and 0.5 ml of Z buffer was also prepared. After the addition of ONPG the samples were placed on ice until all samples had been treated. The samples were placed in a 30°C waterbath and the exact time of the start of incubation was noted. The tubes were checked every three minutes and when a yellow colour began to develop 0.5 ml of 1 M Na_2CO_3 was added. The tubes were vortexed and a note made of the time taken for the colour change to occur. The samples were placed on ice until all samples until a yellow colour developed the other samples. When all the tubes had shown a colour change measure the OD_{420} and OD_{550} of the samples was measured using the control as a blank. The β-galactosidase activity of the samples were calculated and expressed as Miller Units using the equation:

$$\frac{OD_{420} - 1.75 \times OD_{550}}{OD_{600} \times 0.5\ ml \times T} \times 1000$$

Where T = time in minutes for colour change.
0.5 ml is the sample volume taken from the original culture.

Z buffer	Na_2HPO_4	4.26 g
	$NaH_2PO_4.H_2O$	3.11 g
	KCl	0.375 g
	$MgSO_4.7H_2O$	0.123 g
	β–mercaptoethanol	1.35 ml
	SDS (10%)	0.25 ml
	Add distilled water to 500 ml	

2.5.2 Photography of bacterial cells

A Zeiss photo-camera was used to phototgraph bacterial cells . Molten agarose was pipetted onto an ethanol cleaned glass slide using a glass micropipette so that a thin, level layer of agarose covered the surface of the slide. A 10 µl of culture was pipetted onto the agar surface and covered with an ethanol washed coverslip. Cells were photographed through a 100x, phase contrast, oil immersion lens. Ilford HP5 400 film was used.

For photographing immunofluorescence samples a Leitz Metallux photo–camera loaded Kodak Ektapress 1600 colour film was used. Exposure setting was on automatic. For photographing DAPI stained cells, the Leitz Metallux photo–camera loaded with Ilford HP5 400 film was used.

2.5.3 Frozen storage of bacterial strains

Conveniently, *Escherichia coli* can be stored at –70°C without too great a loss of viability. 5 ml of a fresh overnight culture of the strain to be frozen was centrifuged at 4500 rpm for 5 minutes. The supernatant was discarded and the pelleted cells resuspended in 1 ml of Frozen Storage Buffer. The culture was then transferred to a cryogenic vial and placed on ice for three hours before being stored at –70°C.

Frozen storage buffer	50% Bacterial buffer
	50% glycerol (v/v)

2.5.4 Testing UV sensitivity of *recA* strains

Single colonies of the strain being tested were streaked across the surface of an LB agar plate using a sterile toothpick. As a controls samples of *recA*$^+$ and *recA*$^-$ strains were also streaked on the plate. Areas of the streaks were then exposed to UV light calibrated to 600 ergs/mm. A piece of cardboard was used to protect certain areas from UV light. The areas were exposed for different times. Typically these were 0, 10, 20 and 30 seconds. The plates were then incubated overnight and streaks examined for growth on the UV irradiated regions. Typically the *recA* mutants could not grow after 10 seconds of exposure to UV light.

2.5.5 Gene replacement procedure

To replace chromosomal genes with engineered copies a novel method was employed (N. McLennan and M. Masters, pers. comm.). The gene replacement procedure employs high copy number plasmids and the generalised transducing capabilities of bacteriophage P1.

The desired replacement construct was engineered on a high copy number plasmid, such as the pUC series (Yanisch-Perron *et al.*, 1985). To be able to select for the replacement, an antibiotic resistance marker was be cloned into the chromosomal insert on the plasmid. This replacement vector was introduced into a strain with a different antibiotic resistance marker within P1 transducing range (< 2 minutes) of the gene to be replaced. A P1 lysate was grown on the strain and the lysate used to transduce a wild–type strain. Selection was made for the resistance to the antibiotic encoded by the antibiotic resistance gene present in the cloned chromosomal region on the plasmid and the chromosomally linked antibiotic resistance marker. The transductants were screened for the sensitivity to ampicillin (loss of the *bla* on the plasmid backbone). Ampicillin sensitive clones were examined for the replacement of the wild–type gene with the replacement construct by P1 transduction, PCR and Southern blotting.

If the gene to be replaced was thought or known to be essential a complementing copy of the wild type gene was supplied on a compatible plasmid in both the donor and recipient strains.

It is still unclear by what mechanism P1 can transduce a plasmid borne construct from the donor strain to the chromosome of the recipient. Discussion on a possible mechanism can be found in section 5.1.

2.5.6 Immunofluorescence microscopy

The strain of interest was cultured to the desired stage. The cell fixative consisted of 2 µl of 25% glutaraldehyde mixed with 200 µl 16% glutaraldehyde. To this was added 40 µl 1 M $NaPO_4$ (pH 7.0). 1 ml of the culture was added to the fixative and then incubated at room temperature for 10 minutes. The cells in fixative were then placed on ice for 50 minutes. The cells were centrifuged at 14,000 rpm for 2 minutes and the supernatant discarded. The cells were washed in PBS three times and finally resuspended in 500 µl of GTE.

10 µl of 0.1% poly-L-lysine (Sigma) was added to a well of a multi–well slide (ICN). The poly-L-lysine was removed after three minutes and the well was washed twice with 10 µl dH_2O. 25 µl of lysozyme solution (100 µg ml^{-1})was added to 100 µl of cells. 10 µl of the cells/lysozyme

mixture was immediately added to the well. After two minutes the excess cells/lysozyme mixture was removed and the well rinsed three times with PBS. The well was then allowed to dry in a Petri dish. 10 µl of PBS was added to the well to rehydrate the partially lysed cells. After 3 minutes the PBS was removed. 10 µl of PBS/2% bovine serum albumin (BSA) was added to the well. The slide was incubated at room temperature for 15 minutes. The PBS/2% BSA was removed and replaced with 10 µl of a 1:500 dilution of primary antibody in PBS/2% BSA. The slide was placed in a Petri dish and the lid sealed with a strip of Parafilm. The slide was incubated at 4°C overnight. The primary antibody in PBS/2% BSA was removed from the well and the well washed by dunking the slide into a beaker of PBS six times. All extraneous PBS was removed from the slide with a piece of tissue. 10 µl of a 1:250 dilution of secondary antibody in PBS/2% BSA was added to the well. The slide was incubated at room temperature in the dark for 1 hour. The secodary antibody in PBS/2% BSA was removed and the well washed by dunking the slide in a beaker of PBS six times. Excess PBS was removed from the slide with a tissue. 10 µl of Equilibration buffer (PBS/10% glycerol v/v) from the Slowfade kit (Molecular Probes) was added to the well. After 3 minutes the Equilibration buffer was removed and the well rinsed with another 10 µl of Equilibration buffer. 7 µl of the Slowfade reagent (Molecular Probes) was added to the well and a coverslip placed on top of the well. The coverslip was secured in place by spreading a small amount of super glue along one edge of the coverslip. The slide could be stored at –20°C for up to one week before use.

solutions used

GTE	50 mM glucose 20 mM Tris-HCl (pH 7.5) 10 mM EDTA
PBS/2% BSA	2 g bovine serum albumin (Sigma) / 100 ml PBS (q.v.)

2.5.7 DAPI staining of chromosomes

0.5 ml of the required cells was centrifuged at 14,000 rpm for 1 minute. The cells were resupended in 1ml of PBS, vortexed briefly and pelleted as above. The cells were resuspended in 0.5 ml of PBS. 10 µl of the cells was placed on an ethanol washed microscope slied and allowed to dry at room–temperature. Once dried, the slide was immersed in methanol for five minutes. The slide was washed by dunking into a beaker ot tap water six times. The slide was allowed to dry at room temperature. The fixed cells can be stored in a dust-free environment almost indefinitely. Prior to use, 6 ml of a 2.5 µg ml^{-1} solution of 4,6-diamidino-2-phenylindole (DAPI) was pippetted onto the fixed cells and a coverslip placed on top.

CHAPTER 3
MANIPULATION OF *ftsK*

Chapter 3 Manipulation of *ftsK*

A number of plasmids were constructed during this study. A cmoprehensive list of these plasmids is in tabular form in section 2.1.3. Below, in figure 3.0, is a graphic representation of some of the more commonly used plasmids employed in this study.

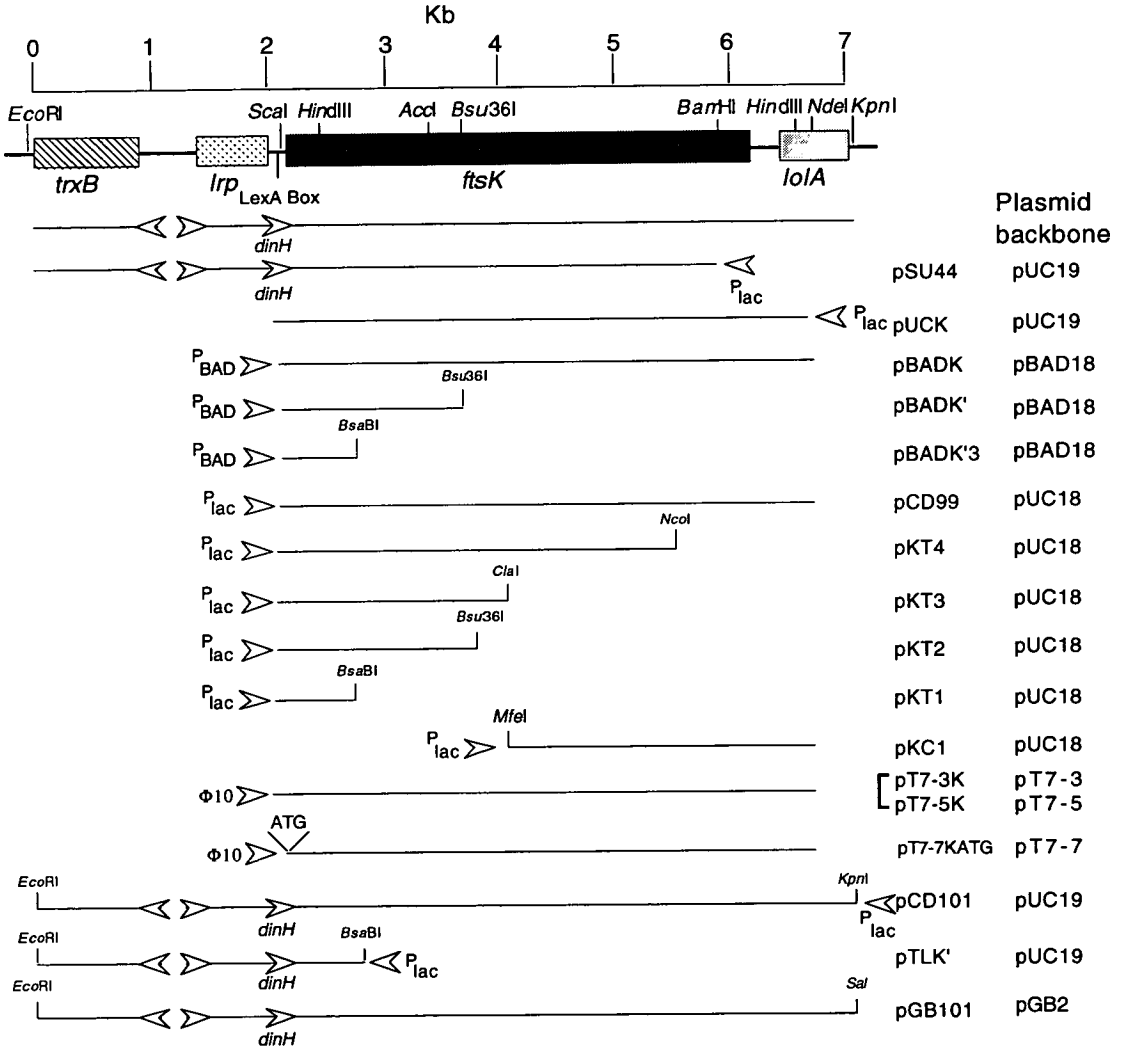

Figure 3.0. A graphic representation of some of the plasmids used in this study. Only the cloned regions are shown. The corresponding region of the *E. coli* chromosome is shown at the top of the diagram

3.1 The 20 minute region

The *ftsK* gene was mapped to the 20 minute region of the *E. coli* chromosome by Begg *et al.* (1995). The region contains the *trxB*, *lrp*, *ftsK* and *lolA* (previously designated *lplA*) genes and is illustrated in Figure 3.1.1 (Begg *et al.*, 1995; Russel and Model, 1985b; Wang *et al.*, 1994, Matsuyama *et al.*, 1995).

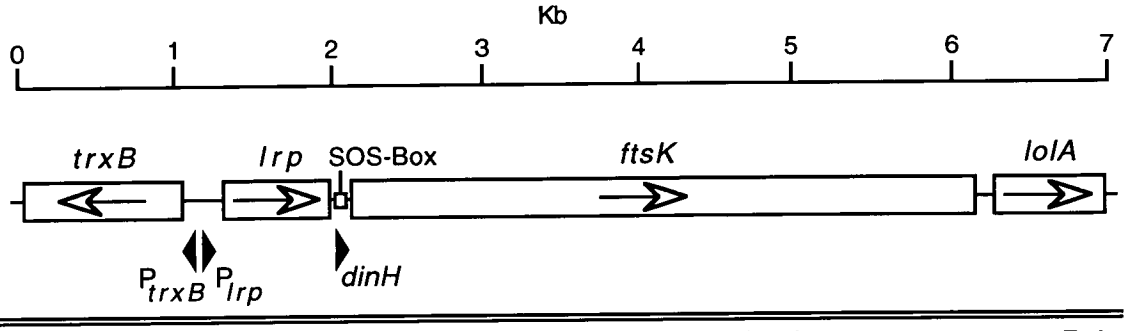

Figure 3.1.1. The 20 minute region of the *E. coli* chromosome. *trxB* is transcribed in the opposite orientation to *lrp*, *ftsK* and *lolA*. *dinH*, the SOS-inducible promoter located by Lewis *et al.* (1992) is indicated.

trxB encodes the thioredoxin reductase protein TrxB (Russel and Model, 1985b; Russel and Model, 1988). Thioredoxin, the product of the *E. coli* chromosomal gene *trxA* (*fip*), is a subunit of bacteriophage T7 DNA polymerase and is essential for filamentous phage assembly (Mark and Richardson, 1976; Lim *et al.*, 1985; Russel and Model, 1983; Russel and Model 1985a). Thioredoxin and thioredoxin reductase are not, however, essential for the viability of *E. coli* (Holmgren *et al.*, 1978; Russel and Model, 1984). *lrp* encodes the leucine responsive regulatory protein Lrp (reviewed in Newman *et al.*, 1996; Tuan *et al.*, 1990). Lrp is a global regulator, a transcriptional activator and/or repressor of a wide range of genes known as the Leucine/Lrp regulon. Despite there being an estimated 35-75 genes regulated by *lrp*, the gene is not essential (Wang *et al.*, 1994). Deletion mutants have a normal morphology but exhibit a variety of metabolic differences to wild type cells (Newman *et al.*, 1996). The *lrp* promoter is autogenously regulated by Lrp and shows growth rate dependent expression (Landgraf *et al.*, 1996; Lin *et al.*, 1992; Wang *et al.*, 1994). *lolA* encodes the 20 kDa (p20) LolA protein involved in the

transport of lipoproteins from the cytoplasmic membrane to the outer membrane via an intermediate step which also involves LolB (Matsuyama *et al.*, 1995; 1997).

3.2 Cloning *ftsK*

pSU44, the partial clone of *ftsK* described by Begg *et al.* (1995), contains a 6.3 kb *Bam*HI fragment of DNA isolated from Kohara λ215 cloned into pUC19 (Kohara *et al.*, 1987; Yanisch-Perron *et al.*, 1985; Figure 3.2.1). This cloned fragment contains a non-coding sequence of 337 bp followed by the 965 bp *trxB* ORF, a non coding sequence of 554 bp, the 455 bp *lrp* ORF, a 134 bp non-coding region containing the SOS-inducible *dinH* promoter (Lewis *et al.*, 1992) and 3794 bp of the 3987 bp *ftsK* ORF. The orientation of P_{lacUV5} promoter on the plasmid backbone is such that it transcribes in the same orientation as *ftsK*.

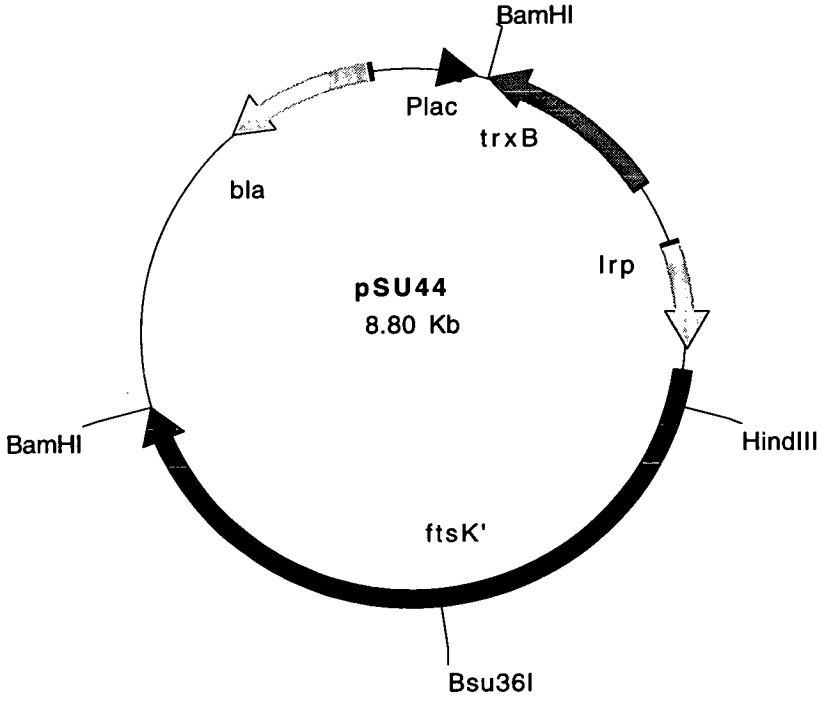

Figure 3.2.1. pSU44, a pUC19 based plasmid that contains *trxB*, *lrp* and 3794 bp of *ftsK*.

pSU44 complements the *ftsK44* mutation at the non-permissive temperature despite encoding only a partial FtsK polypeptide. Begg *et al.*

(1995) describe how this plasmid is not tolerated well in a *pcnB*$^+$ strain (Masters *et al.*, 1993). PcnB is a poly-A polymerase involved in the maintenance of ColE1 plasmid copy number. The replacement of most of *pcnB* with a kanamycin cassette (*pcnB::kan*) reduces the copy number of ColE1 plasmids to about 10% of wild type levels (Masters *et al.*, 1993). The copy number of pUC plasmids have not been determined (M. Masters, pers. comm.). The copy number of pUC19, and hence pSU44, is approximately 500 molecules cell^{-1}, so in a *pcnB::kan* strain the copy number should be reduced to approximately 50 molecules cell^{-1}.

The first step in this study of *ftsK*, continuing from the work described by Begg *et al.* (1995), was to obtain a complete clone of the *ftsK* ORF. DNA was purified from λ214 (see section 2.2.3). The λ214 DNA was digested with *Nco*I and *Nde*I and a 707 bp fragment containing the 3′ end of *ftsK* and the 5′ end of *lolA* was isolated. pSU44 was also digested with *Nco*I and *Nde*I and dephosphorylated. The 707 bp fragment was then ligated into the digested pSU44 and transformed into strain DH5α. After overnight incubation at 37°C no colonies were visible. Several attempts were made to clone the complete *ftsK* ORF in this manner, none of which were successful.

The reason for not isolating any clones of the complete *ftsK* ORF was not known. It was possible that multiple copies of the complete *ftsK* gene was toxic and led to cell death. To test this theory an attempt was made to clone a blunt-ended DNA fragment containing *ftsK* into the high copy number vector pUC19. pUC19 contains the strong *lacUV5* promoter (P_{lacUV5}). If the *ftsK* gene was toxic in high copy, then it should only be possible to clone the gene in the opposite orientation to P_{lacUV5}. There is transcription from P_{lacUV5} even in the absence of the gratuitous inducer, IPTG as there is no *lacI* gene, which encodes the *lac* repressor, in the strain used, DH5α.

The *ftsK* gene was isolated from the purified λ214 DNA by digestion with *Sca*I and *Nde*I. The purified 4.4 kb fragment contained the entire *ftsK* gene, without the *dinH* region upstream of *ftsK* (Figure 3.2.2), and 217 bp of *lolA*.

lrp *lrp* stop
ATTAAGACGCGCTAACACGGAACAGGTGCAAAATCGGCGTATTTTGATTACAC

SOS-Box (LexA Binding) *Sca*I
TC**CTGT**TAATCCAT**ACAG**CAAC***AGTACT***GGGGTAACCTGGTACTGTTGTCCGT

ftsK RBS *ftsK* start
TTTAGCATCGGGCAGGAAAAGCCTGTAACCCCT**GGAG**AGCCTTTC**TTG**AGCCAG

Figure 3.2.2. The *lrp-ftsK* intergenic region. The SOS-box is indicated as is the *Sca*I site used in the construction of pUCK. The proposed TTG initiation codon for *ftsK* is underlined, as is a possible ribosome binding site (RBS) for *ftsK*.

The *Nde*I sticky end was end-filled with Klenow (see section 2.2.9) and the now blunt-ended fragment was ligated into the *Sma*I site of pUC19. The positive clones all had *ftsK* inserted in the opposite orientation to P_{lacUV5}. One of the recombinant plasmids was taken for further study and named pUCK (Figure 3.2.3).

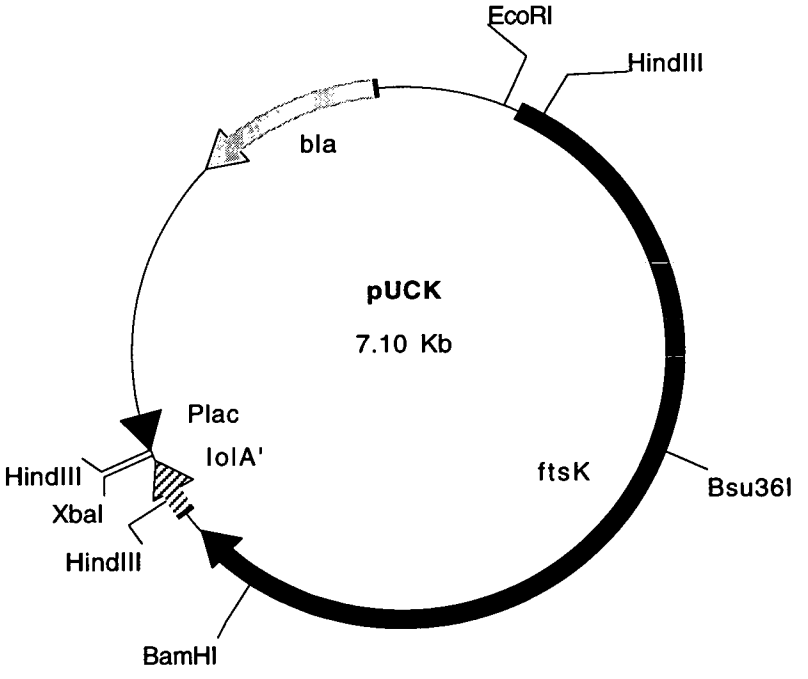

Figure 3.2.3. A 4.4 kb fragment containing *ftsK-lolA'* was cloned into pUC19 to create pUCK. P_{lacUV5} from the vector backbone transcribes in the opposite direction to the *ftsK* ORF.

The lack of any clones with *ftsK* transcribed from P_{lacUV5} agreed with the hypothesis that overproduction of FtsK was lethal to the cell. pUCK was introduced into MGK44. MGK44 was constructed by introducing *aroA*::Tn*10* from MGAT into TOE44 by P1 transduction. Tetracycline resistant transductants were screened for the retention of the *ftsK44* (Ts) allele by testing for temperature sensitivity. A P1 lysate was grown on a tetracycline resistant clone (TOE44 *aroA*::Tn*10*) and this lysate was used to transduce MG1655 to tetracycline resistance. The tetracycline resistant colonies were screened for the co-transduction of the *ftsK44* allele by plating at 30°C and 42°C to test for temperature-sensitivity. The co-transduction frequency of *aroA*::Tn*10* and *ftsK44* was 70%. A tetracycline resistant, temperature-sensitive colony was chosen and named MGK44. The temperature-sensitive mutation in MGK44 was complemented by pSU44, proving that the temperature-sensitive allele introduced into MG1655 was *ftsK44*. pUCK did not complement the *ftsK44* mutation carried by MGK44 at the non-permissive temperature when cultured in liquid or solid media containing no salt, indicating that there was no promoter transcribing *ftsK* or that the promoter(s) present on the cloned region was silenced by the antagonistic transcription of P_{lacUV5} in the opposite orientation.

3.2.1 Construction of pBADK and complementation of *ftsK44*

The next step was to construct a plasmid from which the expression of *ftsK* could be controlled by an inducible promoter. For this, the arabinose-inducible promoter in plasmid pBAD18 was used (Guzman *et al.*, 1995; Figure 3.2.1).

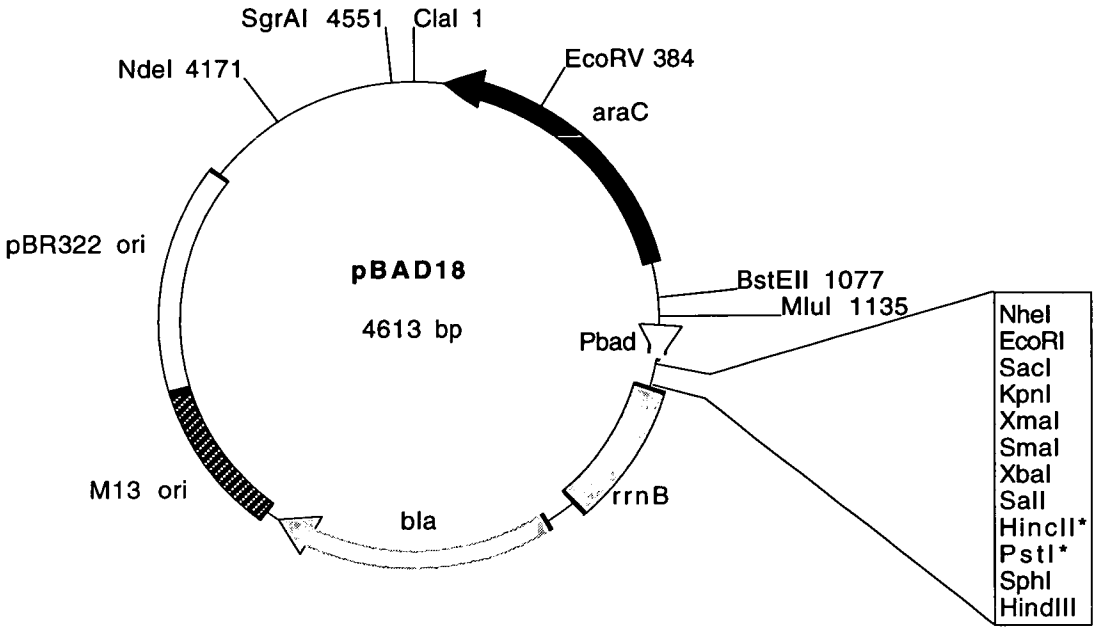

Figure 3.2.1. The arabinose-inducible expression vector pBAD18.

pBAD18 contains the *araC* gene and promoter for the *araBAD* operon, P_{BAD}. *araC* encodes a positive and negative regulator of P_{BAD}. In the presence of arabinose, transcription from P_{BAD} is induced. In the absence of arabinose, transcription from P_{BAD} occurs at low levels. Transcription from P_{BAD} can be further reduced by the addition of glucose (Guzman *et al.*, 1995). It was thought that the tight repression of P_{BAD} by glucose would enable *ftsK* to be cloned without encountering the toxicity problems encountered by having multiple copies of *ftsK* in the cell. The 4.4 kb *Eco*RI-*Xba*I fragment from pUCK containing *ftsK-lolA'* was ligated into [*Eco*RI/*Xba*I]-digested pBAD18. The ligation was transformed into strain DH5α and the transformants plated at 37°C on LB-agar containing 100 μg ml^{-1} ampicillin and 0.2% glucose. Several positive clones were obtained and the plasmid was named pBADK (Figure 3.2.2).

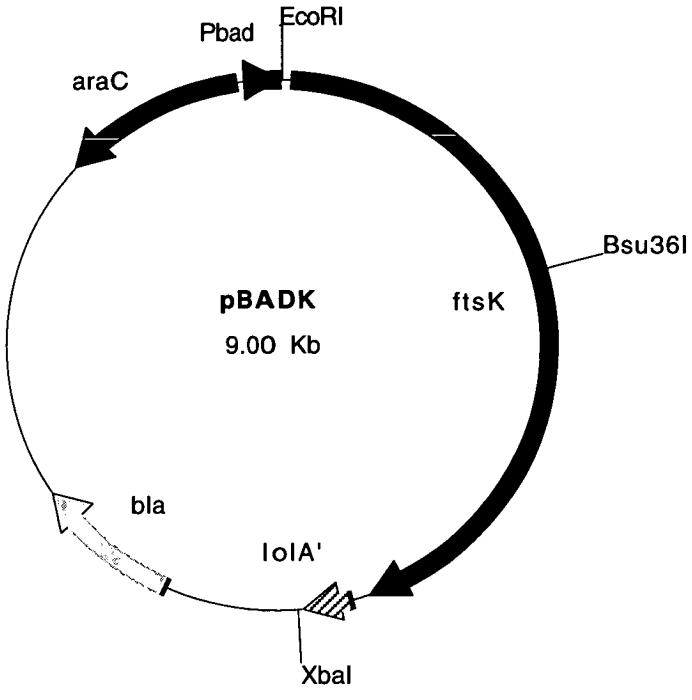

Figure 3.2.2. The arabinose-inducible *ftsK* clone, pBADK.

pBADK was transformed into MGK44 to examine whether the plasmid could complement the *ftsK44* mutation. MGK44 (pBADK) was plated on LBΔNaCl agar in the presence of either 0.2% glucose or 0.2% arabinose at 30°C and 42°C. MGK44 (pBADK) grew at 42°C in the presence of either 0.2% arabinose or 0.2% glucose. A control strain, MGK44 (pBAD18) grew at 30°C but did not form colonies at 42°C on either medium. This indicated either that the very low levels of FtsK produced from pBADK in the presence of glucose was enough to complement *ftsK44* at the non-permissive temperature or that MGK44 (pBADK) had developed a mutation that suppressed *ftsK44*. To resolve this the *pcnB*::*kan* allele from MM38K24 (Masters *et al.*, 1993) was introduced into MGK44 (pBADK) by P1 transduction. The copy number of pBAD18 is estimated to be 40 molecules $cell^{-1}$, therefore the *pcnB*::*kan* mutation would reduce this to approximately 4 molecules $cell^{-1}$. Kanamycin/ampicillin resistant colonies were isolated and the presence of the *pcnB*::*kan* allele was confirmed by plating several potential MGK44 *pcnB*::*kan* (pBADK) isolates on media containing 1000 μg ml^{-1} ampicillin. In a $pcnB^+$ background there is enough β-lactamase produced from the *bla* gene from pBAD18 to confer resistance to this high concentration of

ampicillin. In a *pcnB::kan* background, however, the reduced plasmid copy number means that 1000 μg ml^{-1} ampicillin is lethal because of the significantly lower levels of β-lactamase produced from the plasmids. An isolate that was sensitive to 1000 μg ml^{-1} ampicillin but resistant to 100 μg ml^{-1} ampicillin was selected. MGK44 *pcnB::kan* (pBADK) was plated on LBΔNaCl plates containing 100 μg ml^{-1} ampicillin supplemented with either glucose or arabinose at 30°C and 42°C. Colonies formed on the arabinose plates but not on the plates containing glucose. That pBADK complemented MGK44 *pcnB*$^+$ at the non-permissive temperature even in the presence of glucose was probably due to the leakiness of P_{BAD} and hence low level of expression of multiple copies of *ftsK* is enough to complement *ftsK44*. This suggests that few molecules of FtsK are required for cell division. When the copy number of pBADK is reduced to approximately 10% of wild-type levels there is not enough *ftsK* expression from the repressed clones for complementation, but when induced with arabinose there is sufficient FtsK to complement *ftsK44* at the non-permissive temperature. The wild-type *ftsK* orf contained on pBADK has the potential to recombine with the mutant *ftsK44* allele. This allelic exchange would only occur in a small proportion of the cells, although this would be selected for because the *ftsK44* allele is lethal at the non-pemissive temperature. Arabinose and hence induction of P_{BAD} and expression of FtsK was required for the growth of MGK44 *pcnB::kan* (pBADK) at 42°C, and so it appears that recombination, if any, had little effect on the experiment. This experiment, however, needs to be repeated in a *ftsK44 recA*$^-$ strain to circumvent any problems that may occur with recombination of the suppressing plasmid.

pBADK was introduced into the wild-type strain MG1655 to examine whether overexpression of *ftsK* was toxic to the cells. MG1655 (pBADK) was plated at 30°C, 37°C and 42°C in the presence of 0.2% glucose or 0.02%, 0.2% or 2% arabinose. Growth was observed on all plates. The glucose-containing plates showed the best growth; the arabinose containing plates all showed a slight reduction in colony size but no loss in viability. *E. coli* therefore is sensitive to a slight increase in the level of FtsK.

3.2.2. Construction of pCD99

An attempt was made to subclone the 4.4 kb *Eco*RI-*Xba*I fragment (*ftsK-lolA'*) from pUCK into pUC18. This would place *ftsK* under the control of P_{lacUV5}. No positive clones were obtained when the transformation plates were incubated at 37°C. When, however, the transformation plates were incubated at 30°C for 2 days, tiny colonies appeared that contained the desired clone, pCD99 (Figure 3.2.3).

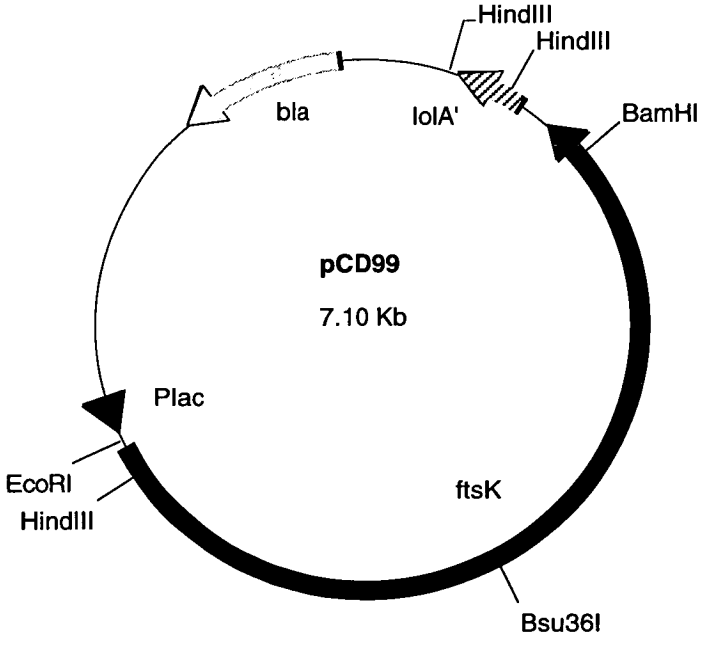

Figure 3.2.3. pCD99, a high copy number vector containing *ftsK* under the control of the *lacUV5* promoter.

pCD99 was transformed into the wild-type strain MG1655. The transformation plates were incubated, as before, at 30°C. MG1655 (pCD99) was plated at 30°C, 37°C and 42°C. No colonies formed at 37°C or 42°C. The level of expression from P_{lacUV5} was enough to overproduce FtsK at toxic levels although growth was seen on the plates incubated at 30°C. In retrospect, this experiment needs to repeated in a strain that overproduces LacI such as a strain carrying the $lacI^q$ allele which has a mutation in the *lacI* promoter which results in increased expression of LacI. The high levels of LacI would be enough to repress transcription from each copy of

P_{lacUV5} present in the cell, enabling the effects of induction of *ftsK* to be observed.

pCD99 was introduced into MGK44 to see if the construct would complement the *ftsK44* mutation. No growth was observed at 42°C. To test whether this was due to the overproduction of FtsK rather than to the lethal effects of the *ftsK44* mutation, pCD99 was transformed into MGK44 *pcnB::kan*. pCD99 complemented *ftsK44* at 42°C to allow growth in this *pcnB::kan* strain. Further attempts were made to characterise the toxic effect of overproduction of FtsK.

3.3 Overproduction of FtsK

The growth rates of MG1655 (pBADK) and MG1655 (pBAD18) in liquid media were then examined. MG1655 (pBADK) and MG1655 (pBAD18) were grown in LB-broth in the presence of 100 μg ml^{-1} ampicillin and glucose at 37°C. After 210 minutes MG1655 (pBADK) and MG1655 (pBAD18) were washed three times with LB-broth and diluted 1:4 into fresh LB-broth containing ampicillin and either glucose or arabinose The growth curve is shown in Figure 3.3.1. Strains containing pBAD18 derivatives were diluted when the OD_{600} reached approximately 0.25 since P_{BAD} is not readily repressed in cultures with an optical density of >0.3 (S. McAteer, pers. comm.).

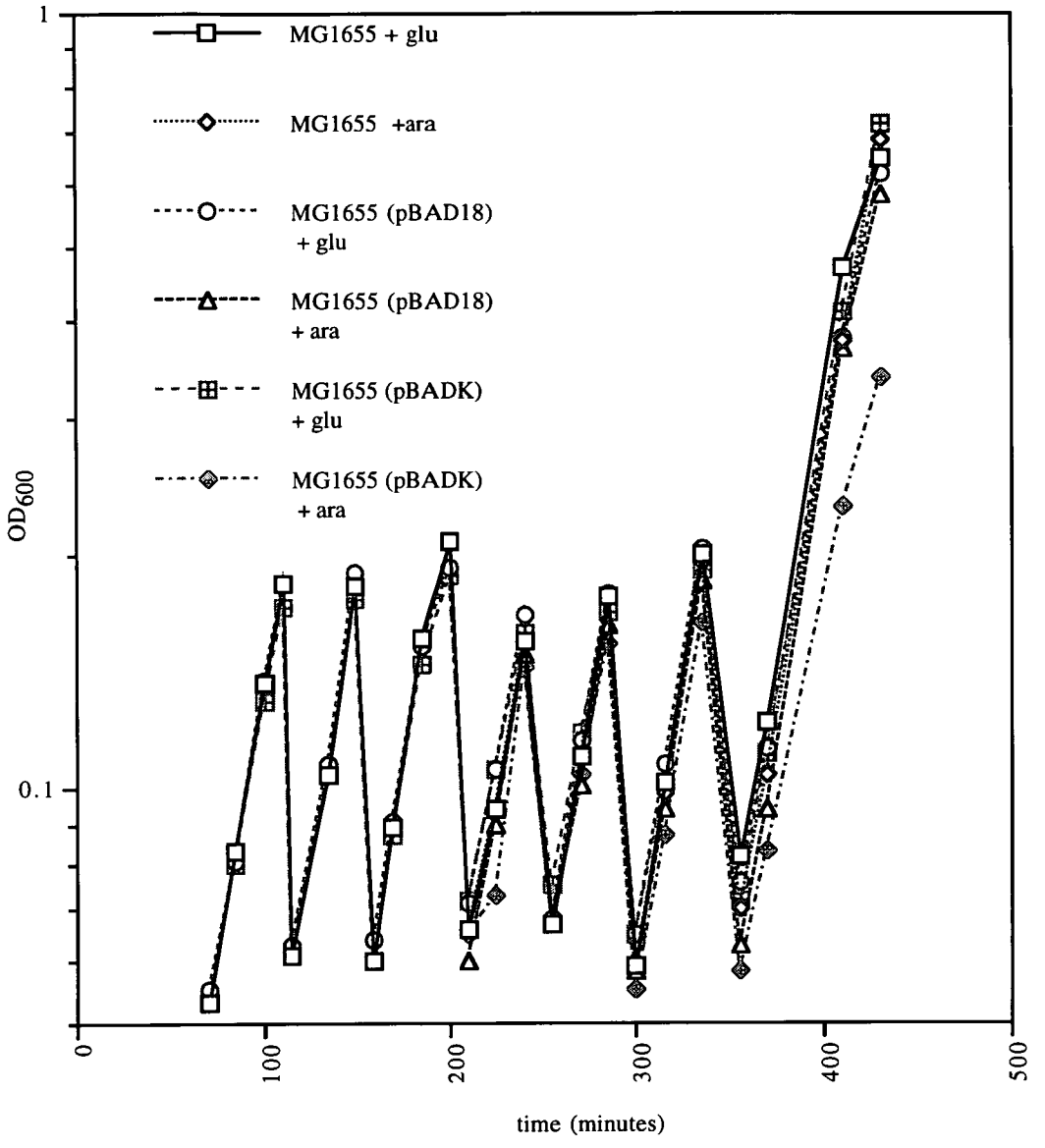

Figure 3.3.1. Growth curves of MG1655 and MG1655 (pBADK)/(pBAD18) grown in the presence of either 0.2% glucose or 0.2% arabinose. Cultures were grown in glucose until t=210, when they were split (after washing) into arabinose or glucose containing media . Three hours after inoculation into LB-broth supplemented with 0.2% arabinose the growth rate of MG1655 (pBADK) was slightly reduced (see below).

Approximately three hours after shifting MG1655 (pBADK) from glucose containing LB-broth into arabinose containing LB-broth the growth rate of the culture was slightly reduced. MG1655 (pBADK) grown in glucose containing LB-broth and MG1655 (pBAD18) glucose/arabinose exhibited similar growth characteristics to the wild-type parent strain,

MG1655. Multiplication of the data presented in Figure 3.3.1 by the dilution factor used, which was 4, results in the growth curve presented in Figure 3.3.2.

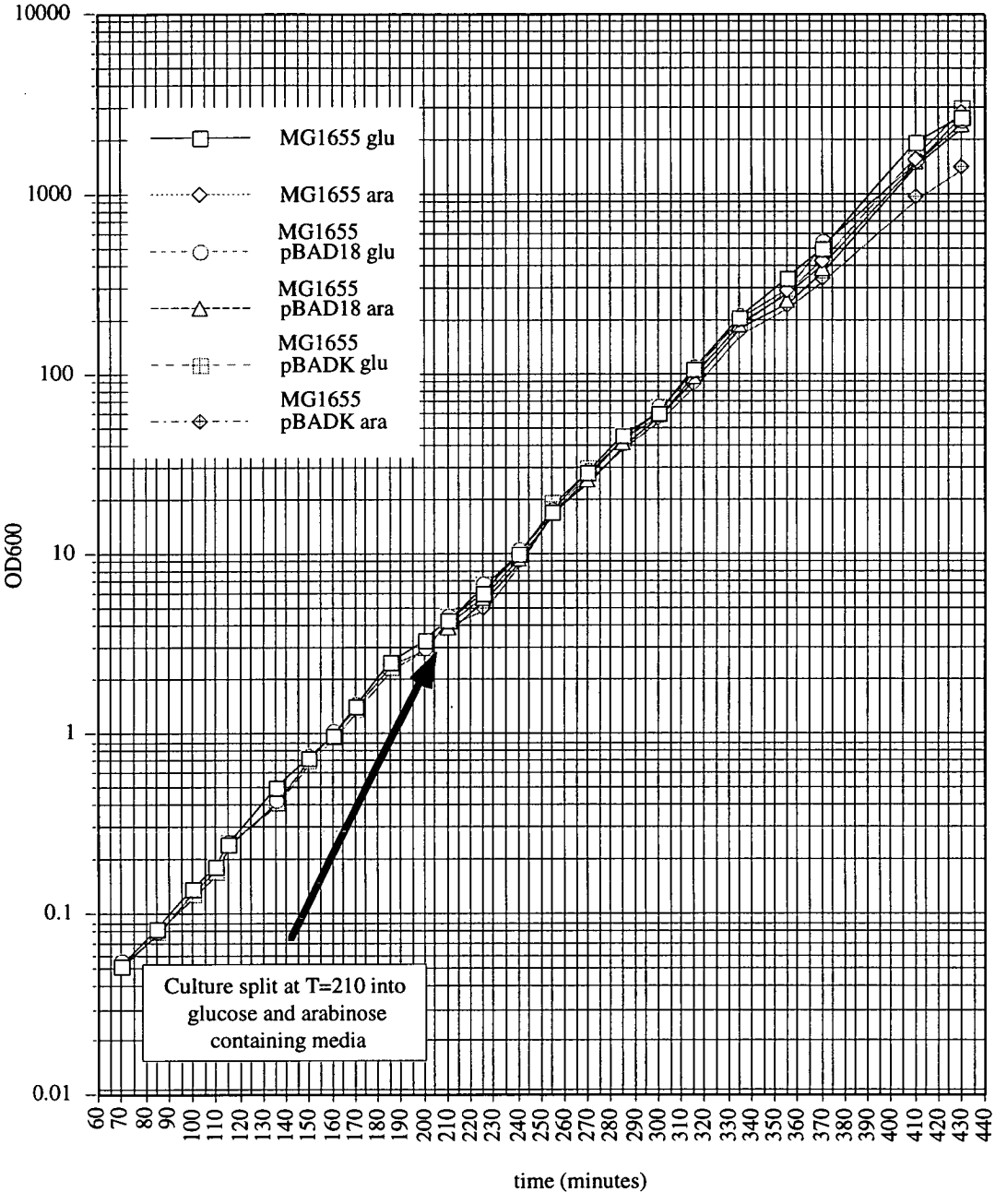

Figure 3.3.2. Growth curve derived from the data presented in Figure 3.3.1. The OD_{600} readings of the cultures were multiplied by the dilution factor, 4.

Table 3.3.1 below contains the data represented in Figure 3.3.2. The indicated strains were grown in glucose containing LB-broth until T=210 then washed three times with LB-broth and subsequently diluted into either LB-broth containing arabinose or glucose as shown.

	OD_{600}					
time (minutes)	MG1655 + glu	MG1655 + ara	MG1655 (pBAD18) + glu	MG1655 (pBAD18) + ara	MG1655 (pBADK) + glu	MG1655 (pBADK) + ara
70	0.053		0.055		0.054	
85	0.083		0.081		0.08	
100	0.137		0.138		0.13	
110	0.184		0.184		0.172	
115	0.244		0.252		0.248	
135	0.51		0.432		0.416	
150	0.732		0.76		0.704	
160	0.96		1.024		0.96	
170	1.424		1.456		1.392	
185	2.496		2.496		2.32	
200	3.328		3.008		3.008	
210	4.224	4.22	4.544	3.84	4.608	4.16
225	6.016	6	6.784	5.76	6.784	5.05
240	9.92	9.92	10.7	9.5	10.176	9.15
255	17.15	17.1	17.40	17.40	19.2	17.40
270	28.16	28.16	29.44	25.85	30.20	26.62
285	45.31	45.31	45.82	41.72	43.26	39.42
300	60.4	60.41	66.56	59.39	66.56	56.32
315	104.4	101.37	110.59	96.25	104.44	89.088
335	205.8	200.7	208.89	189.4	195.58	167.936
355	335.8	286.72	311.29	258.04	294.91	237.6
370	499.7	425.98	544.76	385.02	454.65	339.9
410	1921	1544.19	1556.48	1507.32	1679.36	946.2
430	2650	2813	2547.71	2387.96	2936.83	1388.5

Table 3.3.1. The data from Figure 3.3.1 multiplied by the dilution factor, 4.

The data shown in Table 3.3.1 enables the doubling time (g) of the indicated strains to be calculated using the equation

$$g = \frac{\ln 2\, t}{\ln M - \ln M_i}$$

where t is time, , M_i is the initial concentration of cells in this case represented by OD_{600} and M is final concentration of cells.
For MG1655 grown in the presence of glucose the calculated growth rate was 23 minutes, taking the data from t=70 and i=430. For MG1655

(pBADK) grown in the presence of glucose the doubling time was calculated to be 22.8 minutes, again using the data from *t*=70 and *t*=430. Between time points t=210 and t=430 the growth rate for MG1655 (pBADK) grown in the presence of arabinose the doubling time was 26.2 minutes, however, if the data is separated into two phases, from *t*=210 to *t*=335 and *t*=335 to *t*=430 an indication of the toxic effects of the overproduction can be discerned. The doubling time of MG1655 (pBADK) grown in the presence of arabinose between *t*=210 to *t*=335 was 21.7 minutes and between *t*=335 to *t*=430 was 31.2 minutes. The corresponding growth rates for MG1655 (pBADK) grown in the presence of glucose were 21.4 and 23.2 minutes respectively. Thus, overproduction of FtsK results in a increase in doubling time.

Phase contrast microscopy of MG1655 (pBADK) grown in the presence of arabinose showed that overexpression of *ftsK* resulted in filamentation, although a small number of normally sized cells were apparent (Figure 3.3.3). These small cells could result from rare division events during the filamentation process or might have lost pBADK. These filaments showed no evidence of septation. There were a number of normally sized cells present after three hours incubation in arabinose containing media. Lysis of these filaments could result in the increase in the doubling time of MG1655 (pBADK) noted above after 250 minutes of growth in arabinose containing LB-broth. After >5 hours of such incubation the culture consisted entirely of filaments. MG1655 (pBADK) grown in glucose containing media displayed a normal morphology (Figure 3.3.3). It was decided to examine whether the filamentation was a direct or indirect consequence of overexpression of *ftsK*.

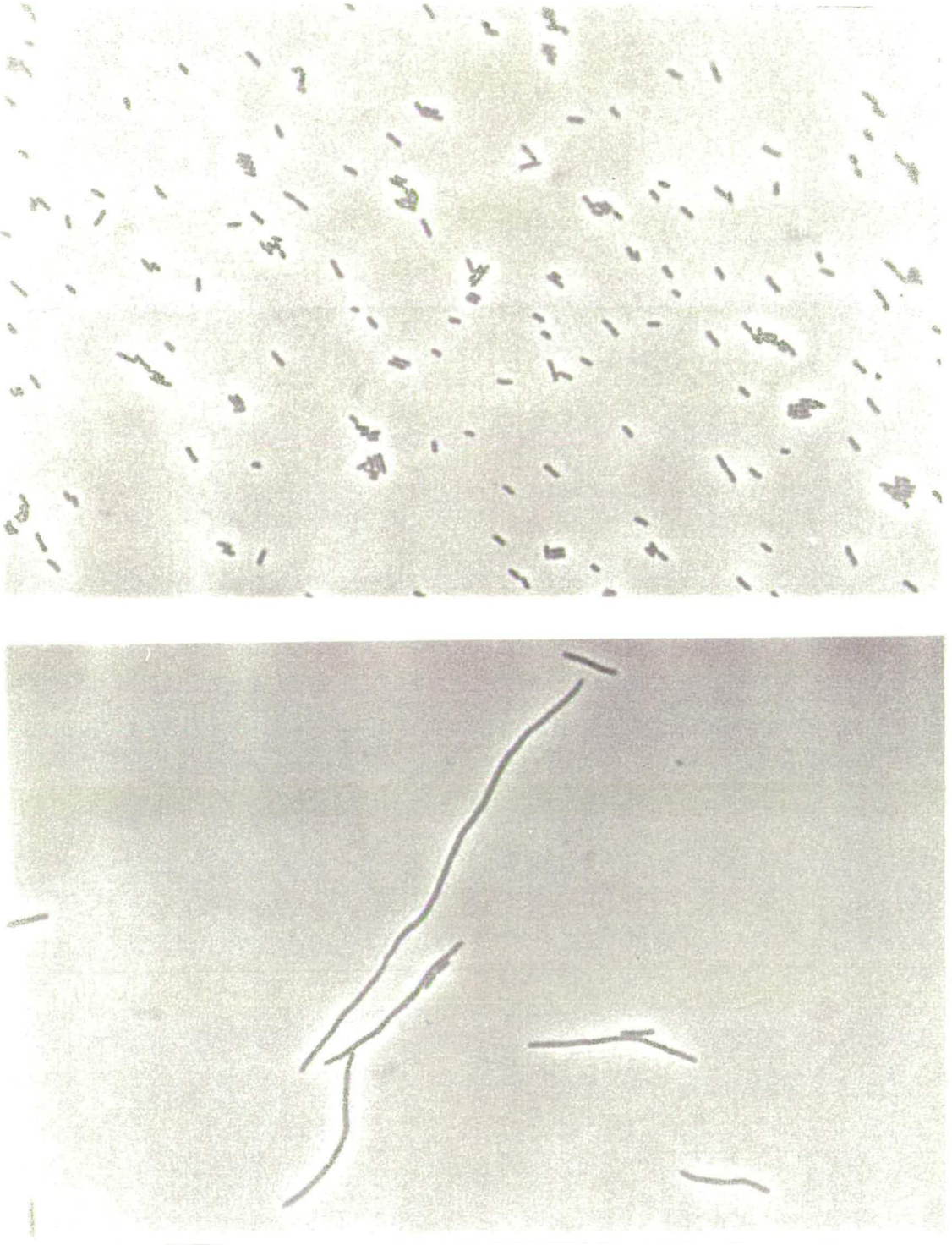

Figure 3.3.3. Phase contrast micrographs of MG1655 (pBADK) cultured in LB 0.2% glucose (top) and LB 0.2% arabinose (bottom) for three hours.

More data could be gleaned from the overproduction studies. A viability count should be performed after increasing times of induction. This would provide information as to whether the filaments observed could recover by dividing. This data could be coupled with Coulter-Counter data that would provide cell count and length data that could provide information as to whether there is a maximum length after which filaments could not recover.

It was decided to test whether the filamentation caused by the overproduction of FtsK could be suppressed by the simultaneous overproduction of FtsQ, FtsA and FtsZ. MG1655 (pBADK) was transformed with pBS58 (which is compatible with pBADK). pBS58 contains the *ftsQ*, *ftsA* and *ftsZ* region cloned into the pSC101-based, low-copy-number vector pGB2 (Bi and Lutkenhaus, 1990a; Churchward *et al.*, 1984). MG1655 (pBADK) (pBS58) was grown in LB-broth containing ampicillin and spectinomycin supplemented with either glucose or arabinose. MG1655 (pBADK) (pBS58) grown in the presence of glucose did not show any sign of filamentation whereas the same strain grown in the presence of arabinose filamented to the same extent as MG1655 (pBADK) cultured in arabinose-containing media. Wild-type strains bearing pBS58 produce three-four times the amount of FtsZ normally present in the cell. This level of overproduction of FtsZ causes the formation of minicells in wild-type strains (Bi and Lutkenhaus, 1990). It is interesting to note that MG1655 (pBADK) (pBS58) cultured in glucose containing media did not form minicells, wheras a control strain, MG1655 (pBAD18) (pBS58) did. The lack of minicell production could be due to the slightly elevated amounts of FtsK produced from pBADK, which even though insufficient to cause filamentation, has an effect on division and prevent the extra divisions caused by the overproduction of FtsZ (Ward and Lutkenhaus, 1985). The control strain, MG1655 (pBAD18) (pBS58) formed minicells and concomitant elongated and normally sized cells. It would be worthwhile to repeat this experiment in a system that overproduces FtsZ to a higher levels than is provided by pBS58.

3.3.1 FtsK overproduction does not induce the SOS-response

Interference with DNA replication induces the SOS-response, a component of which, SulA (SfiA), blocks cell division by preventing the

formation of the FtsZ ring (Bi and Lutkenhaus, 1993; Huisman and D'Ari, 1981). To investigate whether the SOS-response was being induced by the overproduction of FtsK, the activity of the *sulA* (*sfiA*) promoter (P_{sulA})was followed during *ftsK* overexpression. P_{sulA} is normally repressed by the binding of the LexA repressor at a site overlapping the *sulA* promoter (Schnarr *et al.*, 1991). During the induction of the SOS regulon, however, P_{sulA} is derepressed due to the (activated) RecA induced autocatalytic cleavage of LexA. λ p(*sfiA*::*lac*) contains the *sulA* promoter cloned upstream of *lacZ* (Huisman and D'Ari, 1983). Upon induction of the SOS-response the increase in transcription from P_{sulA} can be followed by assaying β-galactosidase activity in a strain that carries λ p(*sfiA*::*lac*) as a lysogen (Miller, 1972).

The activation of the SOS-regulon normally results in the cleavage of the λ *c*I repressor (Roberts and Roberts, 1975; Roberts *et al.*, 1978)). *c*I represses transcription of 'phage genes during lysogeny. When *c*I is cleaved, the phage genes are transcribed, the λ lysogen excises from the chromosome and enters the lytic cycle resulting in cell lysis (Sauer *et al.*, 1982). To prevent lysis of the experimental strain, λ p(*sfiA*::*lac*) encodes a *c*I repressor that is resistant to SOS-mediated cleavage (*c*I *ind*).

λ p(*sfiA*::*lac*) was introduced and lysogenised into TP8503 as described in section 2.4.3. Lysogens were detected by screening for colonies that had a pale blue colour on LB X-Gal plates. TP8503 has the chromosomal region from *lac-proB* deleted and so forms white colonies on LB X-gal plates. The low level of P_{sfiA} expression from λ p(*sfiA*::*lac*) lysogens produces a small amount of LacZ that can function as an aid in screening for lysogens. Some colonies had a stronger blue colour than others; these were assumed to be multiple lysogens and discarded. A pale blue colony was chosen and shown to be immune to superinfection by λ p(*sfiA*::*lac*) but not by λ*vir* indicating that the strain, TPλ, carried λ p(*sfiA*::*lac*) as a lysogen. pBADK, pBAD18, pCD99 and pUC19 were transformed into TPλ. The strains were grown in LB-broth (supplemented with ampicillin where appropriate) at 30°C until mid-log phase was reached (OD_{600}=0.2). TPλ (pBADK)/(pBAD18) were grown in the presence of 0.2% glucose to prevent expression of *ftsK* and create identical conditions for the contol plasmid (pBAD18). The cells were cultured at 30°C to allow growth of TPλ (pCD99) which shows severely impaired growth at 37°C due to the overproduction of FtsK.

TPλ was then subcultured into LB-broth and LB-broth containing nalidixic acid (150 μg ml^{-1}) . Nalidixic acid inhibits DNA gyrase which in turn induces the SOS-response (Gellert *et al.*, 1977; Sugino *et al.*, 1977). The TPλ ± nalidixic acid cultures would act as positive and negative controls respectively. TPλ (pBADK)/(pBAD18) were subcultured into LB-broth containing ampicillin supplemented with either 0.2% arabinose or 0.2% glucose. TPλ (pCD99)/(pUC19) were subcultured into LB-broth containing ampicillin. All cultures were incubated at 37°C. The β-galactosidase activity was measured at 10 minute intervals. The β-galactosidase activity of the cultures is represented in Figure 3.3.4.

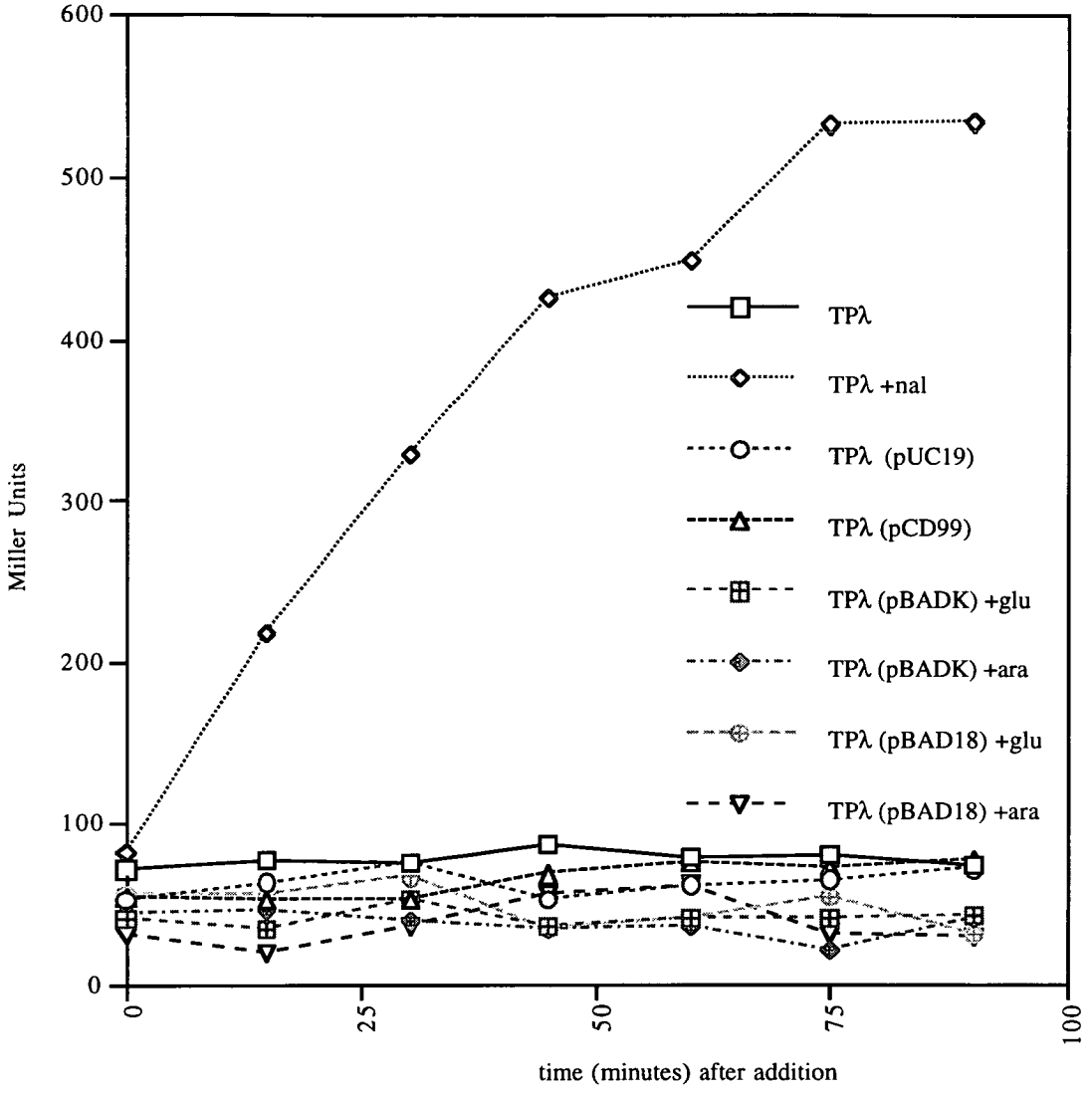

Figure 3.3.4. β-galactosidase activity of TPλ during overproduction of FtsK. Strains were cultured in LB (TPλ), LB +Amp (TPλ (pUC19)/(pCD99)), LB +Amp +0.2% glucose (TPλ (pBAD18)/(pBADK)) at 30°C until OD_{600}=0.2, after which the cultures were split and incubated at 37°C. Nalidixic acid (nal) was added to one of the TPλ cultures to a final conc. of 150 μg ml^{-1}. This control resulted in a high level of SOS induction (represented as β-galactosidase activity). The addition of nal/arabinose/glucose is indicated as time 0. The β-galactosidase activity of TPλ –nal remained at the basal level. Arabinose was added to a washed portion of the TPλ (pBAD18)/(pBADK) cultures, the remaining cultures were grown, as before, in glucose containing media Strains TPλ (pBAD18) arabinose/glucose and TPλ (pUC19) showed no induction of SOS. Overproduction of FtsK from pCD99 or from pBADK in arabinose containing media did not induce SOS.

The data represented in Figure 3.3.4 demonstrates that overproduction of FtsK above the normal cellular levels does not cause the induction of the SOS-response. The level of induction of SOS remains constant irrespective of whether *ftsK* is slightly overexpressed (from pBADK-containing cells grown in the presence of glucose) or greatly overexpressed (pBADK in the presence of arabinose or pCD99). These levels are similar to the levels seen from TPλ when SOS is not induced. The induction of SOS by nalidixic acid occurs rapidly. β-galactosidase activity increases almost immediately after the addition of nalidixic acid to the TPλ culture at time 0. To ensure that cell division had been inhibited during the experiment the cultures were examined microscopically.

TPλ –nalidixic acid, (pBAD18) +arabinose/glucose, (pUC19) and (pBADK) +glucose cells all had a normal morphology. TPλ grown in the presence of nalidixic acid had formed filaments averaging four cell lengths. TPλ (pBADK) grown in arabinose containing LB-broth were filamentous, also averaging four cell lengths. TPλ (pCD99) was extremely filamentous, with some lysis apparent. The filamentation observed by overexpression of *ftsK* was therefore deemed to be due to overproduction of FtsK and not due to SOS-induction.

3.3.2 FtsK overproduction-induced filamentation does not require components of the SOS-response

To confirm this observation *ftsK* was overexpressed in *sulA*, *sfiC* and *recA* mutant backgrounds. The *sulA*::Tn*5* allele was introduced from GC2481 to MG1655 by P1 transduction to form MGS5. pBADK was introduced into MGS5 by transformation. MGS5 (pBADK) was grown in LB-broth containing 100 μg ml^{-1} ampicillin supplemented with glucose. 2 ml of the culture was washed three times in LB-broth then inoculated into LB-broth containing ampicillin supplemented with arabinose. After three hours MGS5 (pBADK) grown in arabinose containing media formed long filaments with no evidence of septation. MGS5 (pBADK) cells grown in L-broth glucose had a normal morphology.

It was possible that MGS5 contained the prophage relic e14 that encodes another SOS-inducible cell division inhibitor, SfiC (D'Ari and Huisman, 1983; Greener and Hill, 1980; Maguin *et al.*, 1986; Maguin *et al.*,

1986). To show that the filamentation caused by overexpression of *ftsK* was independent of both *sfiA* and *sfiC* a *sulA*$^-$ *sfiC*$^-$ strain was constructed. The P1 lysate grown on GC2481 was used to transduce C600 (which is e14$^-$) to kanamycin resistance, creating C6SA5. C6SA5 is both *sulA*$^-$ and *sfiC*$^-$. pBADK was transformed into C6SA5. The transfer of C6SA5 (pBADK) from glucose containing LB-broth into LB-broth supplemented with arabinose resulted in the formation of smooth-sided filaments after three hours of incubation at 37°C.

The filamentation caused by overexpression of *ftsK* was therefore independent of both *sulA* and *sfiC*. It could not be stated at this stage that the filamentation was independent of any component of the SOS-response. This is because the SOS-response is not yet fully characterised. A *sfi*-independent mechanism of division inhibition that is both *lexA* and DNA damage-dependent also exists (Burton and Holland, 1983; Hill *et al.*, 1997). The factors which prevent cell division during *sfi*-independent filamentation are yet to be discovered an could, in theory, be involved in FtsK-induced filamentation.

Experiments with transcriptional fusions have led to the discovery of a number of promoters on the *E. coli* chromosome which are induced in response to interference with DNA replication (Brotcorne-Lannoye and Maenhaut-Michel, 1986; Kenyon and Walker, 1980; Lewis *et al.*, 1994, Lewis *et al.*, 1992; Lundegaard and Jensen, 1994). Not all of these promoters were found to be repressed by LexA (Lewis *et al.*, 1992; Petit *et al.*, 1993). Some of the genes regulated by these promoters have been sequenced, although their actual function, if any, during SOS is unknown (Blattner *et al.*, 1993; Lundegaard and Jensen, 1994). It is possible that there are other uncharacterised SOS-inducible promoters still to be located and the products of the genes controlled by these promoters might have a bearing on the filamentation observed when FtsK is overproduced.

Interestingly, the *dinH* promoter identified by Lewis *et al.* (1992) is located in the 134 bp non-coding region between *lrp* and *ftsK*. Transcription from *dinH* is such that it could cause the expression of *ftsK* (Begg *et al.*, 1995). The possible involvement of *dinH* in *ftsK* expression will be discussed at the end of this chapter.

It was decided to test whether *ftsK* overexpression could cause filamentation in a *recA*$^-$ background. RecA is the protein that stimulates

the induction of the SOS-response and a strain lacking functional RecA should not be able to elicit the SOS-response (Defais *et al.*, 1971) and so the involvement of any genes, as yet not identified as components of the SOS-response, could also be ruled out as factors involved in FtsK overproduction-induced filamentation. A P1 lysate was grown on JC10-240, which contains the *recA56* mutant allele closely linked to *srlC300*::Tn*10*. This lysate was used to transduce MG1655. Transductants were selected for by plating on tetracycline-containing LB agar plates. A number of tetracycline resistant colonies were examined for sensitivity to ultraviolet light to test for the co-transduction of *recA56* as described in section 2.5.4. A tetracycline resistant, UV sensitive isolate was chosen and named MG*recA*. pBADK was transformed into MG*recA*. MG*recA* (pBADK) was cultured in LB-broth containing glucose. 2 ml of the culture was then washed three times in LB-broth and then used to inoculate 9 ml LB-broth containing arabinose. After 3 hours MG*recA* (pBADK) grown in arabinose formed long filaments whereas the cells cultured in glucose had a normal morphology. The filamentation caused by the overexpression of *ftsK* is independent of RecA and hence other components of the SOS-response. Supplementary experiments using *lexA* mutants would also prove informative. The *lexA*(Ind$^-$) mutation results in the expression of a noncleaveable LexA protein that still functions to repress the SOS regulon. Thus, the SOS regulon is not induced even when RecA becomes activated. If FtsK could be overexpressed in a strain bearing the *lexA*(Ind$^-$) mutation, it could be said with even greater certainty that FtsK overproduction can cause a block to cell division in the absence of SOS induction.

3.3.3 Filaments caused by FtsK overproduction do not contain FtsZ rings

The filamentation observed when FtsK was overproduced was independent of any characterised factors involved in SOS-induced filamentation. The cause of the filamentation was unknown, other than it being a consequence of FtsK overproduction. The application of immunofluorescence microscopy (IFM) has shown the presence of many structures associated with cell division in *E. coli* cells (Addinall *et al.*, 1996; Addinall *et al.*, 1997a; Addinall and Lutkenhaus, 1996b; Hale and de Boer, 1997; Pogliano *et al.*, 1997). SulA inhibits the formation of the FtsZ ring,

as does MinCD (Bi and Lutkenhaus, 1993). It was decided to test whether FtsZ rings were present in the filaments formed during the overexpression of *ftsK* and in the filaments formed by growing MGK44 at the non-permissive temperature. MG1655 (pBADK) was cultured at 37°C in L-broth containing 100 μg ml^{-1} ampicillin supplemented with either glucose or arabinose. MGK44 was grown in LBΔNaCl at 30°C and after one doubling of the OD_{600}, the culture was split, with one half incubated at 30°C and the other half at 42°C. The cultures were incubated for a further three hours. After this time, the morphology of the cells were examined. MGK44 grown at 30°C had a normal morphology whereas the cells from the culture incubated at 42°C were filamentous. The cultures were mixed and this mixture of rods and filaments was processed for IFM as described in section 2.5.6. This would allow direct comparison of both cell types on one image. MG1655 was grown in LB-broth as a control. The cultures were incubated for three hours and diluted 1:5 with the appropriate fresh media whenever the OD_{600} reached 0.25. Cells were sampled and fixed and processed for IFM. The primary antibody used was F168-12, a monoclonal anti-FtsZ antibody (described in Voskuil *et al.*, 1994; a kind gift from J. Voskuil and N. Nanninga). The secondary antibody was Cy3-conjugated anti-mouse IgG (Jackson Research). Exponentially growing MG1655 showed a typical pattern of FtsZ ring localization, with most cells possessing one FtsZ ring (Addinall *et al.*, 1996; Figure 3.11). As shown in Figure 3.3.5, MGK44 grown at 30°C also showed normal FtsZ ring formation. MGK44 grown at 42°C formed filaments that had a high level of background fluorescence. FtsZ rings formed in these filaments but there did not appear to be one FtsZ ring per cell length as would be expected if FtsZ ring formation and placement was unaffected (Figure 3.3.5). This phenomenon has been noted by a number of groups and has been proposed to be a result of the fixing procedure or of a reduced pool of FtsZ molecules within filaments or due to reduced FtsZ ring stability in *fts* mutants (Boyle *et al.*, 1997; Pogliano *et al.*, 1997).

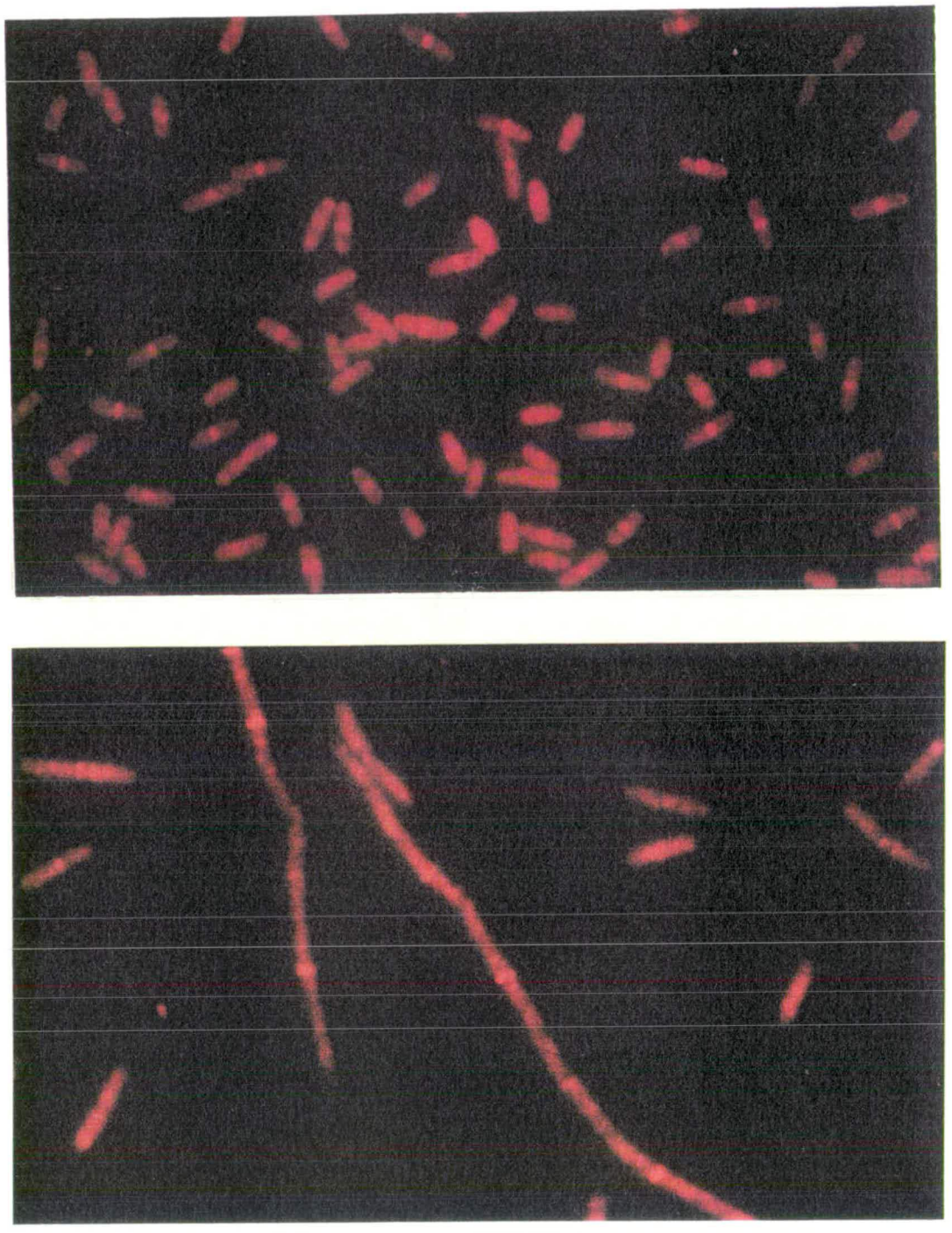

Figure 3.3.5. FtsZ ring formation in MG1655 (top) and MGK44 cultured in LBΔNaCl at 30°C and 42°C (bottom) after three hours growth. The normal sized cells in the bottom image are MGK44 incubated at 30°C and the filaments are from the 42°C culture. The strains all show evidence of FtsZ ring formation.

The filamentation observed at the non-permissive temperature by strains bearing the *ftsK44* (Ts) allele was not due to lack of FtsZ ring formation and was most likely due to the inactivation of the mutant protein. MG1655 (pBADK) cultured in LB-broth glucose had a normal morphology and showed the typical pattern of FtsZ ring formation, similar to that observed in wild-type strains, with FtsZ rings present at both the mid-cell (Figure 3.3.5). MG1655 (pBADK) grown in the presence of arabinose had a filamentous morphology. Interestingly, none of the filaments formed by FtsK overproduction showed evidence of FtsZ ring formation (Figure 3.3.6). The small cells that are present in the culture (see Section 3.3 and Figure 3.3.3) contain FtsZ rings and could have lost pBADK.

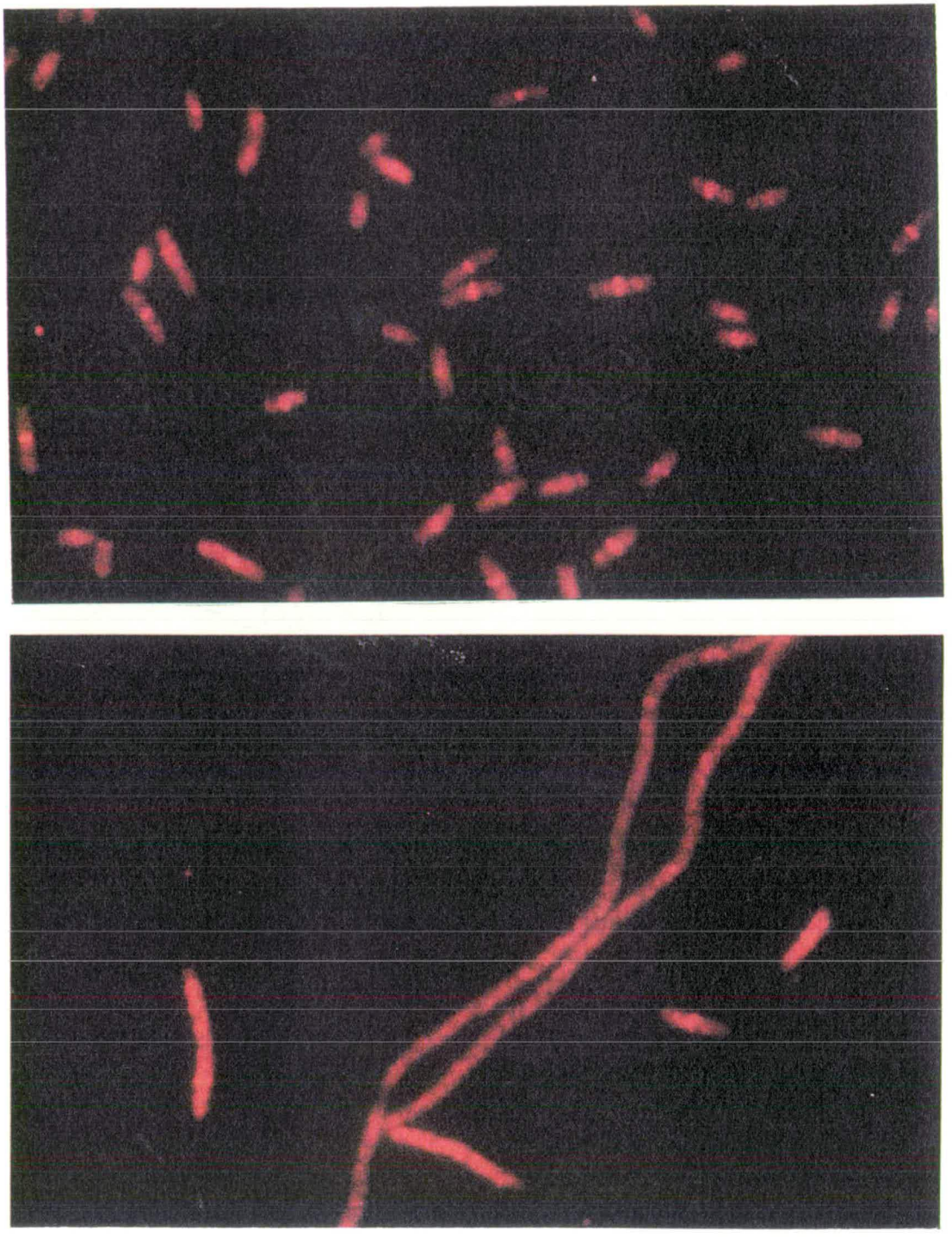

Figure 3.3.6. Overproduction of FtsK results in filaments with no evidence of FtsZ ring formation. Immunofluorescence micrographs of MG1655 (pBADK) cultured in LB-broth containing 100 μg ml^{-1} ampicillin supplemented with glucose (top) or arabinose (bottom) for three hours and then fixed and processed for IFM as described in section 2.5.6.

This experiment was repeated on a number of separate occasions to verify the result. In all cases, the filaments formed by FtsK overproduction did not have FtsZ rings. There are a number of cells in Figure 3.3.6 that are of apparent normal size. This could be because these cells have formed due to a rare division event or that the level of FtsK in the cell is not enough for filamentation to occur. It is worthy of note that these cells show evidence of FtsZ ring formation. In Figure 3.3.3 the bottom panel show the effect of FtsK overproduction. This culture also had a small proportion of small, normally sized cells. Prolonged incubation (> 5 hours) of MG1655 (pBADK) in 0.2% arabinose containing media resulted in a culture consisting entirely of filamentous cells.

It is of some concern that the full complement of FtsZ rings is not visible even in filaments where genetic evidence suggests that FtsZ ring formation should not be affected (Begg *et al.*, 1985). This could be a result of the lysozyme treatment used during the processing of the cells for IFM. The lysozyme step is necessary to partially digest the peptidoglycan layer to enable the antibodies to pass into the cytoplasm. Without lysozyme treatment the antibodies cannot pass through the peptidoglycan layer and into the cytoplasm (SG. Addinall, pers. comm.). Overdigestion with lysozyme leads to cell lysis which may result in the disassembly of the FtsZ rings. Thus, with this relatively new technique, care should be taken not to overemphasise the number of structures seen. Rather, the technique should be seen as qualitative rather than quantitative until the method is further refined.

3.4 Characterization of a partial *ftsK* clone

The work by Begg *et al.* (1995) showed that a partial clone containing the 5′ 1165 bp of *ftsK* was sufficient for complementation of the *ftsK44* (Ts) mutation. It was decided to create a new partial clone of *ftsK* under the control of an inducible promoter. This would allow the study of the effects of overexpression of a truncated FtsK polypeptide and enable the re-examination of the complementation of the *ftsK44* (Ts) mutant by the truncated peptide. A new plasmid, pBADK′ (Figure 3.4.1), was created by digesting pBADK with *Bsu*36I and *Xba*I. The 6142 bp fragment

containing the 5′ 1746 bp of *ftsK* under the transcriptional control of the P_{BAD} promoter was end-filled with Klenow and religated.

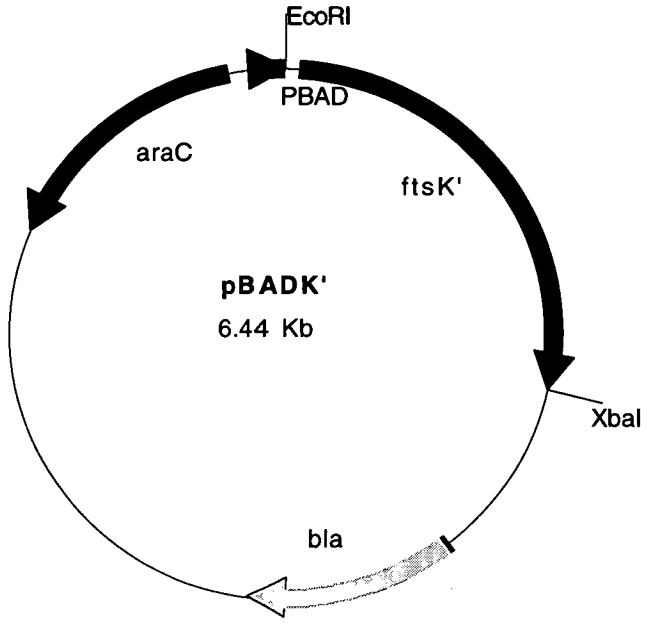

Figure 3.4.1. pBADK′ which encodes 582 aa of the N-terminus of FtsK.

pBADK′ was transformed into MGK44. MGK44 (pBADK′) was plated onto LBΔNaCl plates containing 100 μg ml^{-1} ampicillin supplemented with either arabinose or glucose at 30°C and 42°C. Colonies formed at all temperatures on all media types. The growth seen on glucose containing plates was presumed to be due to the low levels of expression from P_{BAD} allowing the production of small, though sufficient amounts, of the truncated FtsK polypeptide ($FtsK'_{583}$) for complementation. pBADK′ was introduced into MGK44 *pcnB::kan*. MGK44 *pcnB::kan* (pBADK′) was plated onto LBΔNaCl plates containing 100 μg ml^{-1} ampicillin supplemented with either arabinose or glucose at 30°C and 42°C. Growth was observed on the glucose and arabinose containing plates incubated at 30°C and on the arabinose containing plate incubated at 42°C. No colonies formed on the glucose-containing plate that was incubated at 42°C. It was concluded that $FtsK'_{583}$ complemented *ftsK44*. The effects of overproduction of this truncated FtsK protein was investigated.

3.4.1 Overproduction of FtsK′$_{583}$

pBADK′ was transformed into MG1655. MG1655 (pBADK′) was plated onto LB agar plates containing 100 μg ml^{-1} ampicillin in the presence of arabinose or glucose. The plates were incubated at 37°C overnight. Colonies formed on both plates. The morphology of the cells was examined by phase contrast microscopy. The cells grown on glucose containing plates has a normal morphology. The cells from the arabinose containing plate elicited a mixed phenotype. 75% of the cells (543 from 612 examined) had a normal morphology, the remainder formed chains of cells, typically consisting of four cells. MG1655 (pBADK′) was cultured in LB-broth containing 100 μg ml^{-1} ampicillin and either glucose or arabinose. After three hours chains of cells were apparent in the arabinose-containing culture (Figure 3.4.2). This phenotype differs greatly from the overproduction of the wild-type protein, which results in filamentation.

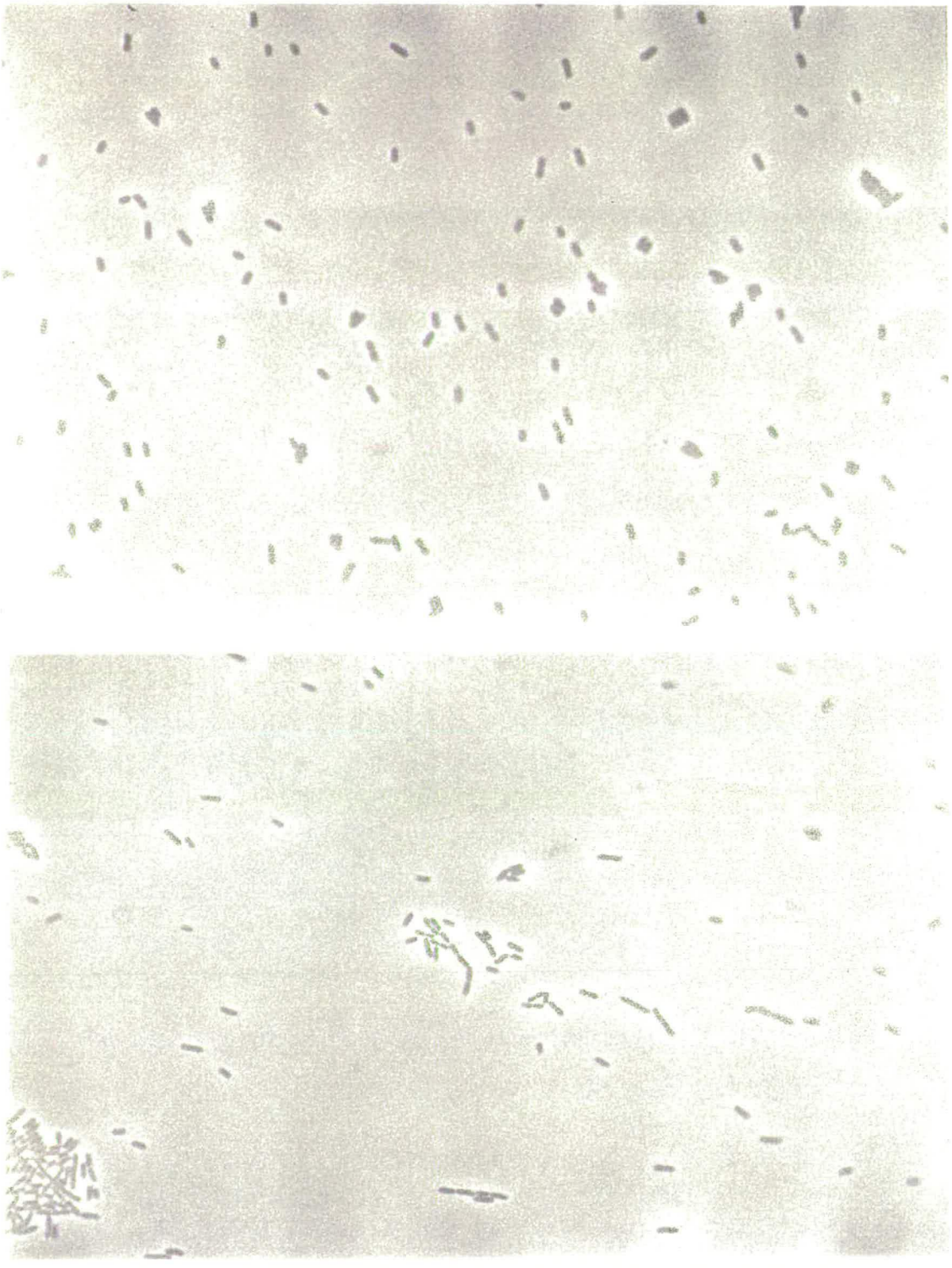

Figure 3.4.2. Phase contrast micrographs of MG1655 (pBADK') cultured in LB-broth 0.2% glucose (top) and 0.2% arabinose (bottom) for three hours. The glucose grown cells had a normal morphology whereas the a proportion of the cells grown in the presence of arabinose formed chains.

The significance of these chains was not immediately apparent. An FtsK-GFP fusion protein locates to the division site during septation but an FtsK44-GFP hybrid localises neither at the permissive nor the non-permissive temperatures (W. Margolin, pers. comm.). Immunofluorescence microscopy with FtsK specific antibodies also shows FtsK located at the septum (J. Lutkenhaus, pers. comm.). The N-terminus of SpoIIIE is thought to localise SpoIIIE to the prespore septum during sporulation (Wu and Errington, 1997). It could be possible, therefore, that the FtsK'$_{583}$ protein also localises to the septum and competes for this position with the full length, chromosomally encoded protein. This presented the possibility that the wild-type protein was present at lower levels than is required at the division site and could not fully carry out a second function, possibly encoded by the C-terminus which resulted in the formation of chains of cells. The C-terminus of FtsK, which shows a high degree of similarity with the SpoIIIE family of DNA translocases, was absent from the peptide encoded by pBADK' (Flannagan *et al.*, 1994; Hagege *et al.*, 1993; Kataoka *et al.*, (unpub.); Kendall and Cohen, 1988; Oswald *et al.*, 1993; Tomura *et al.*, 1993; Wu and Errington, 1994; Wu *et al.*, 1995; Wu and Errington, 1997). There was also the possibility that overproduction of FtsK'$_{583}$ was exerting another, unknown effect on the cell that either directly or indirectly resulted in the formation of chains of cells. It was decided that the function of the N- and C-termini could only be satisfactorily examined with the aid of a null-mutant of *ftsK*.

Microscopic examination of MGK44 (pBADK') cultured in arabinose containing LBΔNaCl broth at 42°C also showed chains of cells, although at a slightly lower frequency than that seen with the overproduction of FtsK' from pBADK' in MG1655 (17% of cells present in the form of chains compared with 25% seen from the overproduction of FtsK'$_{583}$ in MG1655). This finding agreed with the hypothesis that the FtsK'$_{583}$ polypeptide encoded by pBADK' did not possess the full activity of the complete protein and the second proposal that the chain formation was an artefact of FtsK'$_{583}$ overproduction was probably incorrect.

3.8 Discussion

This chapter describes the cloning of *ftsK* and the use of the clones to complement the *ftsK44* (Ts) mutation. *E. coli* requires low levels of FtsK as pBADK complements the *ftsK44* (Ts) mutant at the non-permissive temperature even when repressed. It is evident that overproduction of FtsK causes filamentation. *ftsK* was placed under the transcriptional control of the strong P_{lacUV5} promoter in the high copy number vector pUC18 (pCD99). MG1655 (pCD99) did not form colonies on LB plates at 37°C or 42°C, but did form colonies at 30°C. The experiments should be repeated in a strain bearing a the $lacI^q$ allele, which would result in the repression of P_{lacUV5}, and could be induced with IPTG.

Filamentation caused by the overproduction of FtsK results in (or from) either the inhibition of the formation of FtsZ rings or the destabilization of existing FtsZ rings. This filamentation is not the result of the induction of the SOS-response and is independent of the components of the SOS-response known to block cell division.

ftsK is preceded by the *dinH* promoter which is derepressed upon interference with DNA replication (Lewis *et al.*, 1992). Increasing the amount of FtsK leads to a block to cell division. These observations suggest that FtsK could be a component of the of *sfi*-independent pathway of division inhibition. Filamentation is observed in a *sulA sfiC* double mutant strain when SOS is induced (Burton and Holland, 1983; Hill *et al.*, 1997) and *dinH* appears to be derepressed during SOS (Lewis *et al.*, 1992). The de-repression of *dinH* could lead to an increase in the transcription of *ftsK*. Slight increases in the amount of FtsK in the cell have been shown in this chapter to induce filamentation by preventing the formation of or by destabilising existing FtsZ rings. It cannot be stated that FtsK directly interacts with FtsZ thus preventing FtsZ polymerization, as is the case with SulA (Huang *et al.*, 1996; Higashitani *et al.*, 1995). The filamentation caused by the overproduction of FtsK from pBADK cannot be suppressed by 3-4 fold overproduction of FtsZ from pBS58. As stated in section 3.3, it would be worthwhile to repeat the experiment in a system that overproduced FtsZ to a greater extent. The filamentation caused by SulA during the SOS-response can be suppressed by increasing the levels of FtsZ (Lutkenhaus *et al.*, 1986) and so it could be predicted that there might be a level of FtsZ overexpression that could suppress FtsK-overproduction

induced-filamentation. Alternatively, the de-repression of *dinH* during SOS-induction could lead to the production of a pool of FtsK that might be required for cell division following the repression of the SOS-response once DNA damage has been repaired.

The exact level of overproduction of FtsK in these experiments is not known. The amount of transcription from each of the promoters used in these experiments could be measured (by transcriptional fusions to β-galactosidase, for example) but these levels would be meaningless unless compared to the amount of FtsK per cell. The study of *ftsK* and the protein encoded by it would be greatly enhanced by the availability of specific antibodies to FtsK.

CHAPTER 4
VISUALIZATION OF *ftsK*

Chapter 4. Visualization of FtsK

The *ftsK* gene was sequenced and found to be 3987 bp in length and was predicted to encode a protein of 147 kDa (Begg et al., 1995). There are a number of in frame ATG codons within the *ftsK* ORF which could act as translation initiation codons. This suggested that FtsK could encode for more than one protein. Attempts were made to visualise the protein(s) encoded by *ftsK*.

4.1.1. Construction of pT7 clones of *ftsK*.

The pT7 range of vectors contain the promoter for bacteriophage T7 upstream of a polylinker. *E. coli* RNA polymerase does not recognise the T7 Ø10 promoter. Inhibition of *E. coli* RNA polymerase with rifampicin and induction of the T7 RNA polymerase from λDE3, a lysogenized λ with an IPTG-inducible T7 RNA polymerase gene results in the expression solely of protein(s) under the control of the T7 Ø10 promoter. The plasmid encoded proteins can be visualised by the incubation of the cells with ^{35}S-methionine which is incorporated into the protein(s) and subsequent SDS-PAGE of the protein extracts and autoradiography.

The 4.4 kb *Eco*RI–*Xba*I fragment containing *ftsK* and 227 bp of the 5' end of *lolA* from pUCK was ligated into *Eco*RI/*Xba*I digested pT7-3 and pT7-5 to form pT7-3K and pT7-5K, respectively. pT7-3 and pT7-5 are identical except for the orientation of the *bla* gene. In pT7-3, the *bla* gene is in the same orientation as the T7 Ø10 promoter so *bla* is transcribed and β-lactamase can be expressed when T7 RNA polymerase is present. The *bla* gene in pT7-5 is reversed and β-lactamase is not expressed when T7 RNA polymerase is present. pT7-3K and pT7-5K were transformed into BL21 (λDE3) (pLysS). pLysS is a pSC101 based plasmid that carries the *lysS* gene encoding lysozyme. Lysozyme degrades any T7 RNA polymerase that might be produced due to the leakiness of the IPTG inducible promoter, P_{lacUV5}, which transcribes the T7 RNA polymerase gene contained on λDE3. This prevents the expression of cloned genes before IPTG is added. There is sufficient T7 RNA polymerase expressed when P_{lacUV5} is induced with IPTG to overcome the effects of the lysozyme and transcription from the T7 Ø10 promoter is initiated. The methods for

expression and labelling are described in section 2.3.1. Expression was induced in BL21 (λDE3) (pLysS) (pT7-3K)/(pT7-5K) and protein samples analysed on 7% SDS-PAGE gels as described in sections 2.3.4 and 2.3.5. Figure 4.1.1 shows the results of the BL21 (λDE3) (pLysS) (pT7-5K) experiment.

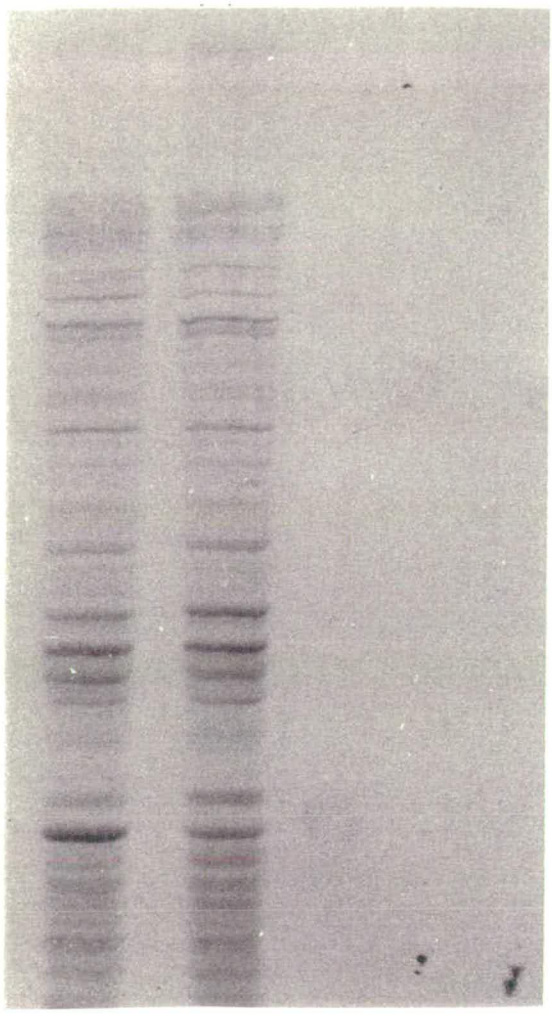

Figure 4.1.1. SDS-PAGE analysis of pT7-5K. The samples not treated with rifampicin are labelled (lanes 1 and 2). The induced (+IPTG) samples do not contain any labelled plasmid encoded proteins (lanes 2 and 4). Lane 1- rif/-IPTG, Lane 2 -rif +IPTG, Lane 3 +rif/-IPTG, Lane 4 +rif/+IPTG.

pT7-5K did not produce a radiolabelled protien that could be detected by autoradiography. β-lactamase was not observed in lanes 3 and 4 of Figure 4.1.1. becuse the *bla* gene is in the opposite orientation to the T7 Ø10 promoter (and cloned *ftsK*) and hence would not be expressed when rifampicin is added. In a separate experiment pT7-3K was shown to express a single peptide with the same apparent molecular weight as β-lactamase. The experiment was repeated several times and neither clone produced a visible radio-labelled protein from the cloned insert. The 4.4 kb insert cloned into pT7-3K and pT7-5K contained *ftsK* and 227 bp of the 5' end of *lolA* which had previously been shown to inhibit cell division when cloned into pBAD18 and pUC18 (sections 3.3 and 3.2.2, respectively). This finding, coupled with the discovery that no peptide(s) was expressed from the cloned insert from pT7 clones of the same region, suggested that *ftsK* was a very poorly translated gene, that FtsK could be subject to proteolysis,had a very short half life or that the secondary structure of the *ftsK* mRNA inhibited translation.

The TTG translation initiation codon predicted to be the start of *ftsK* could lead to reduced levels of translation compared with an ATG translation initiation codon. Although ATG is by far the predominant translation initiation codon (91% of genes compared with 1% TTG) and many mutations wich replace the ATG initiation codon with an alternative initiation codon reduce translation rates, some abundant proteins utilise initiation codons other that ATG (reviewed by Gold and Stormo, 1987). The potential *ftsK* TTG translation initiation codon was mutated to an ATG ($ftsK_{ATG}$) and the gene was cloned downstream of the Ø10 ribosome binding site in pT7-7 in an attempt to optimise the translation of *ftsK* by the following method. A 1.3 kb PCR product was synthesized, using pUCK as the template. The following primer were used

T7090 5'-GGAGAGAATCATATGAGCCAGGAATC-3'
G6689 5'-GGTGGCCAACAAGAC-3'

The melting temperature was 94°C, the annealing temperature 48°C and extension time at 72°C was 90 seconds. Taq DNA polymerase was employed. The introduced *Nde*I site in T7090 is underlined The PCR fragment was ligated into pGEM-T a 3 kb plasmid designed for cloning PCR products (Promega). Taq polymerase exhibits template independent

3' adenylation. pGEM-T is supplied pre-digested and has a single T-overhang at each terminus. This enables the cloning of A-tailed PCR fragments. The resultant plasmid, pGEM-TK was digested with *Sal*I and *Sph*I to release the cloned PCR fragment. This fragment was cloned into *Sal*I/*Sph*I digested pUC19 to form pCD109. pCD109 was digested with *Not*I and *Sal*I, this removed a 1.25 kb fragment, the 5' 54 bases of *ftsK* remained on the plasmid fragment. A 4.35 kb *Not*I-*Sal*I containing fragment bp 54–3987 bp of *ftsK* and 227 bp of the 3' end of *lolA* from pUCK was ligated into the *Not*I/*Sal*I digested pCD109 plasmid fragment. The clone was named pKATG. pKATG had $ftsK_{ATG}$ cloned in the opposite orientation to P_{lacUV5} to avoid the toxic effects of multiple copies of *ftsK*. pKATG was sequenced using the universal primer: 5'-CAGCACTGACCCTTTTG-3' using the T7 Sequencing kit (Pharmacia) as described in section 2.2.18. The region from the –5 to +70 relative to the introduced ATG and site was shown to be error free, save for the intentionally replaced bases. Since the region from bp 54–3987 (the end) of the *ftsK* gene in pKATG was derived from pUCK, the remainder of the insert was known to be functional. The 4.4 kb *Nde*I–*Sal*I fragment from pKATG was cloned into pT7-7 to produce pT7-7KATG. pT7-7 contains the Ø10 promoter cloned upstream of the rbs and the ATG translation initiation codon of T7 Ø10. An *Nde*I site (CATATG) is located overlapping the translation initiation codon to allow transcriptional fusions to the Ø10 rbs, which has been shown to aid the efficient translation of proteins (Studier and Moffat, 1986; Tabor and Richardson, 1988). pT7-7KATG was transformed into BL21 (λDE3) (pLysS) and the expression of $FtsK_{ATG}$ analysed by SDS-PAGE.

pT7-7KATG did not express any polypeptide from the cloned insert. This was unusual since the rbs and start codons for *ftsK* had been optimised. The reason for this could be that FtsK was rapidly degraded, but even if this was the case, the degradation products of $FtsK_{ATG}$ should have been visible after SDS-PAGE and autoradiography.

In vitro transcription/translation of purified λ214 DNA (section 3.2) also did not produce a peptide of the predicted size, although many other proteins, mostly of λ origin, were expressed (Figure 4.2.2).

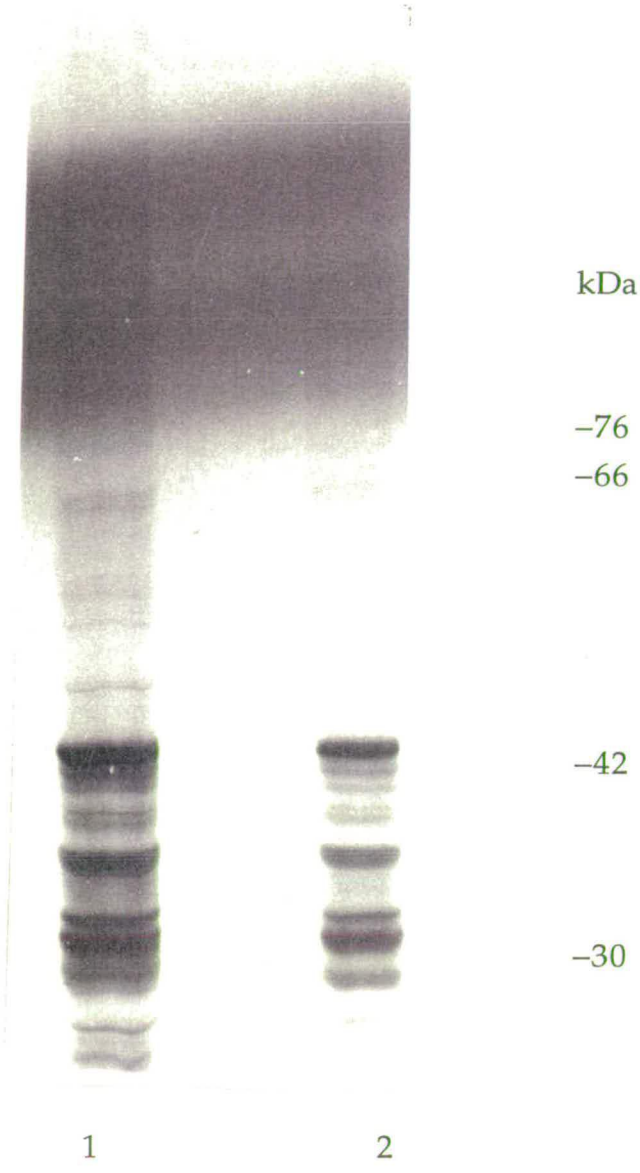

Figure 4.1.2. SDS-PAGE analysis of proteins expressed from the *in vitro* transcription/translation of purified λ214 DNA. The 2 lanes containing radio-labeled protein are from the same sample but twice as much protein was loaded in lane 1 than lane 2. The 147 kDa FtsK protein was not detected, although the smaller proteins, mainly the products of λ genes, could obscure any smaller peptides produced. A number of larger proteins were expressed, probably from the other cloned genes but could also be derived from the *ftsK* ORF.

Neither *in vitro* transcription nor T7-based expression of *ftsK* produced a peptide of the predicted size. Although the T7 system has

been found to be a satisfactory method for the production of some proteins, it is not suitable for the expression and visualisation of others. Indeed, pT7-4 and pT7-6 clones of *mraY* failed to produce a visible MraY peptide (Boyle, 1995). The reasons for the failure to visualise *any* peptides encoded by pT7-3K and pT7-5K are not immediately apparent, although the fragment cloned into these vectors has been shown to complement the *ftsK44* (Ts) allele and cause filamentation when overexpressed in other expression systems (sections 3.2.1 and 3.3, respectively). The problem may lie in the systems employed, which might not be suitable for the production of *ftsK* encoded proteins or that there is a rapid turnover of these peptides once they are produced.

4.2 Use of the Maxicell method to visualise FtsK

Another approach was tried in the attempt to visualise FtsK. The Maxicell method described in section 2.3.2 can be used to visualise proteins expressed from genes under the transcriptional control of promoters that function in *E. coli*. Strain CSR603 was transformed with pUC19, pCD99 and five new plasmids, pKT1, pKT2, pKT3, pKT4 (Figure 4.2.1) and pCD101 (Figure 4.2.2). pKT1-4 are deletion derivatives of pCD99 (section 3.2.2), which have consecutively larger regions of *ftsK* cloned downstream of the strong P_{lacUV5} promoter. With respect to pCD99, pKT1 has the 3.6 kb *Nhe*I–*Xba*I fragment deleted, leaving 719 bp of the 3' end of *ftsK* under the control of P_{lacUV5}. pKT2 is pCD99 with the 2.6 kb *Bsu*36I–*Xba*I fragment removed, leaving 1748 bp of the 3' end of *ftsK* under the control of P_{lacUV5}. pKT3 is pCD99 with the 2.4 kb *Cla*I–*Xba*I fragment removed, leaving 1932 bp of the 3' end of *ftsK* under the control of P_{lacUV5}. pKT4 is pCD99 with the 0.7 kb *Nco*I–*Xba*I fragment deleted, leaving 3652 bp of the 3' end of *ftsK* under the control of P_{lacUV5}.

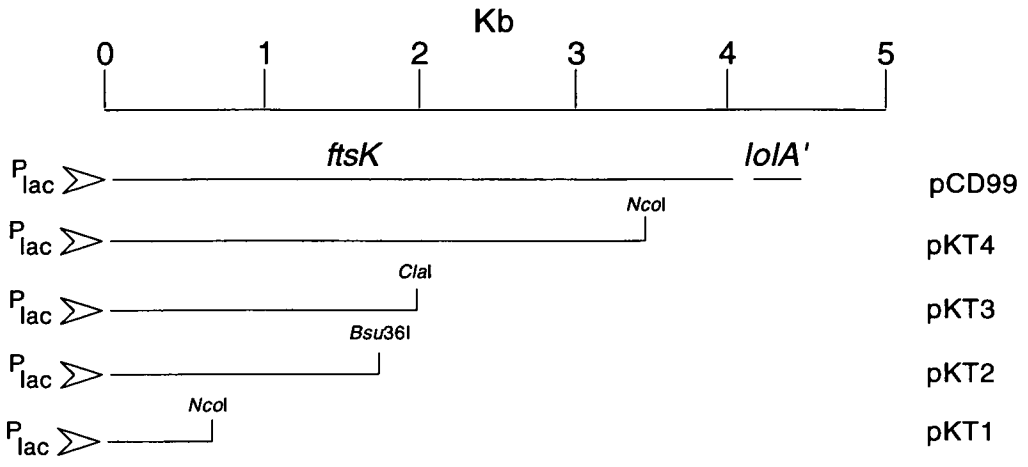

Figure 4.2.1. The regions of *ftsK* present on pCD99 and the pCD99 derived truncation plasmids, pKT1, pKT2, pKT3 and pKT4.

It was hoped that truncated FtsK polypeptides could be visualised from these clones. The predicted molecular weight of the truncated FtsK peptide encoded by pKT1 was 27.1 kDa, by pKT2 was 63.5 kDa, by pKT3 was 70.6 kDa and by pKT4 was 134.3 kDa. pCD101 was constructed by cloning the 7.2 kb *Eco*RI–*Kpn*I fragment from purified λ214 DNA into *Eco*RI/*Kpn*I digested pUC19. The fragment contains the complete *trxB*, *lrp*, *ftsK* and *lolA* genes (Figure 4.2.2).

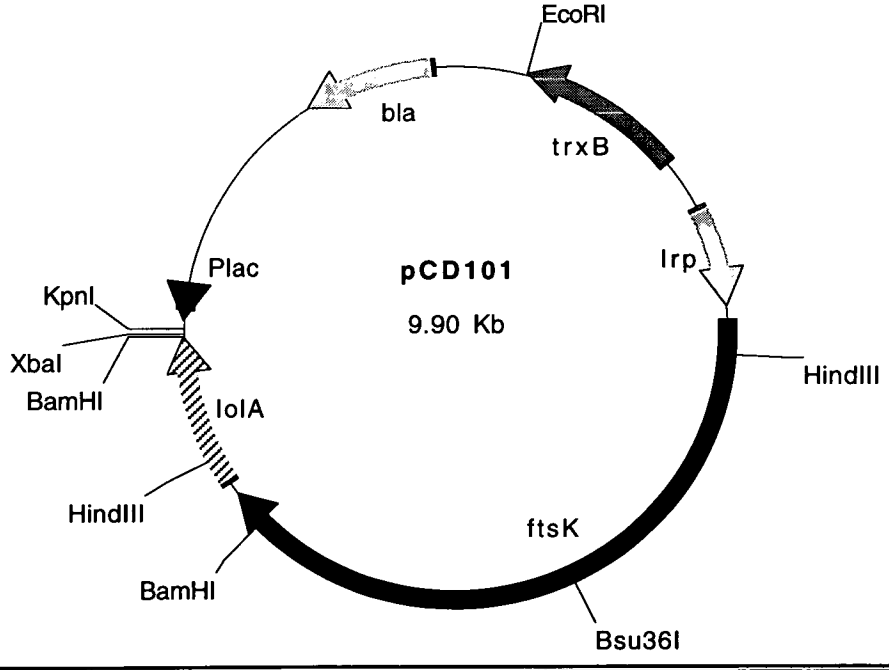

Figure 4.2.2. pCD101 which contains the *trxB*. *lrp*, *ftsK* and *lolA* genes cloned into pUC19.

pCD101 severely impaired growth of the wild-type strain MG1655 at temperatures above 35°C in LB-broth and on LB-agar plates. Phase contrast microscopy revealed that MG1655 (pCD101) formed filaments at all temperatures, but the effect was more striking at higher temperatures. The toxicity of pCD101 was probably due to the presence of *ftsK* on the clone. To show this, a 2 kb *Eco*RV internal *ftsK* fragment was excised and the plasmid religated to form pCD101-ΔRV. MG1655 (pCD101-ΔRV) did not form filaments and formed colonies at all temperatures tested (13°C to 43°C). The toxic effects of pCD101 were due, therefore, to the presence of the cloned *ftsK*.

CSR603 and CSR603 (pUC19)/(pKT1)/(pKT2)/(pKT3)/(pKT4)/(pCD99) and (pCD101) and were processed as described in section 2.3.2 and protein samples from CSR603, CSR603 (pUC19)/(pKT2)/(pCD99) and (pCD101) were analysed by SDS-PAGE and radio-labelled proteins were visualized by phosphoimagery (Figure 4.2.2.)

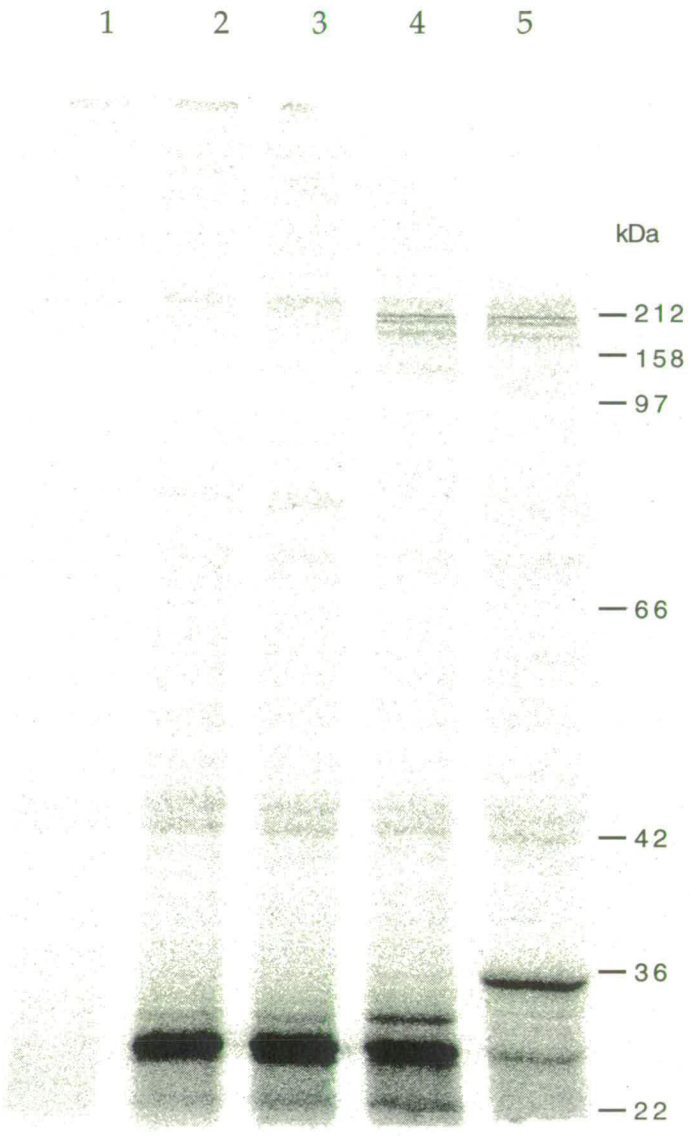

Figure 4.4.2. SDS-PAGE analysis of CSR603, CSR603 (pUC19)/(pKT2)/(pCD99) and (pCD101). CSR603 (lane 1) contains no plasmid and only shows a background level of protein labelling. CSR603 (pUC19) (lane 2) and (pKT2) (lane 3) contains radio-labelled β-lactamase. The truncated FtsK encoded by pKT2 was not visualized. CSR603 (pCD99) (lane 4) produced three radio-labelled proteins from the cloned *ftsK* gene of a similar size which migrated to a position of between 158 and 212 kDa, as well as β-lactamase. CSR603 (pCD101) (lane 5) also produced the three high molecular weight proteins, a 34 kDa protein and β-lactamase. The position of the marker bands is shown.

The three, closely spaced, high molecular weight proteins encoded by pCD99 and pCD101 were probably different forms of FtsK. The experiment was repeated several times and the triplet of bands was always present. These different forms could be formed by proteolysis, instability, phosphorylation or as a result of the expression and visualization procedures. FtsK exhibits abnormal migration, the predicted MW of the protein is 146.7 kDa. The 34 kDa protein expressed from pCD101 was proposed to be TrxB, which has a MW of 34 kDa (Russel and Model, 1995b). The truncated FtsK protein expressed by pKT2 could not be detected. The truncated protein could be unstable or subject to degradation by proteases. SDS-PAGE analysis of the proteins encoded by the CSR603 (pKT1)/(pKT3) and (pKT4) also did not result in the visualisation of the truncated FtsK polypeptides encoded by these plasmids.

It is curious that only the Maxicell method allowed visulalisation of FtsK. Not all expression systems are suitable for the production of all proteins. Even so, all of the above experiments the expressed proteins would be radiolabelled, which should allow detection of even small amounts of expressed protein. As mentioned in section 4.1.1, there are many potential reasons for the lack of expression of FtsK from the pT7 clones of *ftsK* and the *in vitro* transcription/translation of the λ214 DNA. That the *ftsK* regions cloned into the pT7 vectors might not be transcribed by the T7 DNA polymerase is unlikely as the system is well established. Secondary structures in the transcribed mRNA might prevent satisfactory transcription by T7 RNA polymerase but not *E. coli* RNA polymerase, hence the failure of the T7 system and success of the Maxicell method Pulse chase experiments of strains expressing FtsK could help discern whether there is a rapid turnover of FtsK in the cell. Obviously, the generation of antibodies to FtsK would greatly enhance the study of the expression of FtsK.

CHAPTER 5
INACTIVATION OF *ftsK*

Chapter 5. Insertional inactivation and deletion of *ftsK*.

5.1 Gene replacement

To determine whether *ftsK* is an essential gene and to observe the effects of a null-allele of the gene, a strategy to insertionally inactivate and delete the gene was devised. A number of gene replacement strategies exist but in this case a novel method being developed by N. McLennan and M. Masters was employed. This gene replacement method relies on the phenomenon that high copy-number ColE1 based plasmids, such as the pUC range of vectors appear to recombine with the host chromosome via a single cross-over and excise by the same mechanism. It is known that the cross-over requires regions of homology between the plasmid and the chromosome. The excision of the plasmid occurs via homologous recombination at duplicated regions of DNA and could to be promoted by instability caused by the presence of a plasmid origin of replication on the *E. coli* chromosome. It seems that there is a constant exchange of plasmid DNA between the cytoplasm and chromosome (N. McLennan and M. Masters, pers. comm.).

It is possible to direct the location of insertion of the plasmid onto the chromosome and interrupt the gene of interest by cloning the gene, which has been inactivated by the insertion of a selectable marker such as an antibiotic resistance cassette, into the high copy-number plasmid. It is proposed that the plasmid can insert into the locus of interest via a single cross-over between homologous DNA. The amount of flanking DNA required for efficient integration has not yet been determined, although 1 kb of homologous DNA upstream and downstream of the resistance marker has been shown to suffice (N. McLennan, pers. comm.). Upon excision, one of two plasmids can be formed: a molecule identical to the plasmid that was initially introduced into the cells or a plasmid containing the wild-type locus, with the chromosomal ORF now interrupted by the resistance marker (Figure 5.1).

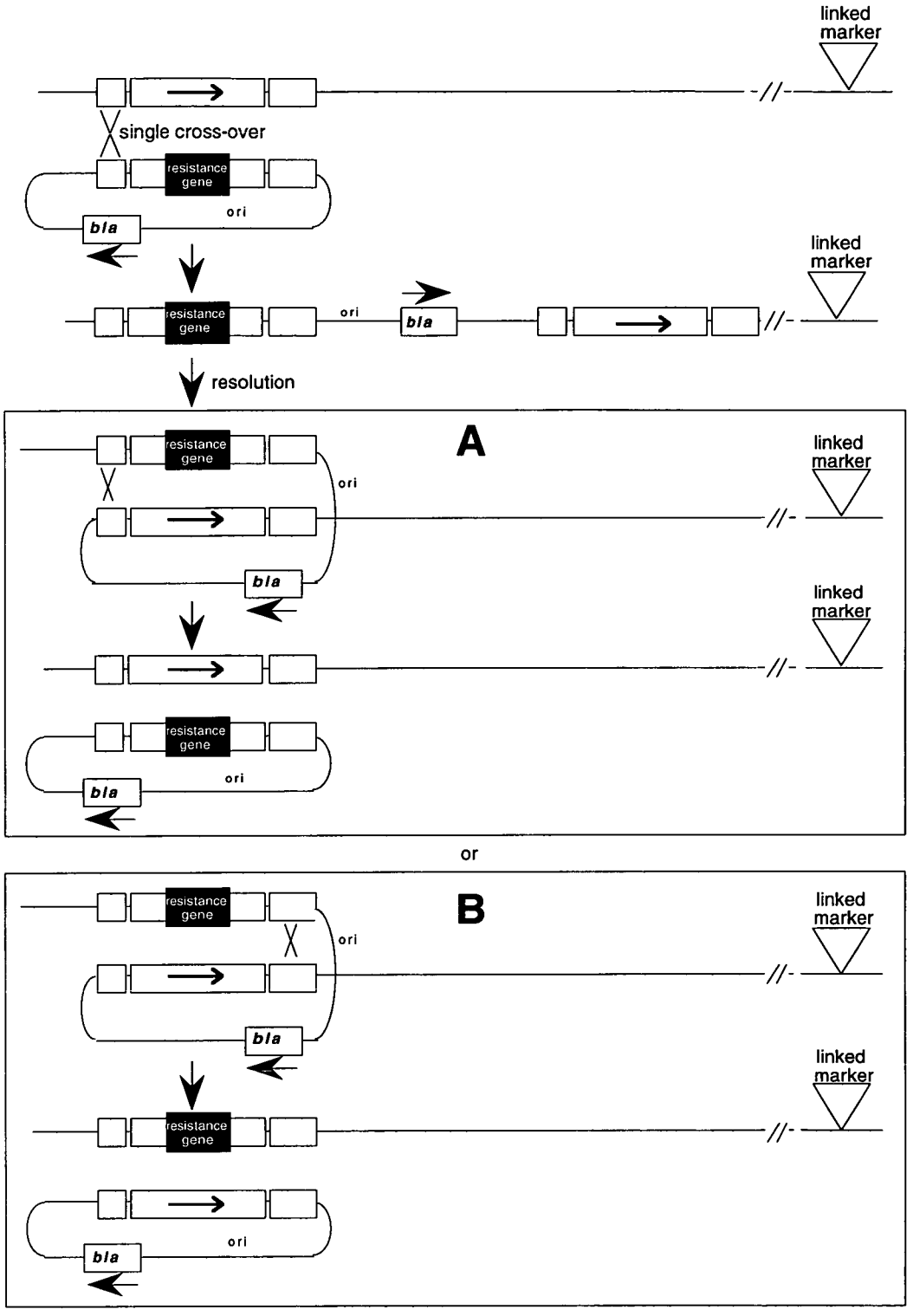

Figure 5.1. The single cross-over of plasmid DNA at a homologous region of chromosomal DNA. Excision of the plasmid can result either in the reformation of the original plasmid or the antibiotic marker can remain on the chromosome and the wild-type locus is transferred onto the plasmid.

A P1 lysate is grown on the strain carrying the plasmid to be used for the replacement. The strain should have an additional selectable marker within transducing range (<2 minutes) of the chromosomal gene being replaced. The transducing particles containing the region of interest will carry three possible arrangements of the chromosome:

- the wild type locus
- the locus with the entire plasmid inserted
- the locus with the ORF of choice interrupted by the antibiotic marker.

The P1 lysate is then used to transduce a wild-type strain, selecting for the insertion marker and the linked chromosomal marker. If necessary, the host can carry a complementing copy of the gene that is being inactivated on a plasmid or at another location on the chromosome. The resultant colonies must then be screened to ensure loss of the original plasmid. This is achieved by screening for loss of the plasmid resistance marker, in the case of the pUC vectors, the loss of ampicillin resistance.

For the replacement of some genes McLennan and Masters have found it necessary to introduce a complementing copy of the gene being replaced in the donor strain prior to growing the P1 lysate. The reason for this is not clear at the moment, although when replacing an essential gene the desired construct would, of course, be lethal unless a complementing copy of the replaced gene were present.

5.2 Construction of CDK1

In the first instance pKBCAT was introduced into MGAT. pKBCAT is derived from pCD101. pCD101 was digested with *Bst*BI, a blunt-ended restriction enzyme which cuts pCD101 once, 677 bp into the *ftsK* ORF. The *cat* gene along with its promoter was excised from pUCAT18 (a gift from N. McLennan) by digestion with *Hin*dII and *Ecl*136II and ligated into the linearised pCD101. The resultant plasmid, pKBCAT has the *ftsK* ORF interrupted with the *cat* gene (*ftsK*$_{677}$::*cat*) and carries both the chloramphenicol and ampicillin resistance markers. The orientation of the *cat* gene is such that it transcribes in the same direction as *ftsK* (Figure 5.2.1).

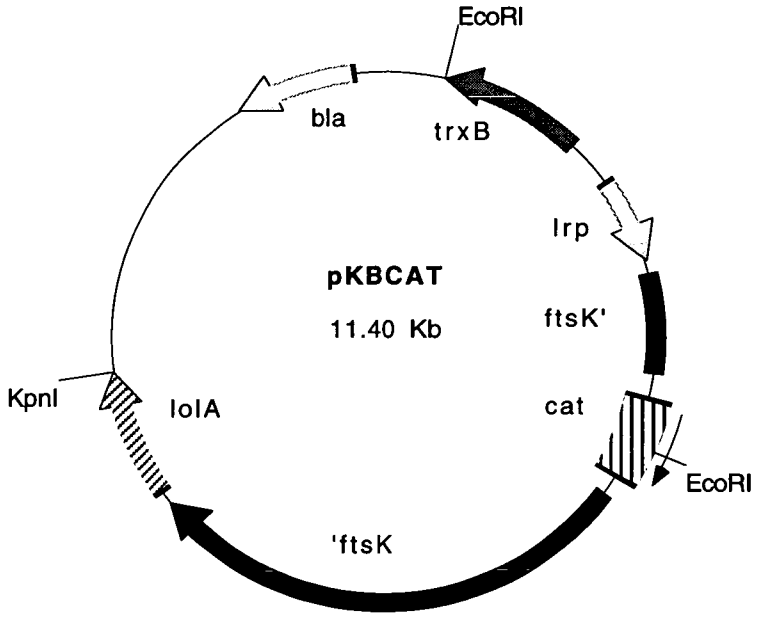

Figure 5.2.1. pKBCAT, the pCD101 based plasmid used for the insertional inactivation of *ftsK*. The *cat* gene is transcribed in the same direction as *ftsK* and is expressed from its own promoter.

5.2.1 The N-terminal 225 aa of FtsK can suppress *ftsK44*

It was thought that the *ftsK*$_{677}$::*cat* construct would abolish FtsK function. To examine whether this was the case, pKBCAT was introduced into MGK44. Interestingly, pKBCAT complemented *ftsK44* at 42°C. The 677 bp of *ftsK* present upstream of the *cat* insertion encodes only 225 aa (17%) of the N-terminus of FtsK. It was also thought possible that the region downstream of the *cat* insertion could encode a polypeptide that could be translated from an mRNA initiated from the *cat* gene or an internal promoter. *In vitro* transcription/translation of a clone containing the 3′ end of the *ftsK* gene produced a protein of between 41.5 and 67 kDa (Diez *et al.*, 1997). To examine whether the 3' end of *ftsK* was involved in the complementation of *ftsK44* by pKBCAT a 4248 bp *Bsa*BI-*Sma*I fragment was excised from pCD101 and the plasmid religated to form pTLK′ (Figure 5.2.2).

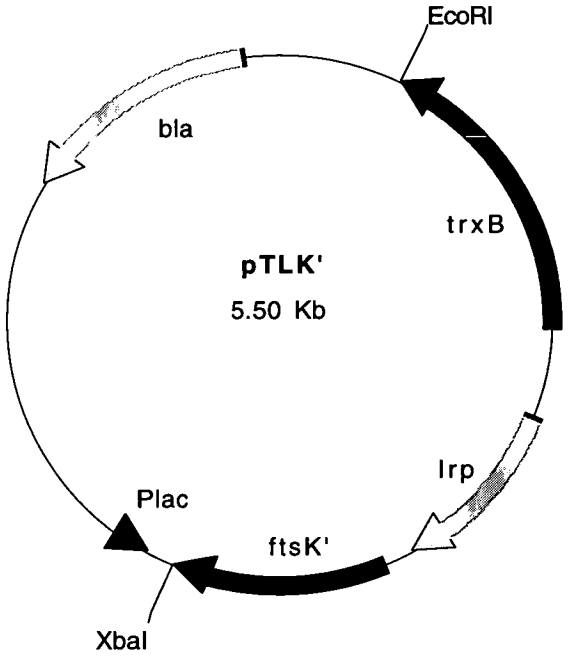

Figure 5.2.2. pTLK', a pUC19 based plasmid, which contains *trxB*, *lrp* and the 5' 677 bp of *ftsK*.

pTLK' was transformed into MGK44. MGK44 (pTLK') was plated on LBΔNaCl at 30°C and 42°C. MGK44 (pTLK') formed colonies at both temperatures indicating that the 5' 677 bp of *ftsK* could complement *ftsK44*. The presence of *trxB* and *lrp* on the clone, however, meant that this 677 bp of *ftsK* might not alone be responsible for the complementation. It could also be possible that the pTLK' could recombine with the chromosome at the *ftsK* locus. Excision of the integrated plasmid could result in the replacement of *ftsK44* with the wild-type gene and the *ftsK44* mutation being placed onto the plasmid. This was addressed by the construction of pBADK'3, which contained the 5' 677 bp of *ftsK* cloned into pBAD18 (Guzman *et al.*, 1995). pBADK'3 was created by partial digestion pBADK with *Bsa*BI. *Bsa*BI cuts pBADK twice, once 677 bp into the *ftsK* ORF and again within the *araC* gene. The restriction digest was subjected to 0.8% agarose gel electrophoresis and the linear molecules isolated. These linear fragments were digested with *Xba*I and end-filled with Klenow enzyme. The 5338 bp fragment which contained the 5' 677 bp of *ftsK* and the plasmid backbone was purified after 0.8% agarose gel electrophoresis and religated to form pBADK'3 (Figure 5.2.3).

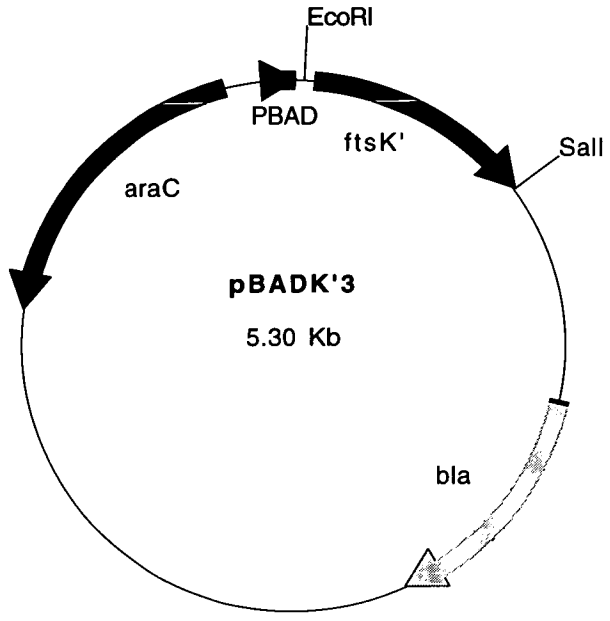

Figure 5.2.3. pBADK'3 which contains the 5' 677 bp of the *ftsK* gene under the control of the arabinose inducible promoter P_{BAD}.

pBADK'3 was transformed into MGK44. MGK44 (pBADK'3) was plated on LBΔNaCl-agar containing 100 μg ml^{-1} ampicillin supplemented with either glucose or arabinose at 30°C and 42°C. Colonies formed on both the arabinose and glucose containing plates at 30°C but only on the arabinose containing plates at 42°C. Again, integration of pBADK'3 into the *ftsK* locus could result in transfer of the *ftsK44* allele to the plasmid and the wild-type gene onto the chromosome by homologous recombination. This was deemed unlikely because MGK44 (pBADK'3) required arabinose for growth at the non-permissive temperature. Replacement of the temperature sensitive *ftsK44* allele with the wild-type gene would not render the cells arabinose dependent. It was concluded that only the amino-terminal 225 aa of FtsK was required for suppression of *ftsK44*. These experiments utilizing fragments of the 5' end of the *ftsK* gene to suppress the lethality of the *ftsK44* (Ts) allele should be repeated in a *recA*$^-$ background due to the possibility that the plasmids might recombine via a single crossover event with the chromosomal copy of *ftsK44*. The excision of the plasmid could result in the exchange of the *ftsK44* allele onto the plasmid and the restoration of a wild-type chromosomal copy of *ftsK*. This would give the impression of

suppression of the lethal Ts allele. The *recA*⁻ allele does not allow recombination to take place and so any suppression of *ftsK44* (Ts) seen would be due to the plasmid-borne fragment of *ftsK*.

The finding that the 225 aa of the N-terminus of FtsK encoded by pKBCAT could complement *ftsK44* raised the question of whether pKBCAT would be a suitable construct for the creation of an *ftsK* null-allele. It was reasoned that because pKBCAT complemented the *ftsK44* temperature-sensitive allele, $ftsK_{677}$::*cat* might also support growth when substituted for the chromosomal *ftsK* gene. This would not allow the question of what happens when FtsK is depleted from the cell to be addressed. Alternatively, $ftsK_{677}$::*cat* might not support growth and that the reason truncated polypeptides from pKBCAT, pTLK' and pBADK'3 could complement *ftsK44* was due to residual action of the temperature-sensitive protein. It was decided to introduce $ftsK_{677}$::*cat* onto the chromosome in place of *ftsK* because even if this construct did not result in the absolute abolition of FtsK activity it would give an invaluable insight into FtsK function.

5.2.2 Replacement of *ftsK* with $ftsK_{677}$::*cat*

Since it was possible that *ftsK* would be an essential gene and that the replacement of the chromosomal copy of *ftsK* with $ftsK_{677}$::*cat* could be lethal, a complementing copy of *ftsK* was introduced into the donor strain by transforming MGAT (pKBCAT) with pGB101, which is compatible with pKBCAT. pGB101 was constructed by digesting pCD101 with *Eco*RI and *Sal*I. A 7.2 kb fragment containing the *trxB*, *lrp*, *ftsK* and *lolA* genes was ligated into *Eco*RI/*Sal*I digested pGB2 (Churchward *et al.*, 1984). pGB101 was transformed into MGK44 and complemented *ftsK44* (Ts) at the non-permissive temperature. A P1 lysate was grown on MGAT (pKBCAT) (pGB101) at 30°C. The reason for growing the P1 lysate at 30°C instead of 37°C was because the desired insertion of the *cat* gene into *ftsK* would result in the chromosomal gene transferring onto the excised plasmid, a plasmid identical to pCD101, the effects of which, as stated in section 4.2, are deleterious to the cell at temperatures greater than 35°C.

The recipient for the transduction was MG1655 (pGB101). pGB101 was present to provide a complementing copy of the *ftsK* gene in case the *ftsK* gene proved to be essential. pGB101 encodes for spectinomycin resistance and would allow the screening for the loss of the ampicillin resistance in the transductants. MG1655 (pGB101) was transduced with the lysate grown on MGAT (pKBCAT) (pGB101). As a control, 1/10 of the transduction mixture was plated onto tetracycline and spectinomycin containing LB-agar to verify the efficiency of transduction. The transductants were incubated at 30°C overnight, selection was for Cmp/Tet/Spec resistance. 176 Cmp/Tet/Spec resistant colonies and approximately 3100 Tet/Spec resistant colonies formed after overnight incubation from 1/10 of the cells from the transduction. The Cmp/Tet/Spec resistant colonies were screened for ampicillin sensitivity and three proved to be Cmp/Tet/Spec resistant and ampicillin sensitive. Phase contrast microscopy of these cells revealed them to have a normal morphology. One of these colonies was taken for further analysis to examine whether the insertion was in the desired locus and whether this insertion of the *cat* gene into *ftsK* resulted in inactivation of the gene. The strain was named K146. A P1 lysate was grown on K146 and this lysate was used to transduce MG1655 (pBADK), selecting for tetracycline and ampicillin resistance in the presence of arabinose or glucose overnight at 30°C. Colonies appeared on both the plates containing arabinose and glucose with the colonies on the arabinose plate appearing smaller after overnight incubation, probably a result of overproduction of FtsK. 200 colonies from both the arabinose and glucose plates where screened for tetracycline and chloramphenicol resistance. 65% of the colonies were both tetracycline and chloramphenicol resistant, in agreement with the co-transduction frequency of two markers 0.4 minutes apart. Cmp/Tet resistant isolates from the arabinose and glucose containing plates were examined microscopically. The cells from the arabinose plate were filamentous whereas cells from the glucose plate had a normal morphology.

It was possible that pBADK was complementing the *ftsK*$_{677}$::*cat* allele even in the presence of glucose. This had been observed previously with the finding that pBADK could complement *ftsK44* in the presence of glucose in a *pcnB*$^{+}$ strain but not in a *pcnB*::*kan* derivative (section 3.2.1). In order to examine this possibility, K146 (pBADK) was transduced with a

lysate grown on the *pcnB*::*kan* strain, MM38K24 (Masters *et al.*, 1993). The insertional inactivation of *pcnB* reduces the copy number of pBADK to approximately 20% of wild-type levels. K146 *pcnB*::*kan* (pBADK) did not show a requirement for arabinose, although this time the glucose and arabinose grown colonies had a similar appearance after overnight incubation at 37°C.

It appeared from these results that either *ftsK* was not an essential gene or, as was originally thought, that the insertion of the *cat* gene at bp 677 of the *ftsK* ORF did not fully inactivate the gene. To demonstrate this, the lysate grown on K146 was used to transduce MG1655 to tetracycline resistance. 200 of the resultant colonies were checked for the presence of $ftsK_{677}$::*cat* by screening for chloramphenicol resistance. 142 of the colonies (71%) were both chloramphenicol and tetracycline resistant. The morphology of the Cmp/Tet resistant colonies was normal, except for the presence of a few chains of cells and a small number of short filaments (Figure 5.2.4) A representative Cmp/Tet resistant isolate was chosen and the strain named CDK1.

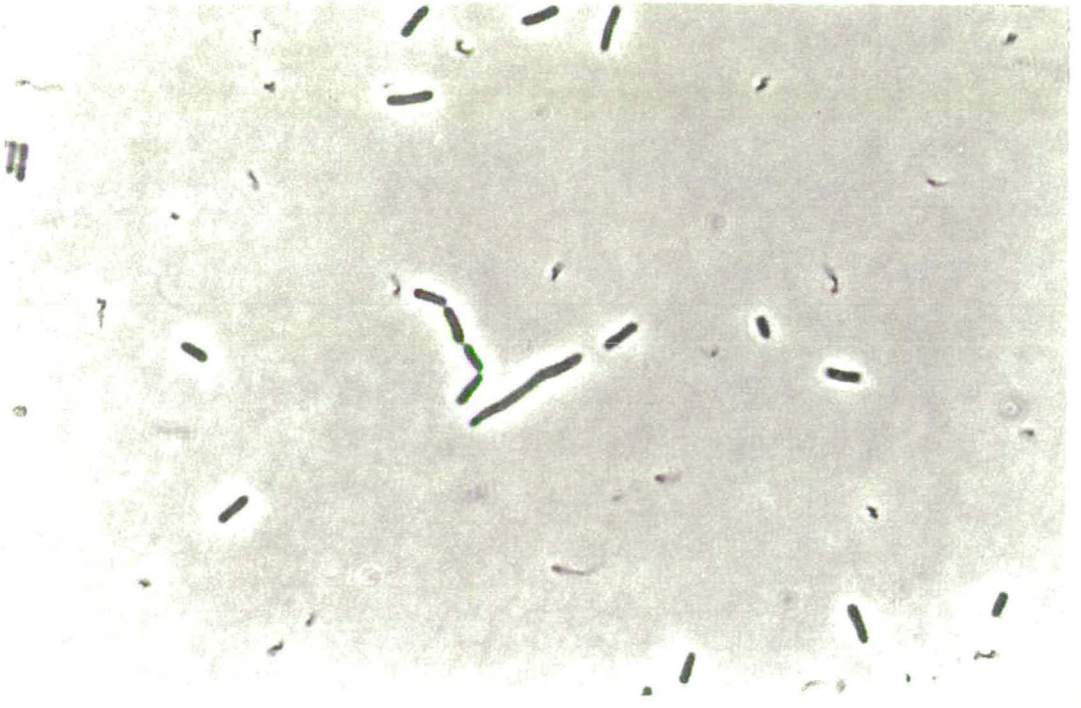

Figure 5.2.4. Phase contrast micrographs of CDK1 cultured in LB-broth containing chloramphenicol and tetracycline.

5.2.3 PCR and Southern blot analysis of CDK1

Two approaches were used to examine whether the replacement of *ftsK* with *ftsK*$_{677}$::*cat* was had taken place correctly. CDK1 and MGAT chromosomal DNA was prepared as described in section 2.2.4. The chromosomal DNAs and pKBCAT was then subjected to PCR analysis. The following primers were used:

- K-up 5′-TTGAGCCAGGAATACATTGAAGAC-3′
- K-rev 5′-GCATCAACAGCGGATGAAGCAGGG-3′

The melting temperature used was 94°C, the annealing temperature 54°C and the extension temperature and time were 72°C and 4 minutes 30 seconds respectively. K-up and K-rev annealed to bp 1-24 and bp 2476-2453 of the *ftsK* ORF respectively. The PCR products were analysed by 0.8% agarose gel electrophoresis, the results of which are shown in Figure 5.2.5.

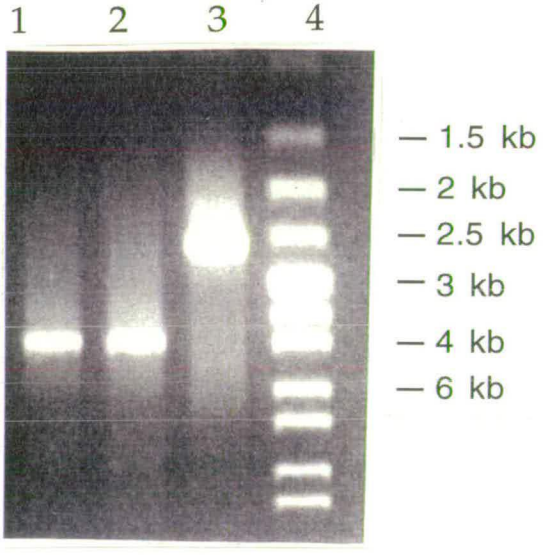

Figure 5.2.5. 0.8% agarose gel electrophoresis of the products derived from PCR analysis of CDK1 (lane 1), pKBCAT (lane 2) and MGAT (lane 3). The markers (lane 4) are MBI 1 kb ladder, sizes are marked.

The predicted products of 3.9 kb from pKBCAT and CDK1 were detected, as was the 2.5 kb fragment from the MGAT chromosomal DNA (Figure 5.6). This result only suggests that CDK1 contains the desired

construct and does not fully show the organization of the *ftsK* locus in CDK1.

To ensure that CDK1 contained $ftsK_{677}$::*cat*, the isolated chromosomal DNA was subjected to Southern analysis. CDK1 and MGAT chromosomal DNA was digested with *Eco*RI/*Bam*HI, *Sca*I and *Pvu*II. The digested DNA was subjected to 0.8% agarose gel electrophoresis as described in section 2.2.10 and blotted onto a positively charged nylon membrane (section 2.2.19). pCD101 was digested with *Bam*HI and a 1165 bp band purified after agarose gel electrophoresis of the digestion products. The fragment contains the 3′ 102 bases of *ftsK* and the entire *lolA* gene. A ^{32}P-labeled probe was produced from the fragment by the random priming method outlined in section 2.2.20. These randomly labeled fragments were used to probe the chromosomal digest filter. It was predicted that the probe would hybridise to the following fragments:

CDK1	*Eco*RI/*Bam*HI	3446 bp
	*Sca*I	5315 bp
	*Pvu*II	5397 bp
MGAT	*Eco*RI/*Bam*HI	3446 bp
	*Sca*I	5359 bp
	*Pvu*II	5427 bp

Figure 5.2.6 shows a photograph of the autoradiograph that resulted from the hybridization of the 1165 bp random labeled probe to the blotted chromosomal DNA.

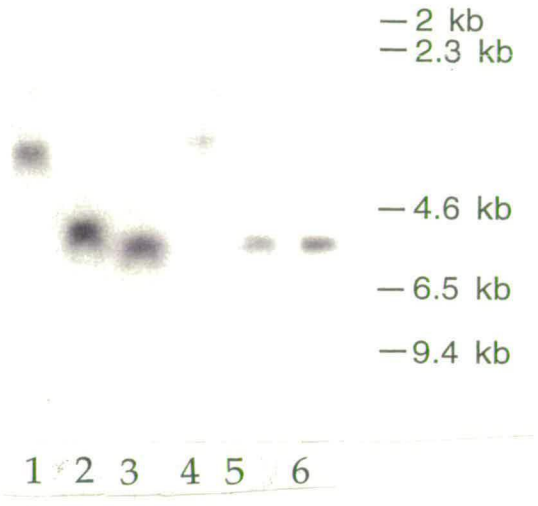

Figure 5.2.6. The autoradiograph resulting from the exposure of the filter probed with randomly ^{32}P-labeled *'ftsK–lolA* fragments to X-ray film for 2 hours. The lane order is CDK1 *Eco*RI/*Bam*HI (lane 1) CDK1 *Sca*I (lane 2) CDK1 *Pvu*II (lane 3), MGAT *Eco*RI/*Bam*HI (Lane 4) MGAT *Sca*I (lane 5) MGAT *Pvu*II (lane 6) lambda *Hind*III digest (lane 7). The fragment sizes of the lambda *Hind*III markers are shown. The smaller, faint band seen in lane 1 is due to star activity of the restriction endonucleases used.

The probed Southern blot shows the correct pattern of hybridization with the exception of a star-activity generated fragment in lanes 1 and 4. To ensure that the *cat* gene was present in CDK1 and not in MGAT, and as a double check that CDK1 contained the correct construct, the filter was stripped of hybridised probe as described in section 2.2.21 and re-probed with a randomly labeled probe produced from a 657 bp *Eco*RI-*Bsu*36I fragment from pUCAT18. This fragment contained the *cat* ORF from bp 214 to the translation termination codon (442 bp) plus 215 bp of pBR325 DNA. The results of a BlastN search (Altschul *et al.*, 1990) revealed that there was no significant regions of homology between the pBR325 DNA contained on the probe fragment and *E. coli* chromosomal DNA and so hybridization with immobilised digested chromosomal DNA was predicted be limited to the *cat* gene region of the probe. The lane order is the same as in Figure 5.2.6. It was predicted that the probe would hybridise to the following fragments:

CDK1	*Eco*RI/*Bam*HI	3822 bp
	*Sca*I	1942 bp and 4948 bp
	*Pvu*II	1097 bp

The probe was not expected to hybridise to MGAT DNA. The autoradiograph resulting from the exposure of the filter probed with the *cat* fragment is shown in Figure 5.2.7.

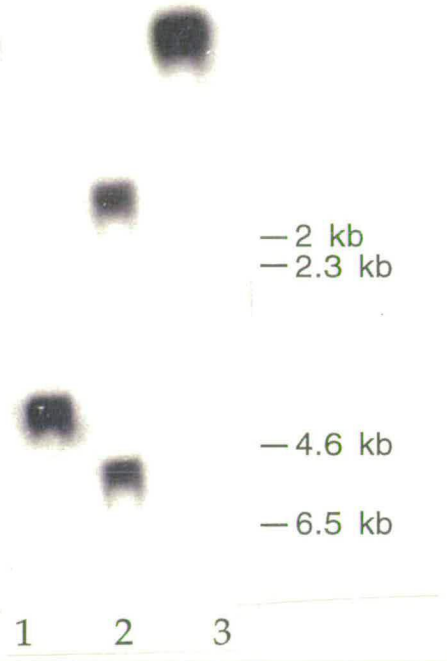

Figure 5.2.7. Image of he autoradiograph resulting from the exposure of the *cat* probed filter to X-ray film for 3 hours The lane order is CDK1 *Eco*RI/*Bam*HI (lane 1) CDK1 *Sca*I (lane 2) CDK1 *Pvu*II (lane 3). The *cat* probe did not hybridise to the MGAT DNA and so has been omitted. The fragment sizes of the λ *Hind*III markers is shown.

The ^{32}P-labeled probe generated from the *cat* gene hybridised to the predicted fragments. The replacement of the chromosomal *ftsK* gene with *ftsK*$_{677}$::*cat* construct was confirmed. The organization of the 20 minute region in CDK1 is shown in Figure 5.2.8.

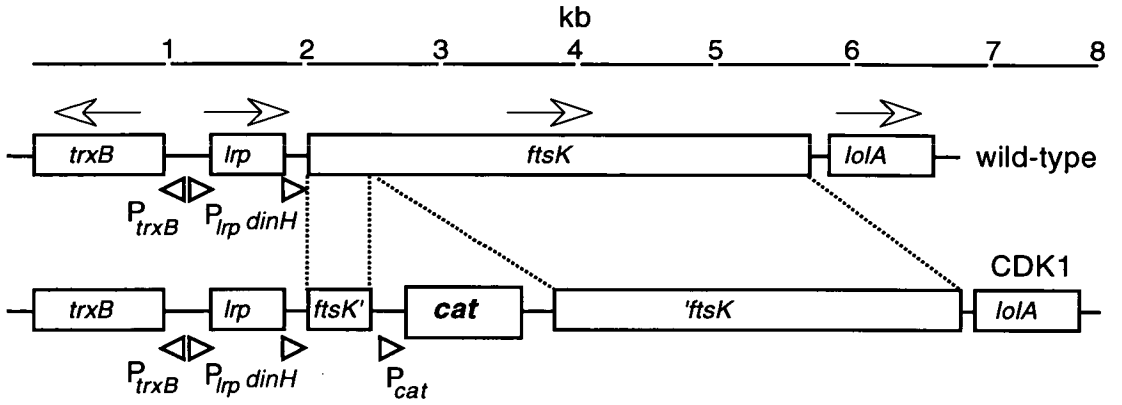

Figure 5.2.8. The organization of the 20 minute region in wild-type *E. coli* and CDK1. The presence of $ftsK_{677}$::*cat* was confirmed by both PCR and Southern analysis.

5.2.4 Expression of the C-terminus of FtsK in CDK1

Diez *et al.* (1997) showed that the insertion of a Tn*10*d-*cat* sequence at bp 2000 of *ftsK* caused a proportion of the cells to form chains. These chains were linked together via a surface structure of unknown composition. The chain formation could be suppressed by supplying bp 1223-3987 of *ftsK* on a plasmid. pGB101, which contains the entire *ftsK* gene and the upstream and downstream genes reversed the chain formation that would otherwise be seen in K146 (see above). To examine whether a clone bearing the 3′ end of *ftsK* could suppress the chain formation seen in CDK1 a new plasmid, pKC1 was constructed. pKC1 was a deletion derivative of pCD99. pCD99 was digested with *Eco*RI and *Mfe*I. A 4.8 kb fragment containing bp 2201-3987 of the *ftsK* gene, 227 bp of *lolA* and the plasmid backbone was purified after agarose gel electrophoresis. This fragment was religated (*Eco*RI and *Mfe*I are compatible sites) to form pKC1 (Figure 5.2.9). There was no external RBS provided in the clone, as was the case in the work of Diez *et al.* (1997), who suggested that there could be an internal initiation codon and RBS within the 3′ end of the *ftsK* ORF.

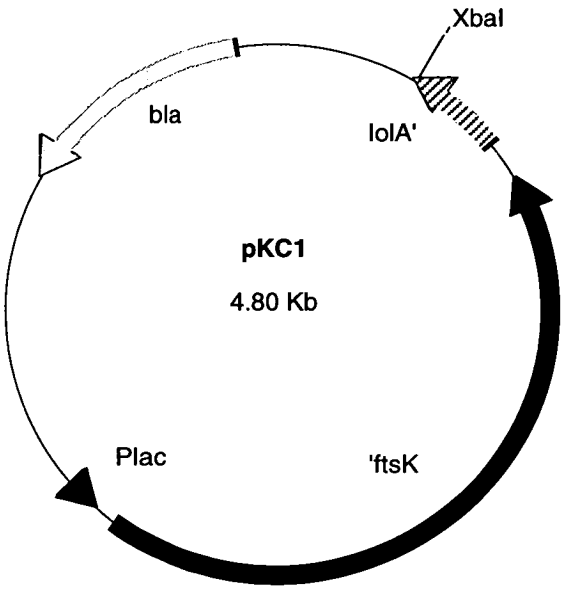

Figure 5.2.9. pKC1, a pUC18 based vector containing bp 2201-3987 of *ftsK* under the transcriptional control of P_{lacUV5}.

pKC1 was transformed into MG1655 and CDK1. MG1655 (pKC1) had a normal morphology whereas CDK1 (pKC1) formed chains. There is disagreement between these results and those described by Diez *et al.* (1997). The chain formation seen by Diez *et al.*, (1997) was suppressed by supplying bp 1223-3987 of *ftsK* whereas in these experiments a smaller fragment from bp 2201-3987 of the *ftsK* gene was provided. It may be important to include the extra region of *ftsK* to achieve the suppression of the chain phenotype. The region of *ftsK* supplied by pKC1 (bp 2201-3987) might not facilitate the expression of a peptide, possible due to the presence of regulatory regions of DNA present in the portion of DNA supplied by Diez *et al.*, (1997) but not in pKC1. It still remains unclear as to whether a peptide is encoded by the 3′ end of the *ftsK* ORF. Specific antibodies to FtsK would help to detect if any smaller peptides are produced from the *ftsK* orf.

The Tn*10*d-*cat* sequence was found to be inserted at bp 2000 of the *ftsK* ORF in the chain forming strain described by Diez *et al.*, (1997) (T. Nyström, pers. comm.). The *cat* gene in CDK1 is inserted at bp 677 of *ftsK* and so the phenotypes of the two insertion mutants, although similar, could be subtly different. Chains of cells are not observed in stationary phase cultures of strains bearing *ftsK*::Tn*10*d-*cat*, whereas chains are

present in stationary phase CDK1 cultures. The two strains were constructed with different goals in mind and it would be extremely useful if these strains were fully examined with the results from both laboratories taken into account.

At this stage it could be concluded that *ftsK* was either an inessential gene or that the insertion of the *cat* gene at bp 677 of the *ftsK* ORF did not fully inactivate the gene and that $ftsK_{677}$::*cat* could support growth. This experiment was so far inconclusive. It was decided to engineer a construct which would better address the question of whether *ftsK* was an essential gene.

5.3 Construction of CDK2

This second construct would have part of *ftsK* substituted with the *cat* gene. The *cat* gene from pBR325 (Bolivar, 1978) was amplified by PCR using the following primers:

- Cat-up 5'-TCAAGGATGCGGCCGCTGTTGAG-3'
- Cat-rev 5'-TCGTCAATTGTTACCTCCACGGG-3'

The introduced *Not*I site in Cat-up is underlined, as is the introduced *Mfe*I site in Cat-rev. Vent DNA polymerase was used for this PCR reaction since Vent has 5'-3' proof-reading activity that would ensure a functional PCR product. The melting temperature used was 94°C, the annealing temperature 50°C and the extension temperature and time were 72°C and 1 minutes 30 seconds, respectively. All salts, residual nucleotides and protein were removed from the PCR reaction by using the Promega DNA Clean-up columns according to the manufacturers instructions. Approximately 0.5 µg of the purified 1492 bp PCR product was subjected to digestion by *Not*I and *Mfe*I. The digested PCR product was electrophoresed through a 0.8% agarose and the fragment purified from the gel using the Qiagen Gel Extraction kit. The *cat* gene, flanked with *Not*I and *Mfe*I ends, was ligated into *Not*I/*Mfe*I digested pCD101, resulting in pCDCAT (see Figure 5.3.1).

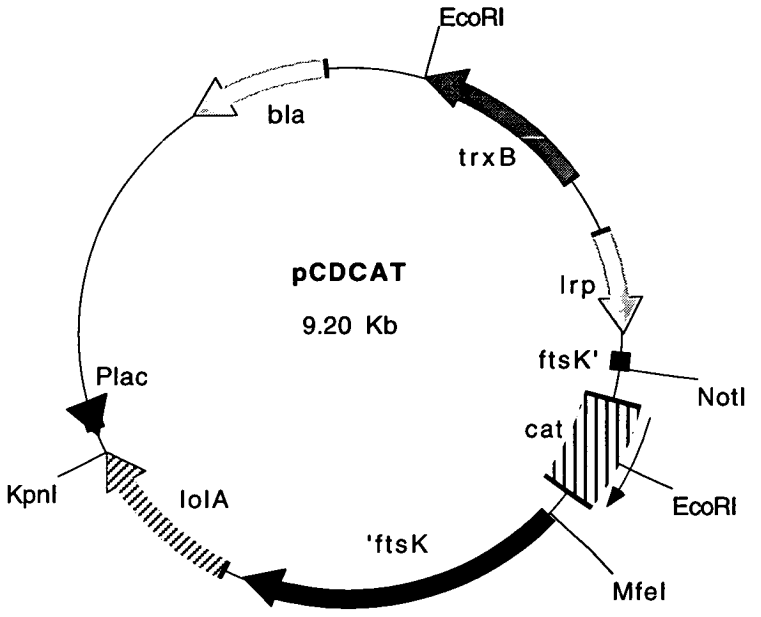

Figure 5.3.1. Map of pCDCAT. The *cat* gene is in the same orientation as *ftsK*.

pCDCAT has bp 54-2201 of the *ftsK* ORF substituted with the *cat* gene from pBR325 ($ftsK_{\Delta 54\text{-}2201}$::*cat*). pCDCAT was introduced into MGK44. MGK44 (pCDCAT) did not form colonies at the non-permissive temperature. This indicated that pCDCAT would be a more appropriate construct for the inactivation of *ftsK* than pKBCAT. pCDCAT was used to replace the chromosomal copy of *ftsK* with $ftsK_{\Delta 54\text{-}2201}$::*cat* in a similar manner to that used for the construction of CDK1.

5.3.1 Replacement of *ftsK* with $ftsK_{\Delta 54\text{-}2201}$::*cat*

pCDCAT was transformed into MGAT (pGB101) by transformation. A P1 lysate was grown on MGAT (pGB101) (pCDCAT) at 30°C. The lysate was used to transduce MG1655 (pGB101). Selection was for Tet/Cmp/$Spec^R$ colonies. 1/10 of the transduction was plated onto Tet/Spec containing LB-agar plates to test for efficient transduction. Approximately 2500 Tet/Spec resistant colonies formed on the control transduction plates. 146 Tet/Cmp/Spec resistant colonies also formed on the replacement transduction plates. These colonies were screened for ampicillin sensitivity. Three of the isolated proved to ampicillin sensitive and Tet/Cmp/Spec resistant. One of these isolates was chosen

and the strain named K2:1. A P1 lysate was grown on K2:1 and this lysate was used to transduce MG1655 (pBADK). Selection was for tetracycline and ampicillin resistance in the presence of arabinose. 200 of the Tet/Amp resistant colonies were plated onto LB Tet/Cmp/Amp plates containing arabinose. 128 colonies were Tet/Cmp/Amp resistant, a co-transduction frequency between the tetracycline and chloramphenicol resistance markers of 64%, consistent of two markers approximately 0.4 minutes apart. A Tet/Cmp/Amp resistant colony was chosen and the strain named CDK2.

As a control, the lysate grown on K2:1 was also used to transduce MG1655 to tetracycline resistance. 100 of the tetracycline resistant colonies were plated onto Tet/Cmp containing LB-agar. No Tet/Cmp resistant progeny were detected. This was the first indication that the *ftsK*$_{\Delta 54\text{-}2201}$::*cat* construct abolished FtsK function and that *ftsK* was an essential gene.

5.3.2 Complementation of CDK2 with pBADK

CDK2 (pBADK) was plated on Tet/Cmp/Amp LB-agar containing either 0.2% glucose or 0.2% arabinose at 37°C. Colonies appeared on both the arabinose and the glucose containing plates. As was observed in the case of CDK1 (pBADK), the colonies formed on the arabinose containing plates appeared smaller than those on the glucose containing plates. This was previously attributed to the toxic effect of FtsK overproduction. Cells from both the arabinose and glucose containing plates were examined microscopically. Cells from the arabinose containing plates appeared filamentous whereas those from the glucose containing plates had a normal morphology. This is consistent with the effects of overproduction of FtsK (see section 3.3).

It was necessary to examine whether the low levels of expression from P_{BAD} was responsible for the viability of CDK2 (pBADK) on plates containing glucose. This phenomenon had been observed previously in the case of MGK44 (pBADK) forming colonies on glucose containing media at the non-permissive temperature(section 3.2.1). An MGK44 *pcnB*::*kan* (pBADK) derivative only formed colonies when grown in arabinose containing media, and not when glucose was substituted. In a similar vein, the *pcnB*::*kan* allele was introduced into CDK2 (pBADK) by transduction with a lysate grown on MM38K24 (Masters *et al.*, 1993).

CDK2 *pcnB*::*kan* (pBADK) was grown on Tet/Cmp/Amp/Kan LB-agar plates containing either arabinose or glucose. Growth was only observed on the arabinose containing plates and not on the glucose containing plates. It appeared, therefore, that *ftsK* was an essential gene and that very little FtsK was required for viability. It was also concluded that the reason that CDK1 was viable was because the $ftsK_{677}$::*cat* allele did not completely abolish FtsK function.

5.3.3 Suppression of the lethality of *ftsK44* and $ftsK_{\Delta 54\text{-}2201}$::*cat*

It was decided to examine whether overproduction of FtsN would suppress the lethal phenotype of *ftsK44* and $ftsK_{\Delta 54\text{-}2201}$::*cat*. FtsN was isolated as a multi-copy suppressor of the *ftsA12* temperature-sensitive mutation (Dai *et al.*, 1993). FtsN overproduction was also found to suppress temperature-sensitive alleles of *ftsI*, *ftsQ* and some alleles of *ftsW* (Dai *et al.*, 1993; M. Khattar, pers. comm.). FtsN overproduction could not suppress the lethal phenotype of a conditional *ftsA* (amber) mutant, suggesting that residual protein (albeit non-functional) was required for the suppression by FtsN. Overproduction of FtsN did not suppress the temperature-sensitive *ftsZ84* allele, so it appears that suppression by FtsN is limited to late acting division genes (Dai *et al.*, 1993)

The FtsN overproducing plasmid pKD140 (Dai *et al.*, 1993) and a control plasmid pBR322 (Bolivar *et al.*, 1977) were transformed separately into MGK44. A second plasmid, pHL1 (a gift from M. Khattar), containing several genes from the 88.5 minute region, including *ftsN*, cloned into pBR322 (Figure 5.3.2) was also transformed into MGK44.

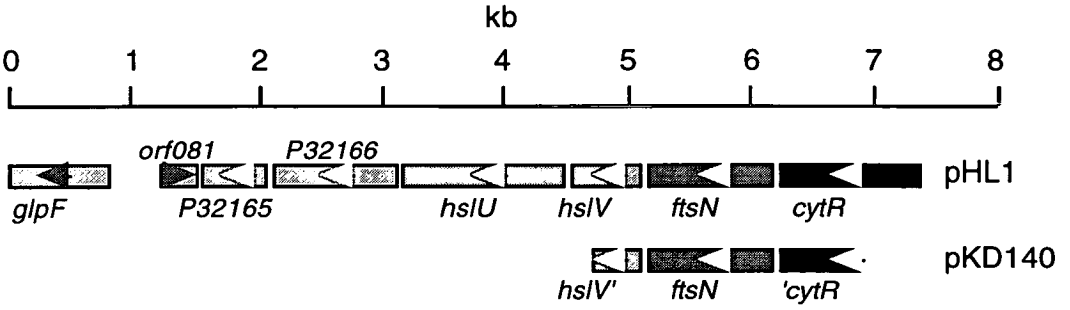

Figure 5.3.2. The chromosomal regions cloned into pBR322 to form pHL1 (top) and pKD140 (bottom).

100 µl of a 1:400 dilution of the exponentially growing cultures (OD_{600}=0.3) were plated on a variety of media. MGK44 was plated onto LB, nutrient broth (NB) and LBΔNaCl at 30°C and 42°C. MGK44 (pBR322), (pKD140) or (pHL1) was plated in a similar manner onto NB and LBΔNaCl plates containing 100 µg ml^{-1} ampicillin (NBA and LBΔNaClA respectively). The number of colonies that formed on each set of plates is indicated in Table 5.1.

Strain	Media	Colonies at 30°C	Colonies at 42°C	Plating efficiency at 42°C (%)
MGK44	LB	231	237	102
MGK44	NB	644	3	0.4
MGK44	LBΔNaCl	594	8	1.3
MGK44 (pBR322)	NBA	662	3	0.4
MGK44 (pBR322)	LBΔNaClA	641	0	0
MGK44 (pHL1)	NBA	450	437	97.1
MGK44 (pHL1)	LBΔNaClA	407	132	25
MGK44 (pKD140)	NBA	1152	1170	101
MGK44 (pKD140)	LBΔNaClA	1209	927	76

Table 5.1. Colony counts on LB-agar (LB), nutrient broth agar (NB) and LBΔNaCl-agar at 30°C and 42°C. Plates with ampicillin added to 100 μg ml^{-1} are indicated by NBA (nutrient broth agar +ampicillin) and LBΔNaClA (LB without salt +ampicillin). Plating efficiency is defined as (no. of colonies on the 42°C plates / no. of colonies on the corresponding 30°C plates) x 100.

MGK44 was suppressed by multicopy plasmids containing *ftsN*. The salt-reversible nature of *ftsK44* (Begg *et al.*, 1995) was confirmed by the 100% plating efficiency observed on LB-agar at 42°C, which contains 1% NaCl (w:v). MGK44 is temperature-sensitive on media with reduced amounts of salt, NB (0.5% NaCl w:v) and LBΔNaCl (no salt), as was described by Begg *et al.* (1995). MGK44 (pBR322) shows similar temperature-sensitive characteristics to MGK44. MGK44 (pHL1) has 100%

plating efficiency on NBA plates but only 25% plating efficiency on LBΔNaClA. This could be due to the extra stringency imparted by the complete absence of salt from the LBΔNaCl plates. MGK44 (pKD140) also showed 100% plating efficiency on NBA plates. MGK44 (pKD140) had a plating efficiency of 76% on LBΔNaClA. The difference between the plating efficiencies of MGK44 (pHL1) and MGK44 (pKD140) on LBΔNaClA differs by 51%. This could be due to the presence of extra genes from the 88.5 minute region in pHL1, some of which are uncharacterized, which might have a slightly deleterious effect on the cell. In pKD140, *ftsN* is cloned into the tetracycline resistance gene of pBR322. It is possible that transcription from the tetracycline resistance gene promoter could lead to extra production of FtsN that might result in the better suppression seen in MGK44 (pKD140) plated on LBΔNaClA media.

It was next decided to examine whether overproduction of FtsN would suppress the lethal effect of $ftsK_{\Delta 54\text{-}2201}$::*cat*. The P1 lysate grown on K2:1 was used to transduce MG1655 (pKD140). Selection was made for Tet/Amp resistant colonies on LB-agar at 37°C. 150 of the Tet/Amp resistant colonies were screened for the co-transduction of the $ftsK_{\Delta 54\text{-}2201}$::*cat* construct. 98 of the 150 (65%) colonies proved to be Tet/Amp/Cmp resistant. It was therefore possible to transduce the $ftsK_{\Delta 54\text{-}2201}$::*cat* allele, which normally required complementation by extra chromosomal copies of *ftsK*, into a strain overproducing FtsN. This is the first report of multicopy *ftsN* suppressing a null-allele. The mechanism of suppression is not known but will be discussed at the end of this chapter.

The insertional inactivation of the *dacA* gene, which encodes PBP5 (see section 1.3.7), was found by Begg *et al.* (1995) to suppress the temperature-sensitivity of *ftsK44*. To examine whether the inactivation of *dacA* would also suppress the lethality of $ftsK_{\Delta 54\text{-}2201}$::*cat*, a P1 lysate was grown on SP1070 (which carries the *dacA*::*kan* allele) and used to transduce MG1655. Selection was for kanamycin resistant transductants. The resultant strain was named MGdak. The lysate grown on K2:1 was used to transduce MGdak. Selection was for Tet/Kan resistant transductants. 100 of the Tet/Kan resistant colonies were screened for the co-transduction of chloramphenicol resistance (from $ftsK_{\Delta 54\text{-}2201}$::*cat*). 46 of the 100 colonies tested were Cmp/Tet/Kan resistant. This co-transduction frequency (46%) was lower than that observed for previous tests for the

co-transduction of the *cat* gene inserted in *ftsK* and *aroA*::Tn*10*. This could be because the insertional inactivation of *dacA* does not suppress the inactivation of *ftsK* as well as does *ftsN* in multi-copy. It was concluded that the insertional inactivation of *dacA* also suppressed the lethal effect of $ftsK_{\Delta 54\text{-}2201}$::*cat*. A Tet/Cmp/Kan resistant isolate was selected and the strain named CDK3.

The possibility that the Tet/Cmp/Kan resistant transductants had developed another suppressor mutation and that the insertional inactivation of *dacA* did not have a suppressing effect was discounted. This was because the number of transductants was deemed too high for all the transductants to have developed other extra- or intragenic suppressors. To check for intragenic suppression in CDK3, MG1655 *pcnB*::*kan* (pBADK) was transduced with a lysate grown on CDK3. Selection was for Tet/Cmp/Kan resistant colonies in the presence of arabinose. 20 of the transductants were plated on Tet/Cmp/Kan plates containing either arabinose or glucose. No growth was observed on the glucose containing plates, indicating that *ftsK* was inactivated in CDK3.

It was also decided to check for suppressors of $ftsK_{\Delta 54\text{-}2201}$::*cat* that might arise when a wild-type strain was transduced with the K2:1 lysate. MG1655 was transduced with the lysate grown on K2:1 and subsequent selection for both tetracycline and chloramphenicol resistance resulted in the formation of only one or two colonies per transduction, not the numbers of transductants seen with the transduction of $ftsK_{\Delta 54\text{-}2201}$::*cat* into MGdak. These isolates were found to be spectinomycin resistant, indicating the co-transduction of pGB101 or that pGB101 was integrated into the chromosome within P1 transducing distance from *ftsK* and *aroA*::Tn*10*. It was concluded that either the presence of *dacA*::*kan* made it more likely for other types of suppressors to develop or, as was thought to be more likely, that the insertional inactivation of *dacA* did suppress $ftsK_{\Delta 54\text{-}2201}$::*cat*. An insight on the mechanism of *dacA* deletion mediated suppression of *ftsK44* was supplied by J. -V Höltje (pers. comm). In the terminal stages of septal closure Dr. Höltje proposes is that the final step requires pentapeptide side chains. These pentapeptide side chains are thought to be required as tripeptide side chains hypothetically cannot span the nearly completed septum. Thus, a role for FtsK in the silencing of PBP5 activity during this septum closing stage could be envisioned. In order for there to be enough pentapeptide side chains to close the gap, the

D-ala:D-ala carboxypeptidase activity of PBP5 needs to be suppressed. As FtsK has already been implicated in the late stage of cell division (Begg *et al.*, 1995) it is possible to see how the inactivation of *dacA* would result in the presence of extra pentapeptide side chains, thus the Ts nature of *ftsK44* could be ameliorated as FtsK activity would not be required for the completion of cell division under these conditions.

Overproduction of FtsQ, FtsA and FtsZ from pBS58 (Bi and Lutkenhaus, 1990) and the overproduction of FtsW from pKHS3 (a gift from M. Khattar) did not suppress $ftsK_{\Delta 54\text{-}2201}$::*cat*, indicating that the suppression of $ftsK_{\Delta 54\text{-}2201}$::*cat* by overproduction of FtsN or the inactivation of *dacA* was specific.

Studies into the suppressors of *ftsK*-null strains need to be expanded and studied in more detail. Perhaps the identification of other suppressors of the inactivation of *ftsK* would lead to greater insight into FtsK function.

5.3.4 PCR and Southern blot analysis of CDK2

Before any further analysis of CDK2 was attempted it was decided to ensure that the chromosomal copy of *ftsK* had been replaced with $ftsK_{\Delta 54\text{-}2201}$::*cat*. Chromosomal DNA was isolated from CDK2 *pcnB*::*kan* (pBADK) and CDK3 as described in section 2.2.4 and these DNAs were subjected to PCR analysis. As with the PCR analysis of CDK1, primers K-up and K-rev (5'-TTGAGCCAGGAATACATTGAAGAC-3') and (5'-GCATCAACAGCGGATGAAGCAGGG-3') respectively, were used. MGAT chromosomal DNA and pCDCAT was subjected to the same analysis. The PCR products were subjected to analysis by 0.8% agarose gel electrophoresis. A photograph of the gel is shown in Figure 5.3.3.

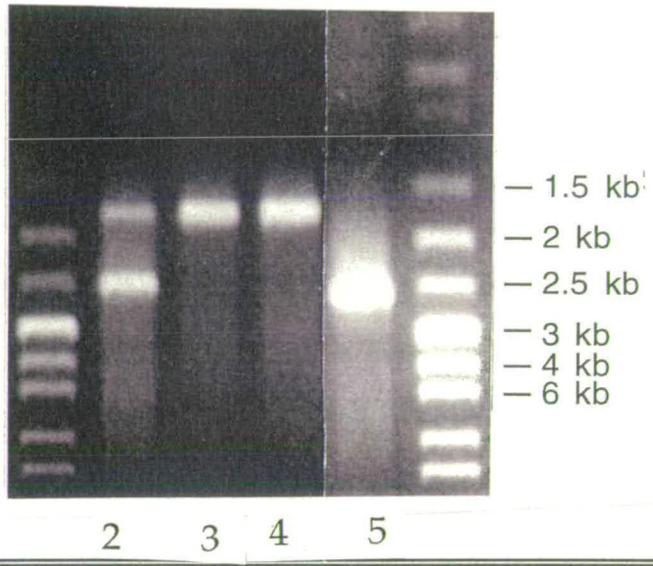

Figure 5.3.3. The results of PCR from CDK2 *pcnB*::*kan* (pBADK) (lane 2), CDK3 (lane 3), pCDCAT (lane 4) and MGAT (lane 5) DNA. The markers are MBI 1 kb ladder, fragment sizes are indicated.

The predicted fragments of 1.8 kb and 2.5 kb resulted from the PCR of CDK2 *pcnB*::*kan* (pBADK) (lane 2). Two fragments are produced because pBADK plasmid DNA is a contaminant of the chromosomal DNA isolated from CDK2 *pcnB*::*kan* (pBADK). The 1.8 kb fragment is derived from the chromosomal construct ($ftsK_{\Delta 54\text{-}2201}$::*cat*) and the 2.5 kb fragment is produced from pBADK. It has been shown previously that the $ftsK_{\Delta 54\text{-}2201}$::*cat* construct is present on the chromosome and not on a plasmid because *aroA*::Tn*10* and $ftsK_{\Delta 54\text{-}2201}$::*cat* can be co-transduced with the expected frequency. CDK3 (MG1655 $ftsK_{\Delta 54\text{-}2201}$::*cat* *dacA*::*kan*) also produced the desired band of 1.8 kb. As controls, PCR of pCDCAT gave a single band of 1.8 kb (lane 3) and PCR of MGAT chromosomal DNA produced a fragment of 2.5 kb. The fragment sizes proved to be as predicted but the organization of the 20 minute region in CDK2 *pcnB*::*kan* (pBADK) could not be deduced from this experiment.

Chromosomal DNA from CDK3 was subjected to Southern blot analysis. CDK3 had previously been shown to carry the $ftsK_{\Delta 54\text{-}2201}$::*cat* allele by P1 transduction and PCR analysis of purified CDK3 chromosomal DNA gave the correct sized band (see above). CDK3 chromosomal DNA was chosen for Southern blot analysis because it lacks the contaminating plasmid DNA present in purified CDK2 *pcnB*::*kan* (pBADK) DNA and would result in clearer autoradiographs. CDK3 and MGAT chromosomal

DNA was digested with *Eco*RI/*Bam*HI, *Sca*I and *Pvu*II. After overnight electrophoresis through a 0.8% agarose gel the DNA was blotted and immobilised onto positively-charged nylon membrane as described in section 2.2.19. In a similar manner to the Southern blot analysis of CDK1 chromosomal DNA, the filter was probed with random ^{32}P-labeled fragments derived from a 1165 bp fragment containing the 3' end of *ftsK* and the entire *lolA* gene (see section 5.2.3). The probe was predicted to hybridise to the following fragments:

CDK3	*Eco*RI/*Bam*HI	3446 bp
	*Sca*I	3362 bp
	*Pvu*II	5232 bp

MGAT	*Eco*RI/*Bam*HI	3446 bp
	*Sca*I	5359 bp
	*Pvu*II	5427 bp

After exposure of the probed membrane to X-ray film the membrane was stripped of hybridised probe as described in section 2.2.21. The filter was re-probed with a random ^{32}P-labeled fragments produced from a 657 bp fragment from pUCAT18 (see section 5.2.3). This probe was predicted to hybridise the following fragments:

CDK3	*Eco*RI/*Bam*HI	2286 bp
	*Sca*I	3362 bp and 1323 bp
	*Pvu*II	5232 bp

The probe was not expected to hybridise to digested MGAT chromosomal DNA. The *cat*-probed membrane was exposed to X-ray film. Photographs of the autoradiographs that resulted from the two different probings are shown in Figure 5.3.4.

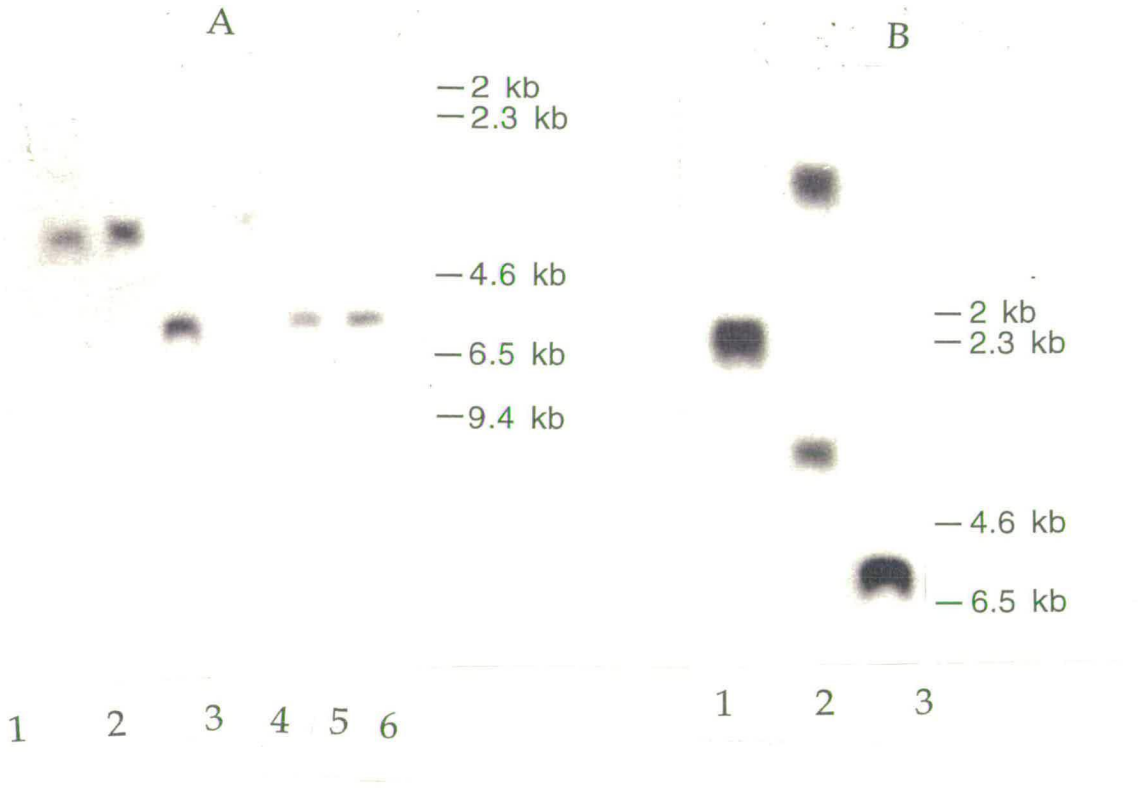

Figure 5.3.4. Photographs of the autoradiographs resulting from probing immobilised digested CDK3 and MGAT chromosomal DNA with (A): a randomly labeled ^{32}P-labeled probe derived from the 1165 bp *Bam*HI restriction product from pCD101 which contains the 3′ 102 bases of *ftsK* and the entire *lolA* gene and (B) a random ^{32}P-labeled probe produced from a 657 bp *Eco*RI-*Bsu*36I fragment from pUCAT18. The lane order in both (A) and (B) is CDK3 *Eco*RI/*Bam*HI (lane 1) CDK3 *Sca*I (lane 2) CDK3 *Pvu*II (lane 3), MGAT *Eco*RI/*Bam*HI (Lane 4) MGAT *Sca*I (lane 5) MGAT *Pvu*II (lane 6). The *cat* probe did not hybridise to MGAT DNA and so have been omitted from (B). The fragment sizes of the λ *Hind*III markers are shown.

The pattern of hybridization was as anticipated. The chromosomal *ftsK* gene had been replaced with $ftsK_{\Delta 54\text{-}2201}$::*cat*. Figure 5.3.5 shows the organization of the 20 minute region in CDK2.

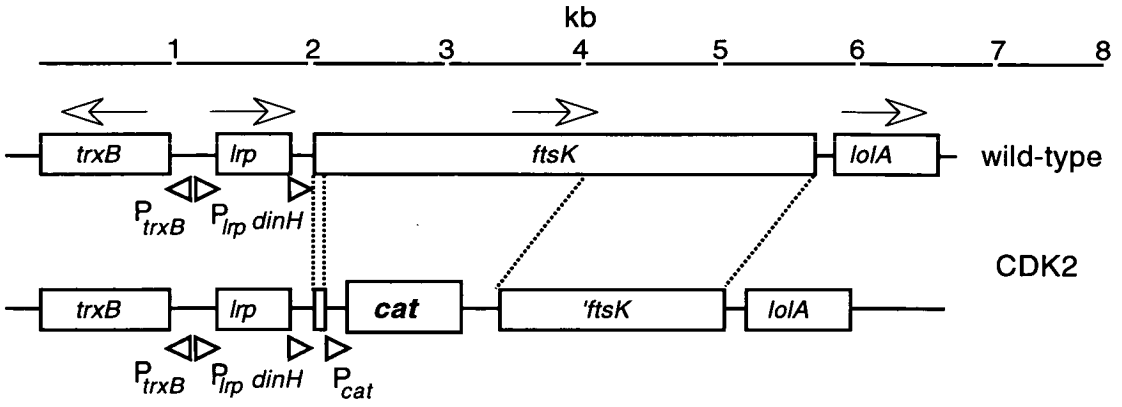

Figure 5.3.5. The organization of the 20 minute region in wild-type *E. coli* and CDK2. The presence of $ftsK_{677}$::*cat* was confirmed by both PCR and Southern analysis.

5.3.5 Depletion of FtsK from CDK2

The effect of depleting FtsK from CDK2 was determined by culturing CDK2 *pcnB*::*kan* (pBADK) at 37°C in LB-broth containing chloramphenicol, ampicillin and kanamycin and arabinose. After 120 minutes the culture was diluted 1:5 into pre-warmed LB-broth with antibiotic supplements containing either arabinose or glucose. The growth curve of CDK2 *pcnB*::*kan* (pBADK) is shown in Figure 5.3.6.

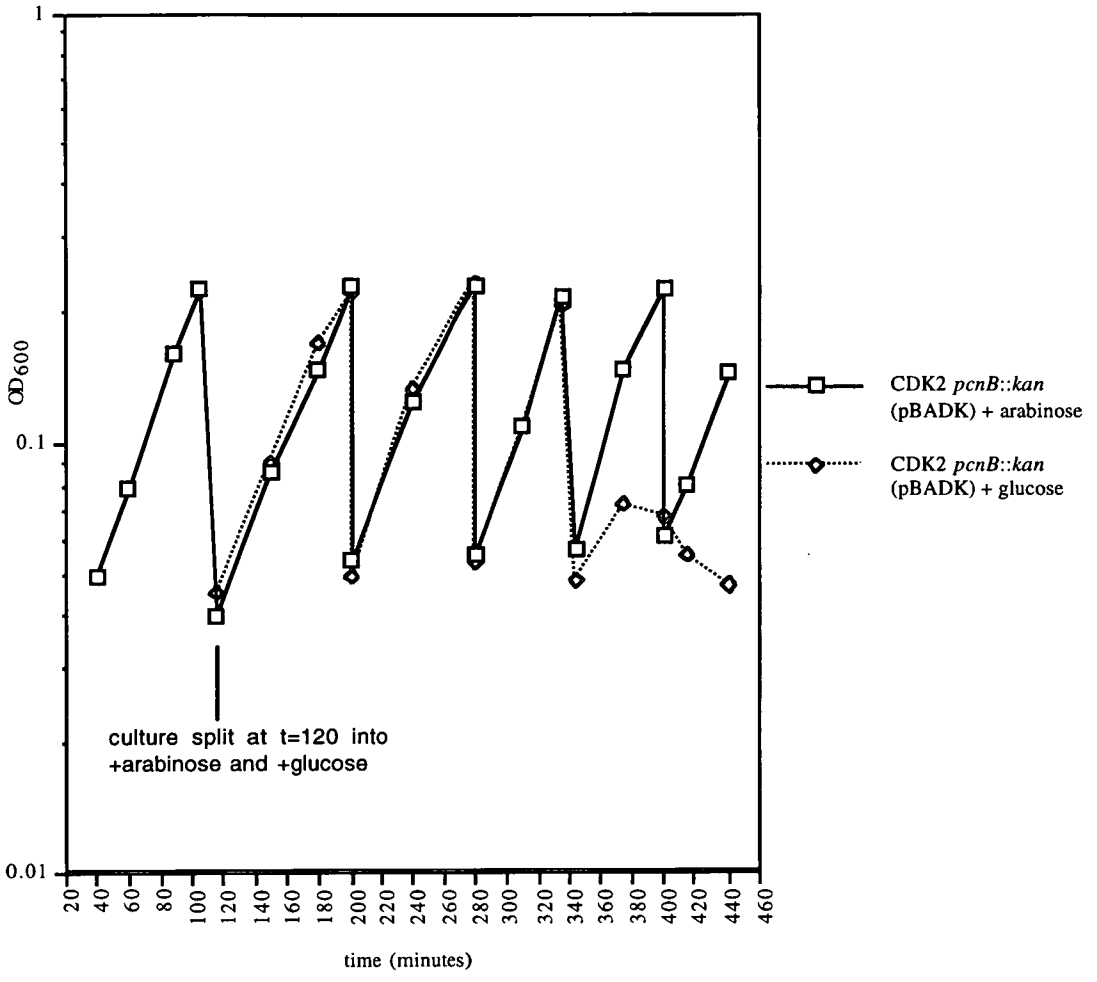

Figure 5.3.6. Growth curve of CDK2 *pcnB::kan* (pBADK) in 0.2% arabinose and 0.2% glucose containing LB-broth. 180 minutes after the switch from 0.2% arabinose containing media to 0.2% glucose containing media filaments were apparent. After 270 minutes of growth in arabinose containing medium the filaments began to lyse.

Microscopic examination of the cultures revealed that CDK2 *pcnB::kan* (pBADK) grown in the presence of arabinose formed a mixture of normal cells and short and long filaments. The filaments could be a result of the loss of pBADK from the cell, which would result in the depletion of FtsK. CDK2 *pcnB::kan* (pBADK) grown in glucose containing media formed filaments with evidence of lysis. This lysis was probably the cause of the drop in the growth rate of CDK2 *pcnB::kan* (pBADK) grown in the presence of glucose seen in Figure 5.3.6. Phase contrast micrographs of CDK2 *pcnB::kan* (pBADK) grown in arabinose and glucose are shown in Figure 5.3.7.

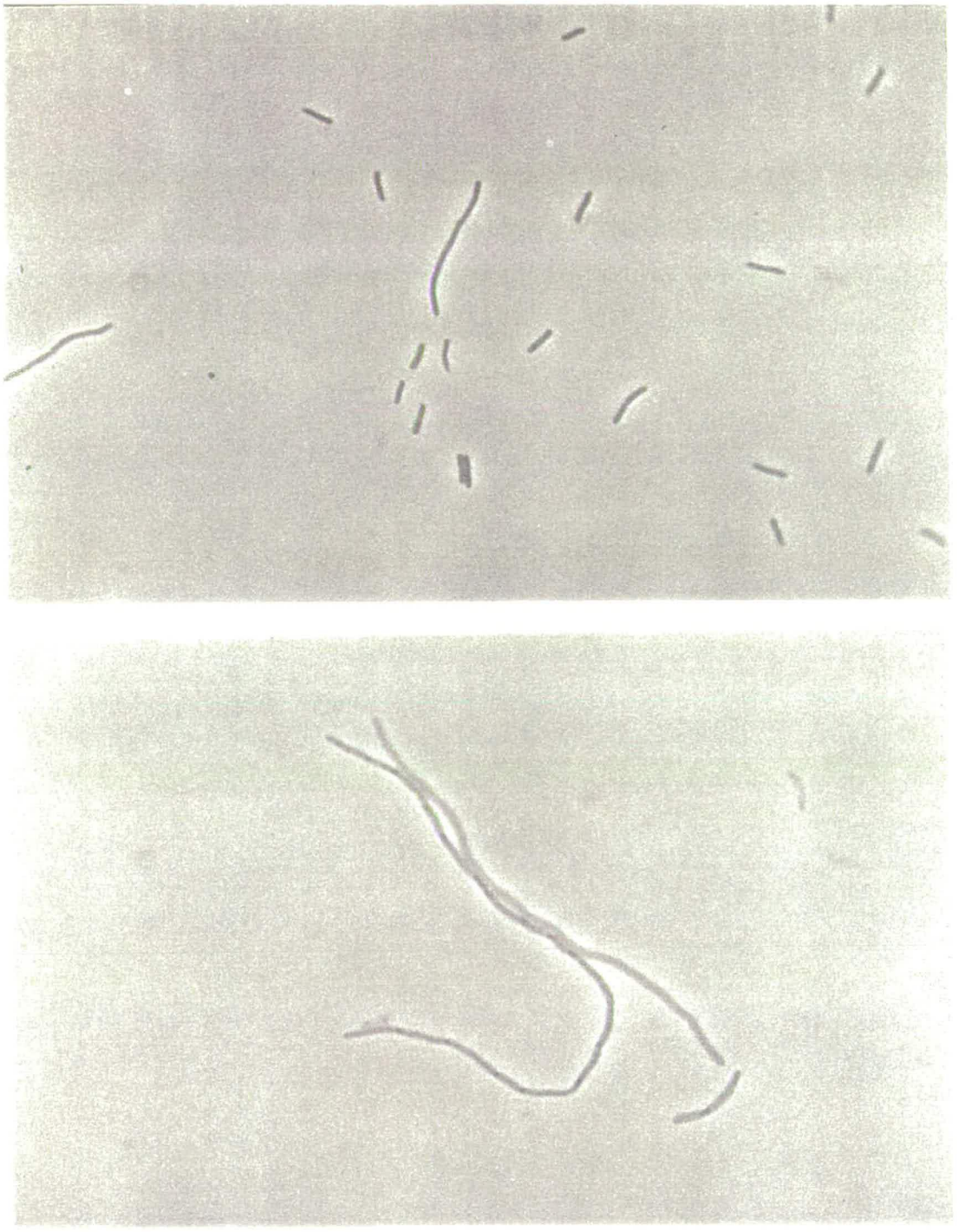

Figure 5.3.7. Phase contrast micrographs of CDK2 *pcnB*::*kan* (pBADK) grown in LB-broth 0.2% arabinose (top) and 3 hours after the switch into 0.2% glucose containing LB-broth (bottom).

FtsK was shown to be essential for cell division as replacement of bp 54-2201 with the *cat* gene from pBR325 resulted in the formation of aseptate filaments that eventually lyse. Whether or not *ftsK* should called an essential gene is a open for debate. In wild-type cells, *ftsK* is an essential gene, as inactivation lethally blocks cell division. The finding that $ftsK_{\Delta 54\text{-}2201}$::*cat* can be suppressed by the insertional inactivation of *dacA* or the overproduction of FtsN leads to the suggestion that mutations in these genes or their regulatory regions could render *ftsK* inessential.

5.3.6 Localization of FtsZ in FtsK depleted filaments

Samples of CDK2 *pcnB*::*kan* (pBADK) cultured in the presence of arabinose and glucose were fixed and processed for IFM and probed with F168-12, an anti-FtsZ monoclonal antibody (Voskuil *et al.*, 1994) and Cy3-conjugated anti-mouse secondary antibody (Jackson Research) as described in section 2.4.6. This would show whether the filaments formed by CDK2 *pcnB*::*kan* (pBADK) cultured in the presence of glucose contained FtsZ rings and hence give an insight as to the mechanism of filamentation. The immunofluorescence micrographs of the cells grown in both arabinose and glucose containing LB-broth are shown in Figure 5.3.8.

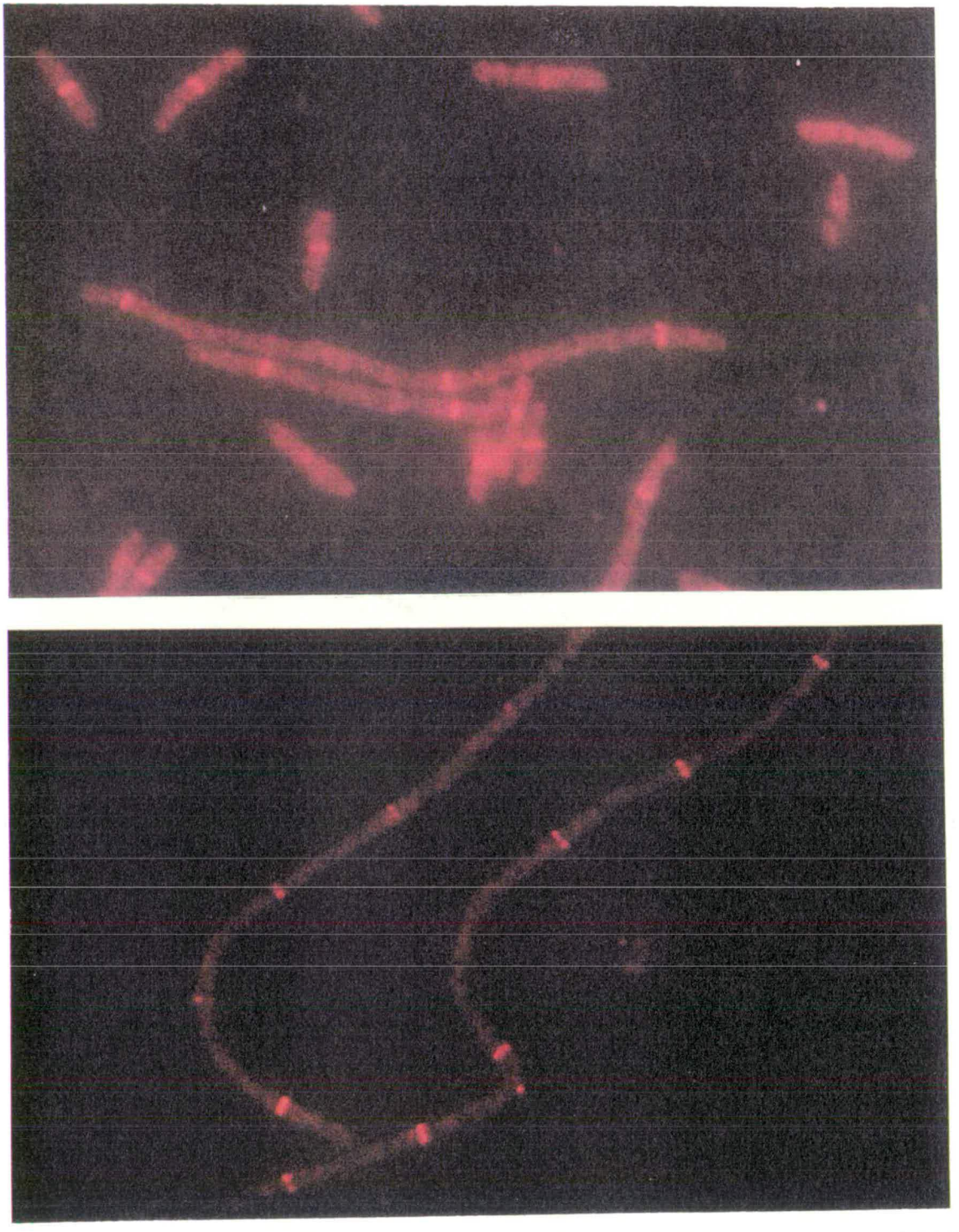

Figure 5.3.8. Immunofluorescence micrographs of CDK2 *pcnB::kan* (pBADK) cultured in the presence of 0.2% arabinose (top) and 0.2% glucose (bottom). FtsZ rings form in cells grown in both 0.2% arabinose and 0.2% glucose containing LB-broth.

FtsZ rings form in cells from both cultures. This was expected from CDK2 *pcnB::kan* (pBADK) grown in the presence of arabinose as most of the cells are actively dividing. Filamentous CDK2 *pcnB::kan* (pBADK) grown in glucose containing LB-broth also possesses FtsZ rings, although fewer than would be expected than if FtsZ ring formation was unaffected. This could be due to the fixing procedure or an actual consequence of FtsK depletion. The formation of the smooth sided filaments by the depletion of FtsK from CDK2 *pcnB::kan* (pBADK) is not, therefore, due to the inhibition of FtsZ ring formation. It appears that the FtsZ rings cannot invaginate due to the lack of FtsK. Deep invaginations do not appear, as would be expected if *ftsK* behaved as a classical late acting division gene (Begg *et al.*, 1985; Begg *et al.*, 1995). This phenotype is similar to that seen with a null-allele of *ftsN*, another late acting cell division gene (Addinall *et al.*, 1997; Dai *et al.*, 1993). An *ftsN::kan* allele is lethal unless extra copies of *ftsN* are supplied on a plasmid and depletion of FtsN from a strain bearing this allele causes the cells to form smooth-sided filaments, much the same as with the depletion of FtsK from CDK2 *pcnB::kan* (pBADK). This apparent contradiction warrants further investigation.

5.3.7 The N-terminus of FtsK exhibits a cell division function

The construction of CDK2 presented the opportunity to investigate the function of the N-terminal membrane spanning domain and the C-terminal SpoIIIE-like regions of FtsK. pBADK′ and pBADK′3 and a new plasmid pKC1 were transformed into MG1655 *pcnB::kan*.

MG1655 *pcnB::kan* (pBADK′), (pBADK′3) and (pKC1) were transduced with the lysate grown on K2:1. Selection was for Cmp/Amp/Kan resistant transductants. In the cases of the transduction of MG1655 *pcnB::kan* (pBADK′) and (pBADK′3) with the K2:1 lysate the selective plates also contained arabinose. Colonies formed on the MG1655 *pcnB::kan* (pBADK′) x K2:1 transduction plates but no colonies formed on the plates from the transductions involving MG1655 *pcnB::kan* (pBADK′3) and (pKC1). Several of the colonies from the MG1655 *pcnB::kan* (pBADK′) x K2:1 transduction were plated onto LB-agar plates containing ampicillin and chloramphenicol supplemented with either arabinose or glucose. Colonies formed on the arabinose containing plates but not on the glucose containing plates. pBADK′ could complement the

ftsK$_{\Delta 54\text{-}2201}$::*cat* allele in a *pcnB*::*kan* background, whereas it appeared that pBADK'3 and pKC1 could not. To examine whether the reduced copy number of pBADK'3 and pKC1 in the *pcnB*::*kan* background had an effect on the transduction pBADK', pBADK'3 and pKC1 were transformed into the wild-type strain MG1655. MG1655 (pBADK'), (pBADK'3) and (pKC1) were transduced with the lysate grown on K2:1 to introduce the *ftsK*$_{\Delta 54\text{-}2201}$::*cat* allele. Selection was for Cmp/Amp resistant transductants. As in the earlier transduction the selective media on which the MG1655 (pBADK') and (pBADK'3) transductants were plated was supplemented with arabinose. This time, transductants appeared on the MG1655 (pBADK') and (pBADK'3) plates [forming CDK2 (pBADK') and (pBADK'3)]. Again, no colonies appeared on the MG1655 (pKC1) x K2:1 transduction plates. The *ftsK*$_{\Delta 54\text{-}2201}$::*cat* allele could not be complemented by a plasmid bearing the 3' 1786 bp of *ftsK*.

CDK2 (pBADK') and (pBADK'3) were plated on LB-agar containing 100 μg ml^{-1} ampicillin and either arabinose or glucose. CDK2 (pBADK') formed colonies on both the glucose and the arabinose containing plates. CDK2 (pBADK'3) formed colonies on the arabinose containing plates but not on the glucose containing plates. CDK2 (pBADK') behaved in a similar manner to CDK2 (pBADK), in that it appeared that transcription from repressed P_{BAD} to complement or suppress the lethality of *ftsK*$_{\Delta 54\text{-}2201}$::*cat*. This had been shown previously, as pBADK' complemented *ftsK*$_{\Delta 54\text{-}2201}$::*cat* in the presence of arabinose but not glucose in a *pcnB*::*kan* derivative of CDK2. pBADK'3 could not support growth in a CDK2 *pcnB*::*kan* strain, as no colonies formed after the transduction of MG1655 *pcnB*::*kan* (pBADK'3) with the lysate grown on K2:1, whether the transductants were plated on media containing arabinose or glucose.

The reason for this copy-number dependent difference in the complementation of *ftsK*$_{\Delta 54\text{-}2201}$::*cat* by the 225 aa truncated FtsK' polypeptide encoded by pBADK'3 (FtsK'$_{225}$) compared to pBADK and pBADK' (FtsK'$_{583}$) could be because FtsK'$_{225}$ is not as stable as wild-type FtsK or FtsK'$_{583}$. Another possibility is that FtsK'$_{225}$ has reduced activity and does not complement the lethality of *ftsK*$_{\Delta 54\text{-}2201}$::*cat* as well as wild-type FtsK or FtsK'$_{583}$ and either more of the truncated peptide or more transcript is required for growth.

Phase contrast microscope analysis of CDK2 (pBADK'3) revealed that when grown in arabinose most of the cells have a normal

morphology, with some (12%) forming chains and filaments (8%) (Figure 5.18). As with depletion of wild-type FtsK from CDK2, depletion of $FtsK'_{225}$ from CDK2 (pBADK'3) resulted in the formation of aseptate filaments, many of which were lysed (Figure 5.3.9). The formation of the filaments in the arabinose culture could be due to loss of the pBADK'3, which would result in the depletion of $FtsK'_{225}$ and hence filamentation

Figure 5.3.9. CDK2 (pBADK'3) grown in the presence of 0.2% arabinose (top) and 0.2% glucose (bottom) after three hours.

These findings suggest that in *E. coli*, only the N-terminal 225 aa of FtsK are required for cell division and viability. It appeared from these results that the C-terminus of FtsK was dispensable. CDK1 and CDK2 both carry large regions of the 3' end of the *ftsK* gene after the insertion point of the *cat* gene (see Figures 5.2.8 and 5.3.5 respectively) and it was not possible to rule out the involvement of any potential peptides produced from this region. The promoters for *ftsK* and *cat* could transcribe this region and there are many potential ATG translation initiation codons in-frame with *ftsK*. *In vitro* transcription/translation of a clone of this region has been shown to produce an polypeptide (Diez *et al.*, 1997). A possible role for this polypeptide (if it is produced *in vivo*) in the phenotypes exhibited by CDK1 and CDK2 could not be ruled out, although with CDK2, the transcription of this region alone could not support cell growth. In order to verify that the chain formation observed in CDK1 and CDK2 *pcnB*::*kan* (pBADK'3) grown in arabinose containing media was solely due to the production of the N-terminal 225 aa of FtsK, a new deletion mutant was constructed.

5.4 Construction of CDK5

The same approach used in the construction of CDK1 and CDK2 was taken to produce a new insertion/deletion derivative of *ftsK*. pCDCAT was digested with *Bsu*36I and *Nru*I. This produced fragments of 1502 and 7741 bp. The 7741 bp fragment was purified and end-filled with Klenow (section 2.2.9). Residual salts, protein and nucleotides were removed using the Promega DNA Clean-up kit and the fragment religated to form pCDCAT2 (Figure 5.4.1)

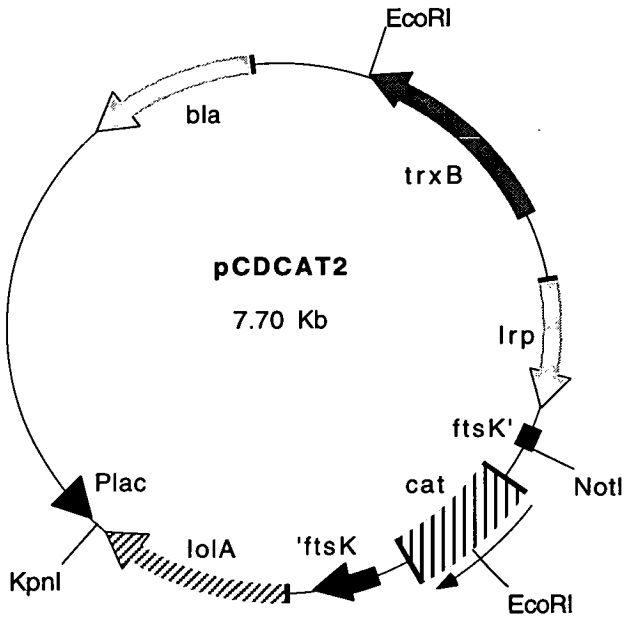

Figure 5.4.1. pCDCAT2, a deletion derivative of pCDCAT. The *cat* gene is transcribed in the same direction as ftsK from its own promoter.

pCDCAT2 has bp 54-3369 of the *ftsK* gene replaced with the cat gene of pBR325 (*ftsK*$_{\Delta 54\text{-}3369}$::*cat*). The *cat* gene transcribes in the same orientation as *ftsK*. The 3' end of the *ftsK* is out of frame with *cat* and the region coding for the nucleotide binding motifs in the C-terminal region of FtsK had been deleted. It was predicted that pCDCAT2 encoded no functional FtsK derived truncated peptides, unlike pKBCAT and pCDCAT.

5.4.1 Replacement of *ftsK* with *ftsK*$_{\Delta 54\text{-}3369}$::*cat*

Like pCDCAT, pCDCAT2 did not complement *ftsK44* (Ts) at the non-permissive temperature. The following transductions and screening was performed with the aid of N. McLennan. pCDCAT2 was transformed into MGAT (pGB101). A P1 lysate was grown on MGAT (pGB101) (pCDCAT2) at 30°C. This lysate was used to transduce W3110 (pGB101). Selection was for Tet/Cmp/Spec resistant transductants. 1/10 of the transduction mixture was plated onto Tet/Spec containing media, this would act as a control and indicate whether efficient transduction had taken place. 16 Tet/Cmp/Spec resistant transductants were isolated along

with approximately 1500 Tet/Spec resistant transductants from the control. The Tet/Cmp/Spec were screened for loss of ampicillin resistance. Two of the isolates proved to be Tet/Cmp/Spec resistant and ampicillin-sensitive. One of these isolates was chosen for further study and named NACK6 (pGB101).

5.4.2 Southern blot analysis of NACK6

Chromosomal DNA was isolated from NACK6 (pGB101) as described in section 2.2.4. The isolated NACK6 chromosomal DNA had pGB101 DNA co-purified as a contaminant. NACK6 and MGAT chromosomal DNAs were digested with *Eco*RI/*Bam*HI, *Sca*I and *Pvu*II. The digested DNA was subjected to agarose gel electrophoresis then blotted and immobilised onto nylon membrane. The immobilised DNA was then probed with the same randomly ^{32}P-labeled *'ftsK–lolA* fragment described in section 5.2.3. The probe was predicted to hybridise to the following fragments:

NACK6	*Eco*RI/*Bam*HI	3446 bp (1165 bp)
	*Sca*I	1900 bp (11.3 kb)
	*Pvu*II	3879 bp (8.1 kb)

The fragment sizes in parentheses are fragments derived from pGB101.

MGAT	*Eco*RI/*Bam*HI	3446 bp
	*Sca*I	5359 bp
	*Pvu*II	5427 bp

A photograph of the resultant autoradiograph is shown in Figure 5.4.2.

The filter was stripped as described in section 2.2.21 and reprobed with the random ^{32}P-labeled probe produced from the cat gene of pUCAT18 (described in section 5.2.3). The probe was predicted to anneal to the following fragments.

NACK6	*Eco*RI/*Bam*HI	823 bp
	*Sca*I	1323 bp and 1889 bp
	*Pvu*II	3879 bp

The probe was not expected to hybridise to the MGAT DNA. The photograph of the resultant autoradiograph is shown in Figure 5.4.2.

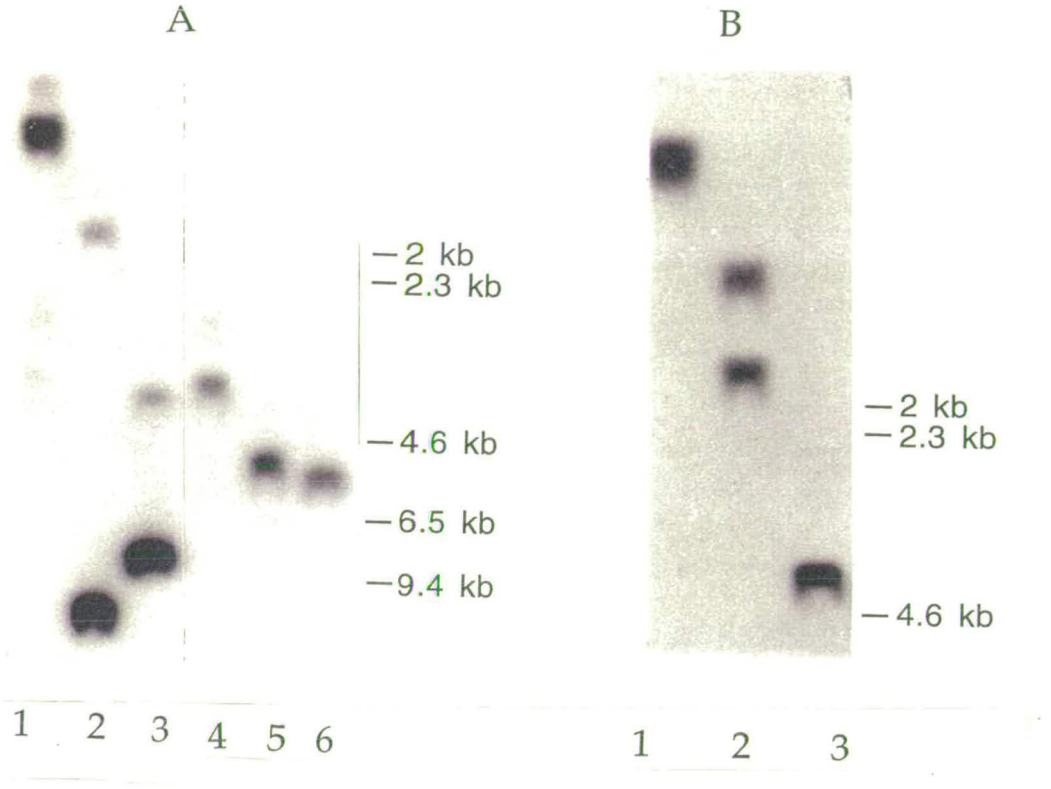

Figure 5.4.2. The photographs of the autoradiographs resulting from probing immobilised digested CDK5 and MGAT chromosomal DNA with (A): a randomly labeled ^{32}P-labeled probe derived from the 1165 bp *Bam*HI restriction product from pCD101 which contains the 3′ 102 bases of *ftsK* and the entire *lolA* gene and (B) a random ^{32}P-labeled probe produced from a 657 bp *Eco*RI-*Bsu*36I fragment from pUCAT18. The lane order in both (A) and (B) is CDK3 *Eco*RI/*Bam*HI (lane 1) CDK3 *Sca*I (lane 2) CDK3 *Pvu*II (lane 3), MGAT *Eco*RI/*Bam*HI (Lane 4) MGAT *Sca*I (lane 5) MGAT *Pvu*II (lane 6). The *cat* probe did not hybridise to MGAT DNA and so these lanes have been omitted. The fragment sizes of the λ *Hind*III markers are shown.

The pattern of hybridization was as predicted, therefore, NACK6 had the wild-type *ftsK* gene replaced with the interrupted *ftsK* gene from pCDCAT2 ($ftsK_{\Delta 54\text{-}3369}$::*cat*). A diagram of the organization of the 20 minutes in NACK6 is shown in Figure 5.4.3.

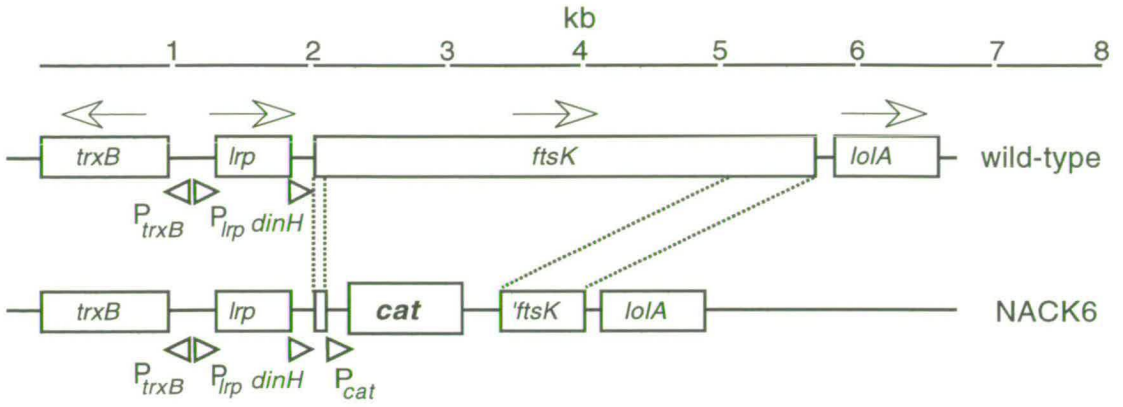

Figure 5.4.3. The organization of the 20 minute region in wild-type *E. coli* NACK6. This was confirmed by Southern blot analysis.

5.4.3 Depletion of FtsK from CDK5

A P1 lysate was grown on NACK6 (pGB101) and used to transduce MG1655 *pcnB*::*kan* (pBADK). Selection was for Tet/Spec/Amp resistant transductants in the presence of arabinose. 100 of the Tet/Spec/Amp resistant transductants were screened for the co-transduction of $ftsK_{\Delta 54\text{-}3369}$::*cat* in the presence of arabinose. 58 (58%) of the isolates proved to be Tet/Cmp/Spec/Amp resistant. One isolate was chosen and the strain named CDK5 *pcnB*::*kan* (pBADK). CDK5 *pcnB*::*kan* (pBADK) was plated onto LB-agar containing Tet/Cmp/Spec/Amp and either arabinose or glucose. Growth was observed on the arabinose- but not the glucose-containing plates.

The effect of depleting FtsK from CDK5 *pcnB*::*kan* (pBADK) was determined by culturing CDK5 *pcnB*::*kan* (pBADK) at 37°C in LB-broth containing chloramphenicol, ampicillin and kanamycin and arabinose. At 100 minutes the culture was washed twice with LB broth and diluted 1:5 into pre-warmed LB-broth with antibiotic supplements containing either arabinose or glucose. The growth curve of CDK2 *pcnB*::*kan* (pBADK) is shown in Figure 5.4.4.

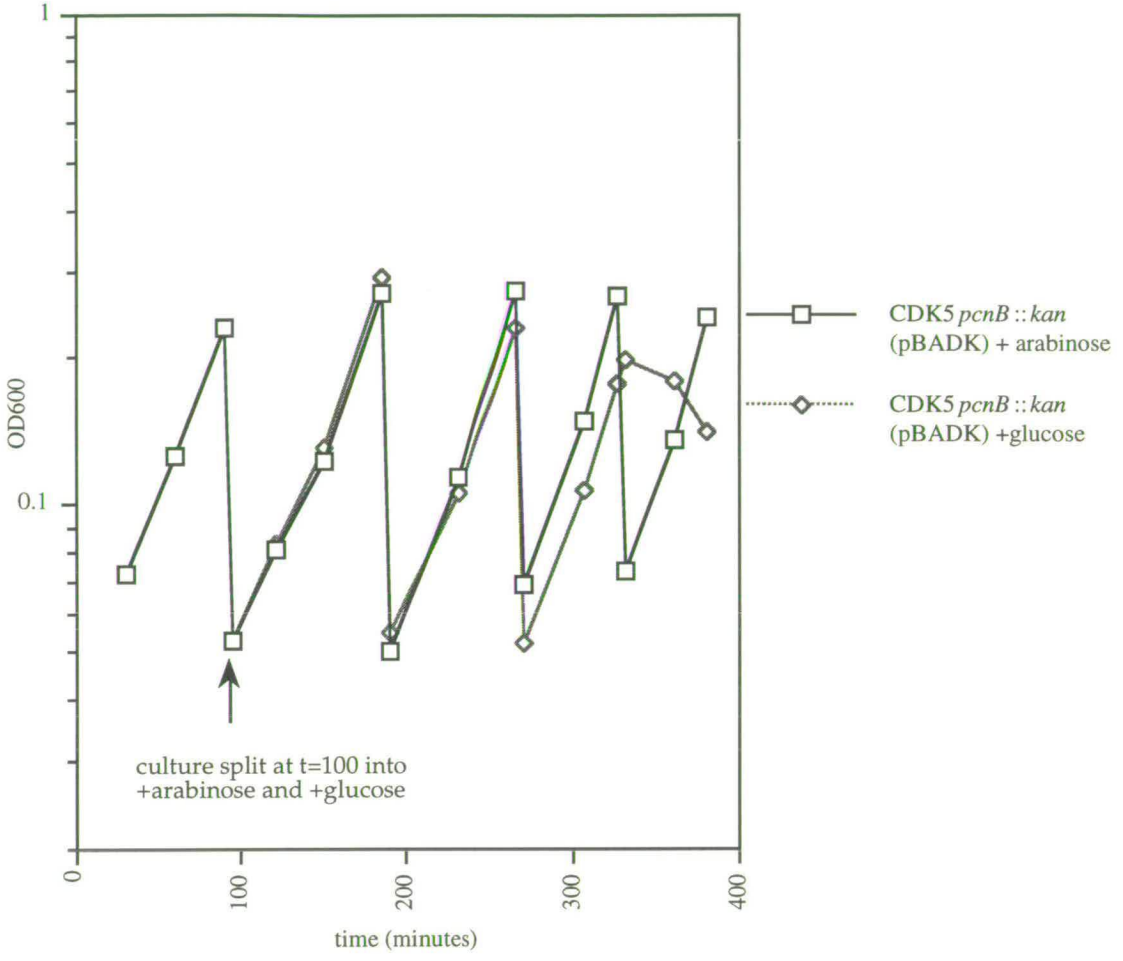

Figure 5.4.4. Growth curve of CDK5 *pcnB*::*kan* (pBADK) in 0.2% arabinose and 0.2% glucose containing LB-broth. 120 minutes after the switch from 0.2% arabinose containing media to 0.2% glucose containing media filaments began to form. After 320 minutes lysis of the filaments was apparent.

The effects of depletion of FtsK from CDK5 *pcnB*::*kan* (pBADK) were similar to the depletion of FtsK from CDK2 *pcnB*::*kan* (pBADK) (Figure 5.3.6; section 5.3.5), which also resulted in filamentation and lysis. Filaments were apparent after 120 minutes and lysis was apparent 230 minutes after the switch into glucose containing media. The lysis was accompanied by a fall on the optical density of the culture. Phase contrast micrographs of CDK5 *pcnB*::*kan* (pBADK) grown in arabinose for six hours and glucose for four hours are shown in Figure 5.4.5.

Figure 5.4.5. CDK5 *pcnB*::*kan* (pBADK) cultured in arabinose-containing LB-broth for six hours (top) and glucose-containing LB-broth for four hours (bottom).

CDK5 *pcnB*::*kan* (pBADK) behaved in a similar manner to CDK2 *pcnB*::*kan* (pBADK). *ftsK*$_{\Delta 54\text{-}3369}$::*cat* could also be suppressed by the overproduction of FtsN from pKD140 (Dai *et al.*, 1993). Interestingly, unlike *ftsK*$_{\Delta 54\text{-}2201}$::*cat*). *ftsK*$_{\Delta 54\text{-}3369}$::*cat* could not be transduced into a strain in which *dacA* had been insertionally inactivated (SP1070) (N. McLennan, pers. comm.). *ftsK*$_{\Delta 54\text{-}3369}$::*cat* and *ftsK*$_{\Delta 54\text{-}2201}$::*cat* therefore, do have different properties. It could be possible that the extra 3' region of ftsK present in *ftsK*$_{\Delta 54\text{-}2201}$::*cat* could be responsible for the suppression of *ftsK*$_{\Delta 54\text{-}2201}$::*cat* by the insertional inactivation of *dacA*. The final steps in the closure of the septum is thought to involve a novel situation where pentapeptide, rather than tripeptide acceptors within the peptidoglycan are required (J.-V. Höltje, pers. comm.). Thus, the D-ala:D-ala carboyxpeptidase activity of PBP5 (encoded by the *dacA* gene) would be detrimental to septum closure. It could be postulated that one of the functions of the N-terminus of FtsK in cell division is to 'silence' the function of PBP5 at the terminal stages of septum formation, resulting in an increase of the amount of pentapeptide acceptor suitable for septum closure. Thus, the *dacA*::*kan* allele could suppress *ftsK44* and *ftsK*$_{\Delta 54\text{-}2201}$::*cat* as there would be reduced degradation of pentapeptide acceptors even in the absence of the proposed PBP5 silencing function of the N-terminus of FtsK. If this is true, then it is surprising that *dacA*::*kan* did not suppress *ftsK*$_{\Delta 54\text{-}3369}$::*cat*. The only difference between *ftsK*$_{\Delta 54\text{-}2201}$::*cat* and *ftsK*$_{\Delta 54\text{-}3369}$::*cat* is the presence in *ftsK*$_{\Delta 54\text{-}2201}$::*cat* of an extra 1168 bp of the 3' of the end of the *cat* gene insertion. This region of DNA could produce a peptide (Diez *et al.*, 1997), that could be important for the proposed *dacA*::*kan* suppression.

To examine whether FtsZ ring formation was affected in CDK5 *pcnB*::*kan* (pBADK), the strain was cultured in both arabinose and glucose containing LB-broth were processed for IFM as described in section 2.5.6. The anti-FtsZ monoclonal antibody F168-12 was used as primary label, followed by Cy3-conjugated anti-mouse secondary antibody. Photographs of the stained cells are shown in Figure 5.4.6.

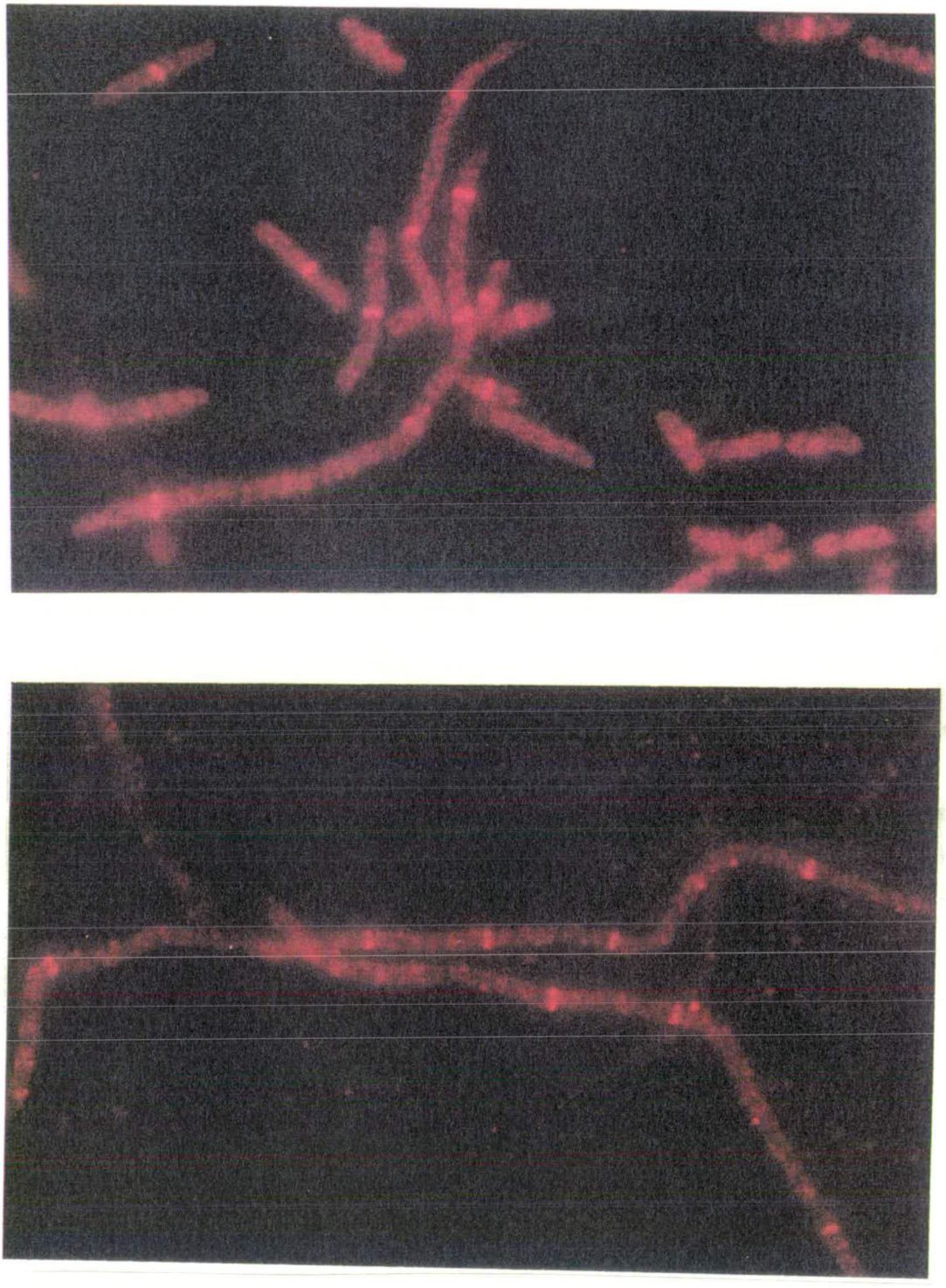

Figure 5.4.6. IFM of CDK5 *pcnB*::*kan* (pBADK) grown in arabinose containing (top) and glucose containing LB-broth for four hours. FtsZ rings appear in both the normal cells and in FtsK depleted filaments.

CDK5 *pcnB*::*kan* (pBADK) grown in the presence of arabinose had FtsZ ring structures. There were a number of filamentous cells in the population, possibly due to the loss of pBADK or because the amount of FtsK expressed from pBADK was insufficient. It is unlikely that the filaments could form due to overproduction of FtsK because FtsZ rings where present, which is not the case when FtsK is overproduced in wild-type cells (section 3.3.3). Filamentous CDK5 *pcnB*::*kan* (pBADK) cultured in glucose containing media also had FtsZ rings, although, as has been observed previously, there were less FtsZ rings seen per cell as would be expected if FtsZ ring formation of stability was unaffected (Pogliano *et al.*, 1997; section 3.6; section 5.3.6). The reason for the filamentous morphology of CDK5 *pcnB*::*kan* (pBADK) grown in glucose was not due to the lack of FtsZ rings. It cannot be stated, however, that FtsZ ring function is unaffected, as it appears that depletion of FtsK prevents FtsZ ring constriction at an early stage.

5.4.4 Chromosome segregation on FtsK depleted filaments

The chromosomes of CDK5 *pcnB*::*kan* (pBADK) and CDK2 *pcnB*::*kan* (pBADK) cultured in either arabinose- or glucose-containing LB-broth for four hours were stained with 4,6-diamidino-2-phenylindole (DAPI). Both CDK5 *pcnB*::*kan* (pBADK) and CDK2 *pcnB*::*kan* (pBADK) were previously shown to produce a mixture of filaments and normal-sized cells in arabinose-containing media (sections 5.3.2 and 5.4.3, respectively). The normal sized cells of both strains (from the arabinose-containing media) showed a standard pattern of chromosome segregation, with one or two chromosomes present per cell (Figure 5.4.7). The filaments present either had normally segregated or obviously mis-segregated chromosomes (Figure 5.5.7). It was proposed in section 5.3.5 that the formation of filaments by CDK2 *pcnB*::*kan* (pBADK) in arabinose-containing media could be due to loss of pBADK and the resultant depletion of FtsK. CDK2 *pcnB*::*kan* (pBADK) and CDK5 *pcnB*::*kan* (pBADK) from the glucose-containing media formed filaments with either a normal or mis-segregated chromosome distribution (Figure 5.4.8). The nucleoids in the cells displaying abnormal chromosome segregation had a condensed, rounded appearance.

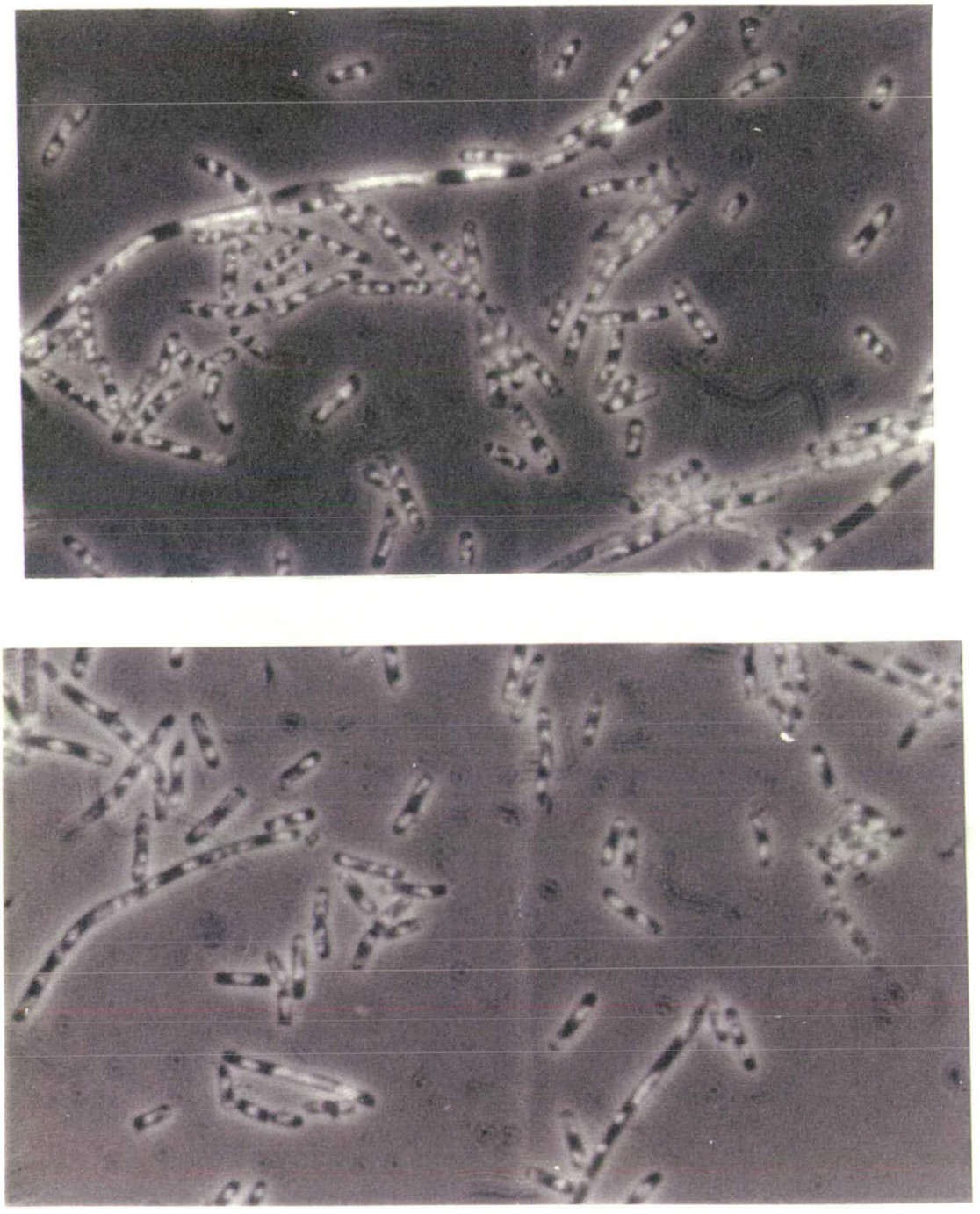

Figure 5.4.7. CDK2 *pcnB*::*kan* (pBADK) (top) and CDK5 *pcnB*::*kan* (pBADK) (bottom) cultured in arabinose containing LB-broth for four hours stained with DAPI. The populations consist of normal sized cells and filaments. The normal sized cells have properly partitioned chromosomes. The filaments display either normal or aberrant nuclear segregation. The nucleoids in the cells displaying abnormal chromosome segregation had a condensed, rounded appearance.

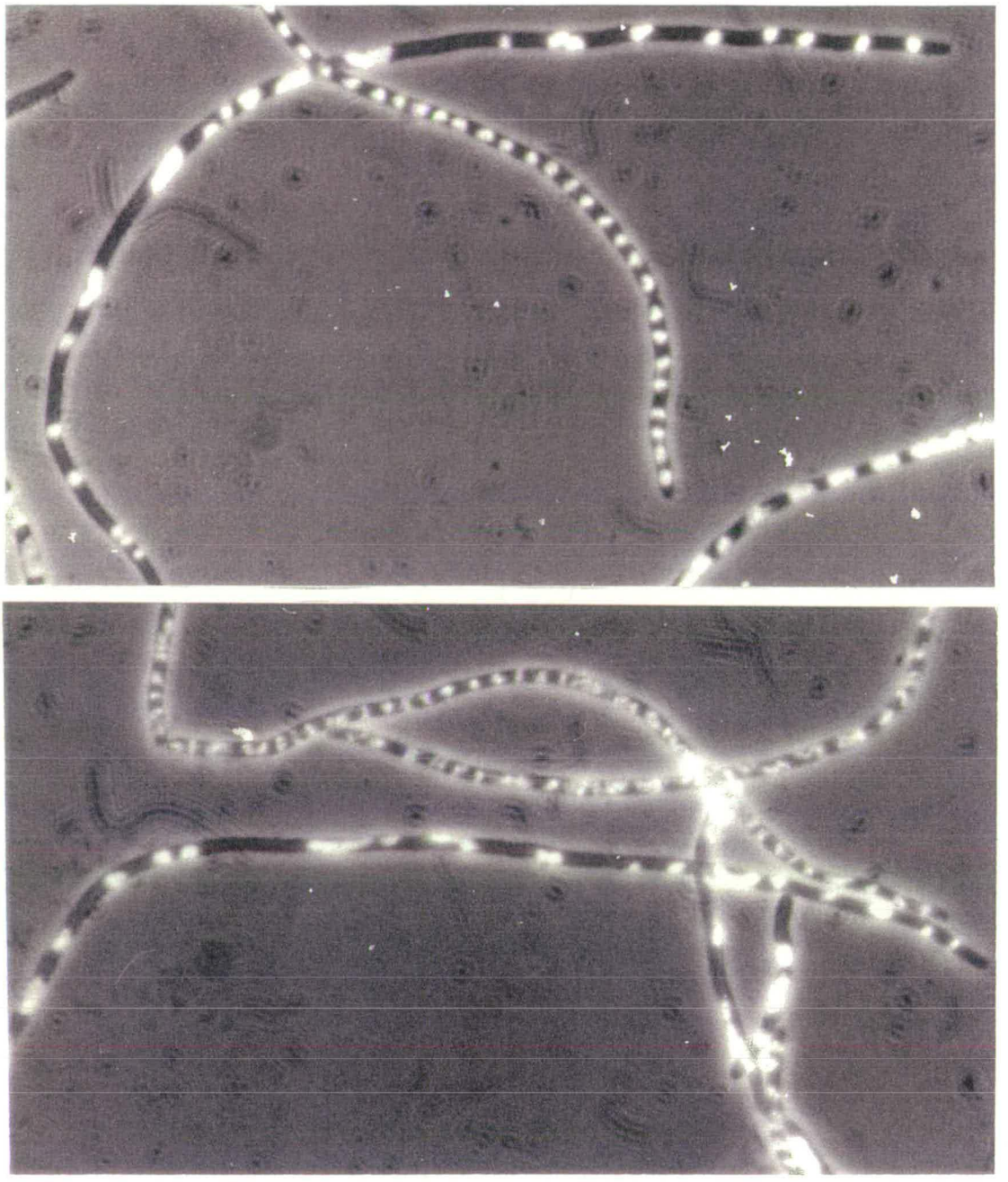

Figure 5.4.8. CDK2 *pcnB::kan* (pBADK) (top) and CDK5 *pcnB::kan* (pBADK) (bottom) cultured in glucose containing LB-broth for four hours stained with DAPI. 70% of the filaments have normally segregated chromosomes. The remaining 30% display a variety of chromosome abnormalities. These abnormalities typically take the form of large, unevenly distributed masses of DNA, small masses of DNA and long strands of DNA. There are also large regions of the filaments that do not contain any DNA. The nucleoids in the cells displaying abnormal chromosome segregation had a condensed, rounded appearance.

Approximately 30% of the filaments displayed some evidence of abnormal chromosome segregation. This took the form of misplaced chromosomes, large masses of DNA and long regions of the filament without any chromosomal DNA. Inactivation of other *fts* genes results in filamentation but not aberrant chromosome segregation (Lutkenhaus and Mukherjee, 1996). One possible explanation for the mixed phenotype of the culture is that FtsK assists chromosome segregation. If FtsK does influence DNA segregation, the function is not required when chromosomes partition without error, as 70% of cells depleted of FtsK show normal patterns of chromosome partition. If there is a partition problem encountered during segregation, FtsK could assist in the partitioning of the mis-segregated chromosomes. For this reason, only a proportion of the cells depleted of FtsK have abnormally segregated chromosomes. A role for FtsK in DNA segregation could be implied by virtue of its sequence similarity to the SpoIIIE family of DNA translocases. Alternatively, FtsK could be part of a system linking chromosome partition and cell division, as the protein has a function in both these processes. Another explanation for the mixed phenotype is that the complementing plasmid carrying *ftsK* could recombine with the *ftsK* locus, thus providing a functional chromosomal copy of *ftsK*,. Excision of the plasmid could result in the mutant allele switching places from the chromosome to the plasmid. This, however, would not result in filamentation because the genotype of the strain would be wild-type with respect to *ftsK*. The plasmids used for the insertional inactivation of *ftsK* (pKBCAT, pCDCAT and pCDCAT2) did not result in filamentation when present in wild-type strains.

The availability of specific antibodies to FtsK would help address the question of the level of FtsK in the depletion strains. Also, repeating the experiment in a *recA*$^-$ strain would prevent any recombination between pBADK and the chromosome.

A third and potentially more troubling reason why only 30% of the cells exhibit a mutant chromosome segregation phenotype was brought to light by Siegele and Hu (1997). The work focused on the application of the P_{BAD} containing expression vectors for tight repression and graduated induction of cloned genes. The green fluorescent protein from *Aequorea victoria* was cloned downstream of P_{BAD} in pBAD18. At low levels of arabinose (0.0016%) it was discovered that a small proportion of the cells

in the population fluoresced brightly. At a higher concentration of arabinose (0.04%) the majority of the cells fluoresced. The authors speculate that the low levels of induction of P_{BAD} by low concentrations of arabinose that was seen by Guzman *et al.*, (1995) was not due to a graduated induction of the promoter, but was rather due to come of the cells in the population being competent for the uptake of arabinose and scavenging the arabinose from the culture medium. This would result in of very few cells being maximally induced, thus giving the impression of low levels of induction by the whole population. This effect is analogous to the induction of the *lac* promoter at subsaturating levels of inducer that was first reported by Novick and Weiner (1957). It appears possible, therefore, that the 70% of the CDK2 *pcnB*::*kan* (pBADK) cells that do not exhibit a chromosomal DNA segregation defect could be scavenging the small amounts of residual arabinose from the culture medium and there is therefore enough FtsK in these cells to support segregation. However, the abnormal cell division phenotype (i.e. filamentation) observed indicates that FtsK is either depleted from these cells or that there is insufficient FtsK present for cell division but enough for proper chromosome partitioning. Guzman *et al.* (1995) noted that P_{BAD} should be repressed by glucose even in the presence of arabinose and as the FtsK depletion was carried out in the presence of glucose we can assume that P_{BAD} is repressed to its fullest extent. Other promoter systems, such as the *lac* promoter or the T7 promoter system could be employed in future studies and the results compared to those found here.

The finding by Steiner and Kuempel (1998) that resolution of chromosome dimers by recombination at *dif* requires cell division raised the possibility that the chromosomal abnormalities observed when FtsK was depleted from the cell could be the result of the lack of resolution of multimerised chromosomes. The chromosome dimers that could potentially form would not be able to resolve at *dif* because cell division had been blocked. This was addressed by comparing the chromosomes in the filaments caused by the depletion of FtsK from CDK5 *pcnB*::*kan* (pBADK) to filaments induced by the inhibition of PBP3 with 10 μg ml^{-1} benzyl penicillin. Some of the filaments formed by the inhibition of PBP3 have slight invaginations, as a result of penicillin insensitive peptidoglycan biosynthesis (PIPS) (Nanninga, 1991; section 1.1.6). Visualization of the nucleoids within these filaments by staining with

DAPI revealed what would commonly be called a normal pattern of chromosome segregation (Figure 5.4.9).

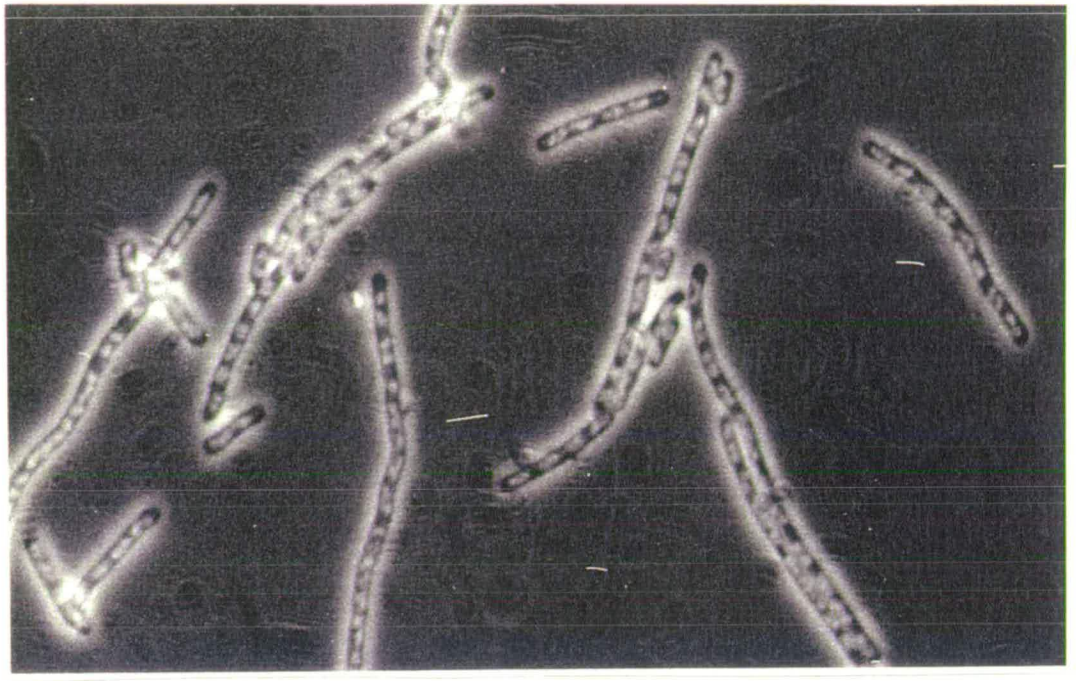

Figure 5.4.9. Nucleoid staining of filaments caused by the inhibition of PBP3. The segregation of chromosomes appears to be unaffected but some of the touching nucleoids could be dimers.

It is reasonable to assume that some of the chromosomes which appear to be touching in the PBP inhibited filaments, which would have previously been attributed to chromosome segregation not yet being completed, could be dimers that could not resolve at *dif* because division had been blocked. However, comparing this pattern of nucleoid segregation to that seen in CDK5 *pcnB*::*kan* (pBADK) filaments depleted of FtsK reveals that there is a considerable difference in the segregation phenotypes of the two strains. As mentioned above, the nucleoid segregation pattern of 30% of the CDK5 *pcnB*::*kan* (pBADK) filaments depleted of FtsK is highly disturbed and is a much more severe phenotype than the PBP3-inhibited filaments. It cannot be stated, however, that the lack of *dif* mediated resolution in CDK5 *pcnB*::*kan* (pBADK) filaments depleted of FtsK does not contribute to partition abnormalities.

The chromosomes of the chain forming strains CDK1 and CDK2 (pBADK'3) and CDK5 (pBADK'3) grown in the presence of arabinose were visualized after staining with DAPI. The chromosomal *ftsK* locus from these strains does not produce functional FtsK. CDK5 (pBADK'3) carries the 228 bp of the 3' end of *ftsK* on the chromosome which is unlikely to produce a peptide. The chromosomes within the chains from all three cultures were found straddling the invaginations or adopted polar locations. A proportion of the normal cells also had nucleoids positioned towards the cell pole. In the cells exhibiting aberrant chromosome segregation the nucleoids had a rounded, condensed appearance (Figure 5.4.10)

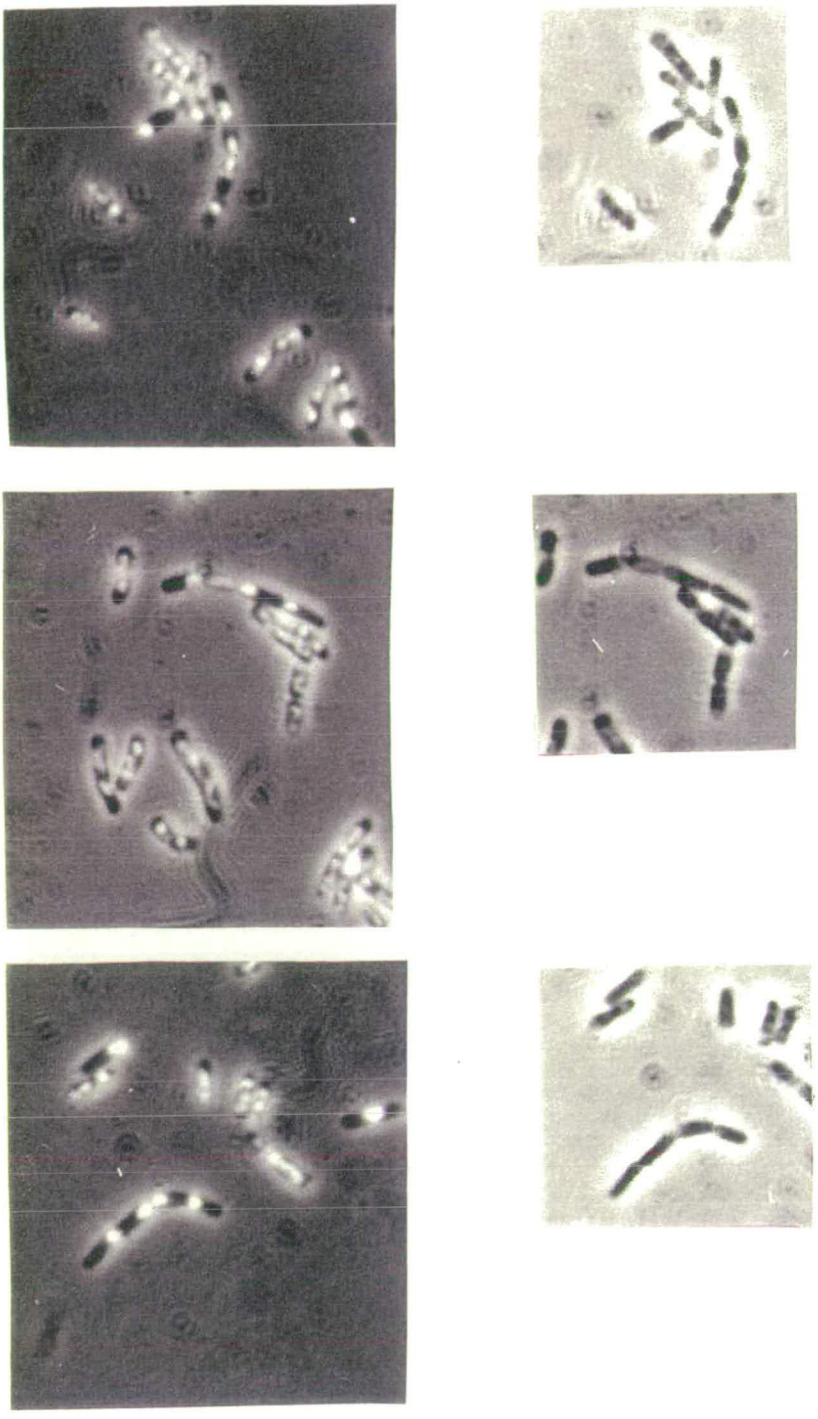

Figure 5.4.10. CDK1 (top) and CDK2 (pBADK'3) (middle) and CDK5 (pBADK'3) (bottom) grown in the presence of arabinose. Between 10–15% of the cells were in the form of chains. The majority of these chains had DNA trapped by the invaginated septum, or had DNA at the poles of the component cells of the chain. Approximately 12% of the normally shaped cells had their chromosomes positioned at the cell poles. The remainder of the cells had normally partitioned chromosomes.

The N-terminal 225 aa of FtsK, which complements the inhibition of cell division imparted by the *ftsK*-null alleles in CDK2 and CDK5 could not complement the chromosome segregation defect. The formation of chains could be due to the presence of chromosomes preventing the completion of the septum. A full-length clone of *ftsK* can suppress this defect (see above). Thus, the N-terminus of FtsK could encode a cell division function and the C-terminus a chromosome partition function. The three cultures display a remarkably similar phenotype. The proportion of chains in each of the cultures was found to be between 10-15%. It appears that the presence of the 3' end of *ftsK* in both CDK1 and CDK2 does not have a noticeable effect, as these strains behave the same as CDK5, which has the most of the 3' end of *ftsK* deleted.

When FtsK'$_{583}$ was overproduced in a wild-type strain from pBADK', chain formation was observed in a proportion of the population (section 3.4.1). Examination of the nucleoid distribution within these chains showed that these chains also has trapped and polar nuclei. Thus, the earlier proposal that FtsK'$_{583}$ could displace wild-type FtsK from the division site (section 3.4.1), leading to the loss of a function due to the absence of the C-terminus of FtsK could be correct. This second, C-terminus encoded function is proposed to be involved in DNA segregation.

5.5 Conclusion and discussion

Only 225 aa of the N-terminus of FtsK is required for cell division. Depletion of this peptide from the *ftsK*-null strains null CDK2 and CDK5 resulted in filamentation and eventual lysis. The N-terminus of FtsK could, therefore, be the region of the protein specifically involved in cell division. The mutation in FtsK44 is located at aa 80 (a glycine to alanine substitution), within the region found to support cell division. When this FtsK'$_{225}$ is supplied from a plasmid in CDK2 and CDK5 a 10–15% of the cells form chains. The chromosomal DNA within these chains was found to be trapped by the septa and at the poles of the cells. Depletion of FtsK or FtsK'$_{225}$ from CDK2 and CDK5 resulted in the formation of aseptate filaments which eventually lyse. the *ftsK*-null alleles in CDK2 and CDK5 could be suppressed by overproduction of FtsN. This is the first

report of FtsN suppressing a null-allele of a cell division gene. Insertional inactivation of *dacA*, which encodes PBP5 has previously been shown to suppress the temperature-sensitive nature of *ftsK44* (Begg *et al.*, 1995). It was shown that insertional inactivation of dacA could suppress the lethality of the $ftsK_{\Delta 54\text{-}2201}$::*cat* allele but not $ftsK_{\Delta 54\text{-}3369}$::*cat*. A role was proposed for the N-terminus of FtsK in silencing the action of PBP5 during the final stages of septum formation, or septum closure. In preventing the D-ala:D-ala carboxypeptidase function of PBP5 during septum closure, the pentapeptide thought to be required for this process could be presented. There is no data to suggest that FtsK completes the septum closure itself.

The nucleoid distribution within these filaments is abnormal in 30% of the population. FtsK, therefore, has at least two functions, cell division and chromosome segregation. The finding that only a proportion of the cells in the population exhibit partition defects is possibly due to FtsK being required when chromosome partition is not carried out correctly. In the *ftsK*-null mutants depleted of FtsK or complemented by $FtsK'_{225}$, there is no C-terminal domain of FtsK present to carry out this 'rescue' function and so the partition defects become apparent. It has recently been shown that cell division is required for *dif*-mediated resolution of dimeric chromosomes (Steiner and Kuempel, 1998). It is unlikely that inhibition of *dif*-mediated dimer resolution that could occur due to the filamentation caused by the depletion of FtsK is the only reason for the partition defect, although it could contribute to the effect. Since FtsK is localised to the septum during division a role for FtsK in both cell division and ensuring correct partition can be envisioned. A role for FtsK in chromosome partition was suggested by Begg *et al.* (1995) because of its sequence similarity to the SpoIIIE family of DNA translocases. The study of the involvement of FtsK in partition could be greatly enhanced by the availability of the *ftsK*-null alleles described in this chapter.

CHAPTER 6
SUMMARY AND FUTURE PERSPECTIVES

Chapter 5. Summary and future perspectives

The conclusion of this work is that *ftsK* is both a cell division and chromosome partition gene. The division function of FtsK is essential in wild-type cells but is not in some mutant backgrounds. FtsK is, therefore, an unsuitable target for anti-microbial agents. Depletion of FtsK from the cell results in filamentation and defective chromosome partition. Complementation of null-alleles of *ftsK* can be achieved with a 225 aa N-terminal peptide, which has the cell division function. Cell division is incomplete in a proportion of these cells, resulting in the formation of chains. It is proposed that this chain formation is due to trapping of nuclei at the incomplete septa which would prevent closure of the septum. The C-terminus of FtsK is dispensable, but its omission from the *ftsK*-null strains also results in chain formation with trapped nuclei at the uncompleted division site. The identification of more suppressors of both the *ftsK* (Ts) and null strains would lead to a greater insight into FtsK function.

Overproduction of FtsK results in lethal filamentation. This filamentation is independent of the SOS response. It is possible that FtsK could be involved in the *sfi*-independent pathway of division inhibition, which is *lexA* dependent and requires DNA damage for induction. *ftsK* is preceded by an SOS-inducible promoter that could result in an increase in FtsK levels during the SOS-response.

There is much work still to be done on *ftsK*. The transcriptional organization of the region is an important area of study that needs to be investigated. The findings by Begg *et al.* (1995) that a region upstream of *lrp* was required for complementation of the *ftsK44* (Ts) mutant and the presence of *dinH* upstream of *ftsK* (Lewis *et al.*, 1992) hints at the complex transcriptional controls that could be involved in the regulation of *ftsK* expression. The topology of the protein and subcellular localization of FtsK are also targets for further research.

Investigation of the interactions of FtsK with other cell division proteins would give an important insight into the co-ordination of division proteins at the division site. Purification of FtsK and the characterization of its biochemical properties would be informative and give a more detailed picture of the overall function of FtsK.

The influence of FtsK on partition also needs to be more fully characterized. Whether FtsK has a direct or indirect role in chromosome segregation is still not fully known. The mechanism by which the C-terminus of FtsK affects cell division might be elucidated further by mutagenesis of this region.

CHAPTER 7
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