

A study of the *Escherichia coli* cell cycle

**A dissertation
presented for the Degree of Doctor of Philosophy**

**By
Guowen Liu**

**Institute of Cell and Molecular Biology
The University of Edinburgh**

March 2000

DECLARATION

I declare that the composition of this thesis was all my own work, except where stated.

Guowen Liu

March 19, 2000

Abstract

The study of *Escherichia coli* cell cycle

Guowen Liu

2000

The *ftsK* gene of *Escherichia coli* encodes a 147kDa peptide, which consists of three distinct domains; namely a conserved N-terminal domain, a proline and glutamine rich region, and a C-terminal domain with consensus nucleotide-binding pocket (Begg *et al.*, 1995). The N-terminus is essential for cell division (Begg *et al.*, 1995; Draper *et al.* 1998; Wang and Lutkenhaus 1998) and targets the whole protein to the septum (Yu *et al.*, 1998 and Wang and Lutkenhaus, 1998). The C-terminal domain of FtsK has high sequence similarity to SpoIIIE, a protein involved in transferring chromosomal DNA from mother cell to pre-spore in *Bacillus subtilis* (Begg *et al.*, 1995; Wu and Errington, 1994). In order to determine whether the whole FtsK protein or its C-terminal region has a function in chromosome partition in *E. coli*, a strain named GLC600 was constructed. In this strain the N-terminal part of FtsK was transcribed from its native promoters, but the expression of the whole protein was under the control of the arabinose-inducible *P_{BAD}* promoter. It was found that depletion of the whole protein resulted in the appearance of about 15% of cells with abnormally located chromosomes and also in cell division-dependent SOS induction. Collaborating with Peter Kuempel's lab in USA we found that the C-terminus (also called FtsKc in this thesis) was required for chromosomal dimer resolution. By comparing the effects of inactivation of FtsKc or XerC (a protein required for dimer resolution) on SOS induction, the evidence suggests

that FtsKc is required only for chromosomal dimer resolution. These results suggest that chromosome dimers are resolved only during cell division and at the septation site.

The phenomenon of cell division-dependent SOS induction in FtsK-depleted GLC600 cells indicated that improperly located chromosome DNA could be guillotined by the septum, suggesting that chromosome partition mutants should have continuous SOS induction. Based on such a consideration, a method was developed to isolate putative chromosome partition mutants. A study of the mutants obtained in this way showed that the RuvABC proteins, which are required for the resolution of recombination intermediates, are involved in chromosome partition in wild type cells. However, over-expression of RuvAB caused inhibition of cell division in both wild type cells and in *recA*⁻ cells in which recombination intermediates cannot form. Together with the report that RuvAB acts at arrested replication forks (Seigneur *et al.*, 1998), this suggested that cell division was coupled with chromosome replication. It was found that over-expression of RuvAB could slow down the elongation of chromosome replication and also cause the repression of transcription of cell division genes in the 2min region of the *E. coli* chromosome. To confirm that cell division and chromosome replication are coupled, nalidixic acid treatment and thymine starvation were used to block chromosome replication and it was found that blocking chromosome replication caused the repression of transcription of cell division genes in the 2min region. Thus, cell division is coupled with chromosome replication (possibly elongation) at the level of transcription.

It was also found that over-expression of DsbB, a protein required for protein disulfide bond formation in *E. coli* (Bardwell *et al.*, 1993), resulted in the loss of the rod shape of *E. coli* cells. Over-expression of another gene named *friL*, which is very close to *dsbB* on the *E. coli* chromosome, caused inhibition of cell division at a very early stage. It is proposed that FriL may be a regulator of cell division.

A study of the *Escherichia coli* cell cycle

**A dissertation
presented for the Degree of Doctor of Philosophy**

**By
Guowen Liu**

**Institute of Cell and Molecular Biology
The University of Edinburgh**

March 2000

Acknowledgements

First, I wish to thank my adviser, Professor William D. Donachie, without whom this thesis would not have been possible. I thank him for his great patience, support, advice and encouragement throughout the course of my study in Edinburgh University, Scotland. He also told me a lot about the history of Scotland.

I wish to thank my second supervisor, Dr. David R Leach for giving me advice, encouragement and strains. I learned a lot on the recombination pathways in *E. coli* from him.

I also wish to thank Dr. Ken Begg for giving me encouragement, advice and strains. During this period he also gave me many helps concerning the life of my family. I have enjoyed his novels very much.

A special thanks to my colleague in Lab 820, Darwin Building. I thank Sean for helping prepare solutions, providing strains and many other help; Auste, Dave, Peter, Dawn and John for being nice labmates and sharing joys; Utah, Neil, Medhat and Craig for spending some time together in the same lab. I would also like to thank Millie for holding exciting parties in her nice home.

I also want to thank many other people in the Darwin Building, especially Laura and other persons in the medium room on 8th floor; Nicola on 7th floor for helping sequence DNA; and many, many other nice people.

I thank Prof. Ken Murray in Darwin Trust for generously providing financial support to pay my tuition fees and living expenses, which makes my study in Edinburgh University possible.

Finally and most importantly, I wish to take this opportunity to thank my mother, Keru Yuan, for her great love, patience, support and inspiration. Without her hard work I do not think I can enter any university. I also want to thank my father and brothers for their deep affection. I am very appreciated to my dear wife's support, understanding and deep love and also thank her for waiting late at night.

Table of Contents

Declaration	i
Abstract	ii
Title	iv
Acknowledgements	v
Table of contents	vii
Chapter 1. Introduction	
Cell division	1
Chromosome segregation	14
Chapter 2. Studies on the <i>ftsK</i> gene of <i>E.coli</i>	
Identification of a new cell division mutant as an <i>ftsK</i> mutant	17
The C-terminal domain of FtsK is involved in chromosome partition in <i>E.coli</i>	26
Location of the C-terminus of FtsK protein to the cytoplasm	47
The C-terminal domain of FtsK is required, and probably required only for, chromosome dimer resolution during cell division	53
Chapter 3. Isolation of potential chromosome segregation mutants in <i>E.coli</i>	
Introduction	70
Materials and Methods	72
Results	76
Summary	90
Chapter 4. RevAB is required for both chromosome replication and segregation in <i>E.coli</i>	
Introduction	91

Materials and methods	94
Results	97
Summary	104
Chapter 5. Overexpression of <i>dsbB</i> and <i>friL</i> genes	
Overexpression of <i>dsbB</i> causes cells to become spherical	113
Overexpression of <i>friL</i> causes cells to become filamentous	119
Chapter 6. Cell division is co-ordinated with chromosome replication at transcriptional level in <i>E.coli</i>	
Introduction	129
Materials and methods	131
Results	133
Summary and discussion	138
References	153
Appendix	184

Chapter 1. Introduction

This introduction is intended to provide background information about the cell cycle of *Escherichia coli*, especially on cell division and chromosome partition.

1. Cell division

Briefly, the cell cycle of wild type *E. coli* includes several continuous steps (Fig. 1.1). First, the newborn cell increases its mass and volume and this causes the elongation of the rod-shaped bacterium. When the ratio of cell volume to replication origins reaches a particular constant value, new replication is initiated and the synthesis of daughter chromosomes continues steadily. After the new born chromosomes have been segregated the cell will constrict in the middle of its long axis and finally divide into two new-born cells (Donachie, 1993).

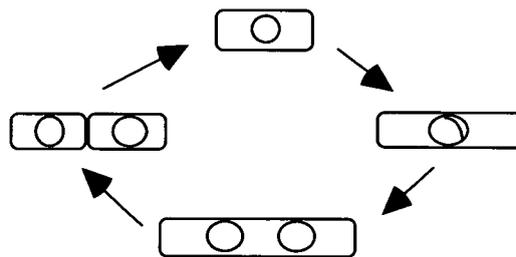


Figure 1.1. Diagram showing the *E. coli* cell cycle.

If cell division is blocked in the cell cycle, the cell will continue its elongation to form a long filament, before eventually lysing. Most of the cell division genes were discovered by isolating *fts* (filamentous of temperature sensitive) mutants, where division was blocked at non-permissive temperature, but neither DNA replication nor segregation was affected. Division proteins act in a coordinated way to bring about the ingrowth of cytoplasmic membrane, peptidoglycan and outer membrane. Together with the septum-specific peptidoglycan synthesis enzymes, the concerted action of these proteins results in the formation of a covalently linked double layered septum. The hydrolysis of the bonds between the two layers followed by the invagination of the outer membrane will at last complete the separation of the two daughter cells (Donachie, 1993).

Several cell division genes have been identified. At least nine of them have been confirmed and well studied. The nine cell division proteins encoded by these genes are called FtsZ, FtsA, FtsQ, FtsW, FtsI, FtsL, FtsN, FtsK and ZipA.

1.1. Cell division proteins.

1.1.1. Cell division proteins encoded at the 2 min region.

Among the nine cell division genes, six are clustered in the 2min region (also named *mra* or *dcw* cluster) of the *E. coli* chromosome. The genetic structure of the 2min region is shown in Fig. 1.2.

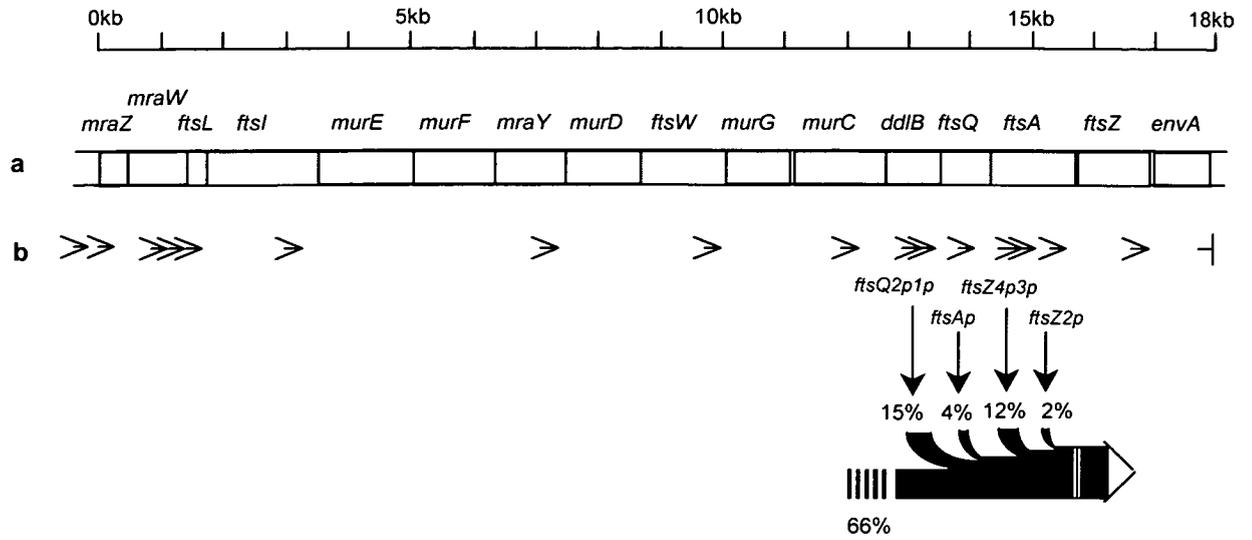


Figure 1.2. Genetic organization of the *mra* cluster at 2min on the *Escherichia coli* chromosome map (a) and contributions of individual promoters to the transcription of *ftsZ* (b). (Vicente *et al.*, 1998; Flårdh *et al.*, 1998; Lutkenhaus and Mukherjee, 1996). Arrows represent detected or putative promoters.

FtsZ:

Among all the cell division genes, *ftsZ* (formerly also called *sfiB* or *sulB*) gets the most attention. It was mapped between *ftsA* and *envA* in the *E. coli* chromosome (Lutkenhaus *et al.*, 1980). By immunoelectronmicroscopy it was found that FtsZ protein formed a ring structure at the cell division site (Bi and Lutkenhaus, 1991). FtsZ was the first protein to be located at the cell division site. Its division site location has been further confirmed by immunofluorescence and GFP fusions (Addinall and Lutkenhaus, 1996; Ma *et al.*, 1996). It is estimated that in *E. coli* there are 5,000-20,000 molecules of FtsZ per cell (Bi and Lutkenhaus, 1991; Pla *et al.*, 1991). This is enough to encircle the cell midpoint roughly 10 to 40 times. Although other division proteins are also localised at the cell centre (see below), FtsZ appears to be the first one (on which all the others depend for subsequent localization), except that it is not clear if FtsW acts earlier than FtsZ (Begg and Donachie, 1985; Addinall *et al.*, 1996; Boyle *et al.*, 1997; Wang *et al.*, 1998).

FtsZ is a soluble cytoplasmic protein that can polymerize *in vitro* to form a variety of ordered structures (Mukherjee and Lutkenhaus, 1994; Bramhill and Thompson, 1994). These structures are composed of linear arrays of FtsZ molecules similar to the protofilaments formed by tubulin. FtsZ is a GTP binding protein and a GTPase (RayChaudhuri and Park, 1992; de Boer *et al.*, 1992). Its polymerization is GTP-dependent (Mukherjee and Lutkenhaus, 1994; Bramhill and Thompson, 1994; Erickson *et al.*, 1996; Mukherjee and Lutkenhaus, 1998; 1999), but the hydrolysis of GTP is not required for polymerization (Mukherjee and Lutkenhaus, 1994).

FtsA:

FtsA acts at a stage in cell division after FtsZ has assembled (Begg and Donachie, 1985). Later studies showed that FtsA is localized at the septum in a ring pattern as previously shown for FtsZ. The localization of FtsA is completely dependent on the localization of FtsZ. It was found that under a variety of conditions that prevented formation of the Z ring, FtsA was unable to localize. In mutants in which FtsZ forms structures in addition to Z rings, or in a strain in which FtsZ is overexpressed and FtsZ

forms spiral tubules that span the length of the filamentous cell, the pattern of FtsA duplicates these structures. These results suggest that the FtsZ ring recruits FtsA to the septum (Ma *et al.*, 1996; Addinall and Lutkenhaus, 1996). The amino acid sequence of FtsA indicates that it is related to the actin/HSP70/sugar kinase family of ATP-binding proteins (Bork *et al.*, 1992), which provides a basis to model its structure (Sanchez *et al.*, 1994). It has been shown that FtsA is present in different cell compartments depending on its phosphorylation state. The FtsA fraction isolated from the cytoplasm is phosphorylated and capable of binding ATP, while the membrane-bound form is unphosphorylated and does not bind ATP. A variant protein FtsA104, which cannot be phosphorylated because the predicted phosphorylatable residue has been replaced by a non-phosphorylatable one, although is unable to bind ATP *in vitro*, is able to complement the reversible *ftsA2*, the irreversible *ftsA3* and, almost with the same efficiency, the *ftsA16* amber alleles. This suggests that phosphorylation and ATP binding may not be essential for the function of FtsA. Alternatively they may have a regulatory role on the action of FtsA in cell division (Sanchez *et al.*, 1994).

FtsW:

ftsW is an essential cell division gene located in the 2 min region (Ishino *et al.*, 1989; Boyle *et al.*, 1997). FtsW is localized at the septum (Wang *et al.*, 1998) and is an integral membrane protein (Ikeda *et al.*, 1989; Khattar *et al.*, 1994). It seems that FtsW acts at an early stage in cell division (Begg and Donachie, 1985; Khattar *et al.*, 1994) and plays a role in the stabilization of the FtsZ ring (Boyle *et al.*, 1997). However, it is not clear if FtsW acts before or after FtsZ during septation (Boyle *et al.*, 1997; Wang *et al.*, 1998). FtsW is highly homologous to RodA in *E. coli* and to SpoVE in *B. subtilis* (Ikeda *et al.*, 1989).

FtsQ:

The *ftsQ* gene product is essential for the formation of the cell septum and is made in very small amounts, about 22-25 molecules per cell (Barondess *et al.*, 1991; Carson *et al.*, 1991). The study of alkaline phosphatase fusions of FtsQ showed that the

FtsQ protein is a simple cytoplasmic membrane protein with an approximately 25-amino-acid cytoplasmic domain and an approximately 225-amino-acid periplasmic domain (Barondess *et al.*, 1991; Carson *et al.*, 1991; Storts *et al.*, 1989)

The localization of cell division protein FtsQ in *Escherichia coli* wild-type cells was studied by immunofluorescence microscopy with a specific monoclonal antibody (Buddelmeijer *et al.*, 1998) and with a *gfp-ftsQ* fusion by fluorescence microscopy (Chen *et al.*, 1999). Both studies showed that FtsQ was localized at the division site. GFP-FtsQ localization to the septum depends on the cell division proteins FtsZ and FtsA, but not on FtsL and FtsI, indicating that FtsQ is an intermediate recruit to the division site (Chen *et al.*, 1999). Replacing the cytoplasmic domain and the transmembrane domain of FtsQ with alternative membrane anchors did not prevent localization of FtsQ at the septum (Buddelmeijer *et al.*, 1998; Chen *et al.*, 1999), whereas replacing the periplasmic domain did (Chen *et al.*, 1999). This indicates that the periplasmic domain of FtsQ is necessary and sufficient for septal targeting, as has also been shown for the periplasmic domain of FtsN (see below).

FtsI:

FtsI, also called penicillin-binding protein 3 (PBP3), is a septation-specific peptidoglycan transpeptidase (Ishino and Matsubishi, 1981; for a review see Neu, 1983). FtsI has a simple membrane topology with almost all of the protein (residues 36 to the carboxy-terminus) exposed on the periplasmic side of the cytoplasmic membrane (Bowler and Spratt, 1989). Immunofluorescence microscopy shows that FtsI is localized to the septum (Weiss *et al.*, 1997; Wang *et al.*, 1998). Septal localization of FtsI depends on an intact membrane anchor and also depends upon FtsZ, FtsA, FtsQ, and FtsL, indicating that FtsI is a late recruit to the division site (Weiss *et al.*, 1999). Replacement of the cytoplasmic domain and/or membrane-spanning segment shows that these domains are required to support cell division (Guzman *et al.*, 1997).

FtsL

FtsL, a 13.6-kDa cytoplasmic membrane protein with a cytoplasmic amino terminus, a single membrane-spanning segment and a periplasmic carboxyl terminus, is essential for cell division and is estimated to be present at about 20 to 40 copies per cell (Barondess *et al.*, 1991; Guzman *et al.*, 1992). It is found that FtsL localizes to the cell septum and this localization is dependent upon the function of FtsZ, FtsA and FtsQ, but not FtsI, suggesting FtsL is a relatively late recruit to the ring structure that mediates septation (Ghigo *et al.*, 1999). Both the membrane-spanning segment and the periplasmic domain of FtsL are required for localization to the division site (Ghigo and Beckwith, 2000). Mutagenesis of the periplasmic heptad repeat motif severely impaired both localization and function as well as the ability of FtsL to drive the formation of sodium dodecyl sulfate-resistant multimers *in vitro*. It is believed that most of the FtsL molecule is a helical coiled coil involved in FtsL multimerization (Ghigo and Beckwith, 2000).

1.1.2. Other cell division proteins: ZipA, FtsN and FtsK

ZipA:

ZipA was isolated as an FtsZ binding protein (Hale and de Boer, 1997). It is an integral inner membrane protein and essential for cell division. With a functional ZipA-Gfp fusion protein, it is shown that ZipA is located in a ring structure at the division site (Hale and de Boer, 1997). Its location requires FtsZ, but not FtsA or FtsI, indicating that it is recruited by FtsZ. Normal Z rings are formed in the absence of ZipA, suggesting that ZipA acts after Z ring formation. The interaction between FtsZ and ZipA occurs through their carboxy-terminal domains (Liu *et al.*, 1999). Genetic and morphological evidence show that a 2-fold higher dosage of the division gene *zipA* suppresses thermosensitivity of the *ftsZ84* mutant by stabilizing the labile FtsZ84 ring structure *in vivo*. Purified ZipA promotes and stabilizes protofilament assembly of both FtsZ and FtsZ84 *in vitro* and cosediments with the protofilaments (RayChaudhuri, 1999).

FtsN:

The *ftsN* gene is an essential cell division gene and is located at 88.5 min on the *E. coli* chromosome. It was first isolated as a multicopy suppressor of the *ftsA12*(Ts) mutation (Dai *et al.*, 1993). Multicopy *ftsN* can also suppress *ftsI23*(Ts), *ftsQ1*(Ts), but not the *ftsZ84*(Ts) mutation (Dai *et al.*, 1993). A recent study showed that *ftsK* mutations and deletions can also be suppressed by overexpression of *ftsN* (Draper *et al.*, 1998). Immunofluorescence microscopy shows that FtsN protein is located at the septum and examination of FtsN localization in *fts* mutants indicates that FtsN is incorporated into the ring of proteins after FtsZ, FtsA, FtsQ and FtsI at a late stage in cell division (Addinall *et al.*, 1997).

The 36 kDa FtsN protein is a membrane protein (Dai *et al.*, 1993). Protease accessibility studies of FtsN in spheroplasts and inverted membrane vesicles showed that this protein has a short cytoplasmic N-terminus, a single membrane-spanning domain and a large C-terminal periplasmic domain (Dai *et al.*, 1996). Its cell division function only requires the carboxy domain and the N-terminal cytoplasmic and transmembrane domains of FtsN are required only for transporting the large C-terminus to the periplasm (Dai *et al.*, 1996). Study of a MalG-FtsN fusion where the N-terminal cytoplasmic and transmembrane domains were replaced by a MalG membrane-spanning domain indicates that the information for FtsN localization is supplied by its periplasmic domain (Addinall *et al.*, 1997).

FtsK:

ftsK is located at 20min on the *E. coli* chromosome (Begg *et al.*, 1995). The FtsK protein, a homologue of SpoIIIE in *B. subtilis*, is a large protein consisting of 1330 amino acids with three structural domains (Begg *et al.*, 1995). Only the N-terminal domain of FtsK is required for cell division (Begg *et al.*, 1995; Draper *et al.*, 1998; Wang and Lutkenhaus, 1998). By fusing the N-terminus of FtsK to GFP, it was shown that FtsK localizes to the septum and that an N-terminal fragment as short as 15% of the

protein is sufficient for localization (Yu *et al.*, 1998). FtsK localization requires the function of FtsZ and FtsA but not FtsI and FtsQ (Wang and Lutkenhaus, 1998).

1.1.3. Possible cell division proteins encoded by the terminus region.

It was reported that there is a cluster of cell division genes mapping to the *terC* region of E.coli chromosome, between 33.5 and 36 min (Ben-neria *et al.*, 1995; Dewk *et al.*, 1984). They divided their mutants into eight classes, basing on their map positions and phenotypes at restrictive temperature. These temperature sensitive mutants could replicate and segregate DNA at non-permissive temperature (except group VII, where chromosome replication was blocked), but could not continuously grow after were shifted from 30°C to 42°C. Their phenotypes at 42°C were varied: they formed lemon-shaped cells, long filaments, strings of beads or very small cells or became lysed. The variety of phenotypes displayed by the mutants is similar to those displayed by mutants of the 2min region genes. However, it is still possible that these genes are just regulators of cell division.

1.1.4. Assembly order of cell division proteins at the division site.

According to the above data on cell division proteins, they are probably assembled at the division site in the order FtsZ > FtsW(?) > ZipA > FtsA > FtsK > FtsQ > FtsL > FtsI > FtsN.

1.2. Selection of cell division site.

The mechanism of selective positioning of the Z ring at the mid-cell site is still unknown. Since FtsZ is a cytoplasmic protein, localization of the Z ring and then the division machinery underneath the inner membrane probably needs the assistance of transmembrane protein(s). The location signal that is recognised by FtsZ protein is still unknown. However, important progress has been made from studies on minicell producing mutants. The *min* locus has three genes (*minC*, *minD* and *minE*) and the coordinate action of their gene products is required for proper placement of the division septum (de Boer *et al.*, 1988; 1989). Deletions of *minC*, *minD* or *minCDE* together cause the minicell phenotype, whereas inactivation of *minE* alone causes general inhibition of cell division.

Studies of the effects of expression of the three genes, alone and in all possible combinations, indicates that the *minC* and *minD* gene products act in concert to form a nonspecific inhibitor of septation that is capable of blocking cell division at all potential division sites, and that the *minE* gene codes for a topological specificity factor that prevents the division inhibitor from acting at internal division sites while permitting it to block septation at polar sites in wild-type cells (de Boer *et al.*, 1989). MinC is a general division inhibitor and a proximate cause of the septation block. With a MalE-MinC fusion that retains full biological activity, it was found MinC interacts with FtsZ and prevents its polymerization (Hu *et al.*, 1999). MinD is a membrane-associated ATPase. MinD activates the MinC septation-inhibition-function and is also required for the sensitivity of the MinC system to the MinE topological specificity factor (de Boer *et al.*, 1989; 1992). Its ATPase binding domain is required for the activation of MinC (de Boer *et al.*, 1991). The 88-amino acid MinE protein has two different functional domains. The N-terminal domain (residues 1-22) is responsible for suppression of MinCD inhibitor. The carboxy terminal domain (residues 31-88) is required for the topological specificity of MinE function and affects the site specificity of cell division even when separated from the N-terminal domain. The C-terminal domain is also responsible for MinE homodimerization (Zhao *et al.*, 1995; King *et al.*, 1999). By using a functional

fusion of MinE-Gfp it is shown that MinE forms a ring structure near the middle of young cells. Formation of the MinE ring is MinD-dependent, MinC-independent and importantly, FtsZ-independent (Raskin and de Boer, 1997). The MinE ring can be seen in very young cells but not in deeply constricting cells. Surprisingly, functional Gfp-MinD accumulates alternately in either one of the cell halves in what appears to be a rapidly oscillating membrane association-dissociation cycle imposed by MinE (Raskin and de Boer, 1999). In the absence of the other Min proteins MinD associates with the membrane around the entire periphery of the cell. MinE is capable of redistributing the membrane-associated MinD to either one of the halves of the cell (Raskin and de Boer, 1999; Rowland *et al.*, 2000). By using a functional GFP-MinC fusion protein, it is found that MinC displays a very similar oscillatory behavior which is dependent on both MinD and MinE and independent of FtsZ. GFP-MinC is a cytoplasmic protein in the absence of the other Min proteins. The addition of MinD results in GFP-MinC appearing on the membrane. In the presence of both MinD and MinE, GFP-MinC oscillates rapidly between the halves of the cell (Raskin and de Boer, 1999b; Hu and Lutkenhaus, 1999). These results suggest a model in which the formation of the FtsZ ring at midcell point is directed by the Min system. At an early stage of cell division, MinE forms ring at or near the middle of the cell and this causes rapid relocation of MinC and MinD in between the two halves of the cell to prevent the formation of Z ring in the halves of the cell. The presence of MinE ring at midcell prevents the MinCD inhibitory activity at this point and thus allows the formation of the Z ring only at the middle point of the cell. Other cell division proteins, in an probable order of FtsW(?) > ZipA > FtsA > FtsK > FtsQ > FtsL > FtsI > FtsN, can then be recruited to the Z ring, leading to the synthesis of a new septum. Nevertheless, the Min proteins cannot be the sole means of localising the site of FtsZ polymerization because FtsZ rings form in the centres of *min* mutant cells that lack all of these proteins. In such mutants, FtsZ rings may also form near the cell poles, causing the production of minute "minicells". The function of the Min proteins seems therefore to be to prevent this abnormal localization of cell division proteins, rather than to provide spatial information about the "correct" location at the

cell centre. Both FtsZ and MinE must therefore respond to some other spatial signal, which is as yet unknown.

1.3. Regulation of the transcription of cell division genes *ftsQ*, *ftsA* and *ftsZ* during the change of growth phase.

***SdiA*:**

It is well known that bacteria can modulate their growth according to the environment they are living in. Recent studies have shown that many prokaryotes can even sense cell density by production of extracellular signaling molecules. One of the most famous cases is that of the luminous marine bacteria. (References can be found in Ulitzur and Dunlap, 1995; and Sitnikov *et al.*, 1996.) Luminescence in these bacteria is controlled by autoinduction, a regulatory mechanism that activates light production at high population density and suppresses light production at low population density. Several genetic, physiological and environmental factors contribute to autoinduction. *Bacillus* uses peptides to induce competence and sporulation (Perego and Hoch, 1996), while *Myxococcus* uses a complex mixture of amino acids and fragments of peptidoglycan to control sporulation, social and asocial motility (Kaplan and Plamann, 1996; Kaiser and Losick, 1993). However, the signal molecules most widely used to effect cell density-dependent gene regulation in prokaryotes may be N-acyl-L-homoserine lactones, called autoinducers, which accumulate in a population density-dependent manner (Bainton *et al.*, 1992; Swift *et al.*, 1993). The gene that produces such an inducer in *Vibrio fischeri* is called *luxI*. Autoinduction also needs a transcriptional activator protein, which is LuxR in *V. fischeri*. The activator together with autoinducer activates transcription of the LuxR homolog-dependent transcription (Bainton *et al.*, 1992; Swift *et al.*, 1993). A homolog of LuxR in *E. coli* is SdiA. However, no *luxI* homologue has been found in *E. coli* so far.

SdiA was isolated as multicopy suppressor of cell division inhibition caused by over-expression of MinC/MinD (Wang *et al.*, 1991). It has been shown that SdiA can activate transcription of *ftsQAZ* by acting on the *ftsQ2p* promoter, but not on the *ftsQ1p* promoter or the promoters inside the *ftsQAZ* gene cluster (Wang *et al.*, 1991; Sitnikov *et al.*, 1996). There are contradictory reports about whether conditioned media (media that had previously supported growth of *E. coli*) will up-regulate (Sitnikov *et al.*, 1996) or down-regulate *ftsQ2p* (GarciaLara *et al.*, 1996). However, even in the latter report the down-regulation of *ftsQ2p* coincided with the down regulation of *sdiA* itself. One possible reason for this discrepancy may be the differences between the experimental conditions. It has also been proposed that the expression of *sdiA* is regulated by an extracellular factor (GarciaLara *et al.*, 1996).

RpoS:

Three different kinds of promoter are defined according to their dependence on the growth rate: the 'house-keeper' promoter of many metabolic genes, the stringent promoter found at several rRNA and ribosomal protein genes, and the 'gearbox' at genes whose products are required at higher relative amounts at lower growth rates. RpoS, which encodes the stationary phase sigma factor Sigma S, is one of the sigma factors for gearbox transcription (for reviews see Vicente *et al.*, 1991; Loewen *et al.*, 1998). The sigma S subunit of RNA polymerase (encoded by the *rpoS* gene) is the master regulator in a complex regulatory network that controls stationary-phase induction and osmotic regulation of many genes in *Escherichia coli* (for a review see Hengge-Aronis, 1996).

It was found that disruption of *rpoS* can lower the protein level of FtsZ (Cam *et al.*, 1995) and *ftsQ1p* is sigma S-stimulated (Sitnikov *et al.*, 1996). The most potent promoters in the *ddlB-ftsA* region of the *mra* cluster have been analysed for sigma S-dependent transcription. It was found that only the gearbox promoter *ftsQ1p* is transcribed in vitro by RNA polymerase holoenzyme coupled to sigma S (EsigmaS). This dependency on sigma S is also found in vivo when single-copy fusions to a reporter gene were analysed in *rpoS* and *rpoS*⁺ backgrounds. The rest of the promoters

assayed, *ftsQ2p* and *ftsZ2p3p4p* are preferentially transcribed by EsigmaD, the housekeeper polymerase (Ballesteros *et al.*, 1998).

2. Chromosome segregation

After the termination of chromosome replication, sister chromosomes are partitioned to daughter cells with high accuracy. In wild type *E. coli* strains, anucleate cells are produced at a frequency of less than 10^{-4} (Hiraga, 1992). By visualizing the cellular location of the replication origin (*oriC*), it has been found that chromosome partition is an active process in both *E. coli* (Gordon *et al.*, 1997) and *B. subtilis* (Webb *et al.*, 1997; Lewis and Errington, 1997). Although many genes affecting chromosome partition have been isolated, their roles in this process remain to be further characterized.

In *E. coli*, *mukB*, *mukE* and *mukF* mutants all produce anucleate cells at a high frequency and are temperature sensitive for colony formation (Niki *et al.*, 1991; Yamanaka *et al.*, 1996). These three genes are located at 21min in *E. coli* chromosome. MukB is a large protein (1534 amino acids) and predicted to have five distinct domains: an amino-terminal globular domain which can bind to ATP and GTP, a central region containing two alpha-helical coiled-coil domains and one globular domain, and a carboxyl-terminal globular domain which is rich in Cys, Arg and Lys and required for DNA binding (Niki *et al.*, 1991, 1992; Saleh *et al.*, 1996). MukB is now known to have an "SMC"-like structure with the 2 monomers lying antiparallel (*i.e.* both ends of the dimer consist of 1 N-terminus and 1 C-terminus (Melby *et al.*, 1998). MukF has a leucine zipper structure and an acidic domain and both are required for its function. Overproduction of MukF protein in wild-type strain cause the production of anucleate cells, while overproduction of either MukE or MukB did not cause the defect (Yamanaka *et al.*, 1996). Mini-F and P1 plasmids can be maintained normally in *mukB* mutants (Ezaki *et al.*, 1991; Yamanaka *et al.*, 1996). By co-immunoprecipitation and

sucrose gradient sedimentation experiments, it was found that MukB forms a complex with MukF and MukE. MukF and MukE bind the C-terminal globular domain of MukB. MukF is indispensable for an interaction between MukB and MukE, while MukF itself is able to associate with MukB even in the absence of MukE (Yamazoe *et al.*, 1999).

Structural analysis shows that MukB protein belongs to the structural maintenance of chromosomes (SMC) protein family (Melby *et al.*, 1998). It is also shown that *mukB* and *seqA* are mutual suppressors. A *seqA* null mutation can suppress temperature sensitivity, anucleate cell production and poor nucleoid folding seen in the *mukB* strain, whereas inactivation of the *mukB* gene can suppress filamentation, asymmetric septation and compact folding of the nucleoids observed in the *seqA* strain. Membrane-associated nucleoids were studied by sedimentation. It was found that the *mukB* mutation caused unfolding of the nucleoid, and that the *seqA* mutation led to a more compact packaging of the chromosome, whereas the *mukB seqA* double mutant regained the wild-type nucleoid organization, suggesting they have opposing influences on the chromosome (Weitao *et al.*, 1999). Thus it is possible that MukB is just involved in chromosome folding.

In *B. subtilis*, much progress has been made in understanding the mechanism of chromosome partition. It has been found that Spo0J is required for normal chromosome partition as well as for sporulation. *spo0J* null mutants produced a significant proportion of anucleate cells during vegetative growth (Ireton *et al.*, 1994). Subcellular localization of Spo0J protein shows that it is associated with the origin-proximal region of the chromosome, forming discrete stable foci. Spo0J is located near the cell pole both in growing cells dividing symmetrically and in sporulating cells, suggesting the existence of a dynamic, mitotic-like apparatus responsible for chromosome partitioning in bacteria (Lin *et al.*, 1997; Glaser *et al.*, 1997). It has also been found that Spo0J is a site-specific DNA-binding protein that recognizes a 16 bp sequence. Allowing two mismatches, this sequence occurs ten times in the *B. subtilis* chromosome, all in the approximately 20% around the origin. Such a site can also stabilize plasmid in a Spo0J-dependent manner (Lin and Grossman, 1998). Soj, a suppressor that normally antagonizes the action of

SpoOJ in sporulation, plays a role in organization or compaction of Spo0J-*oriC* complexes and possibly other regions of the nucleoid (Marston and Errington, 1999). Visualization of the rapid movement of the *oriC* region provides further evidence for a mitotic-like motor responsible for segregation of the origin regions of the chromosomes (Webb *et al.*, 1998; Sharpe and Errington, 1998). Studying the movement of *oriC*/SpoOJ foci shows that soon after duplication, sister *oriC*/Spo0J foci move rapidly apart to achieve a fixed separation of about 0.7 microns (Sharpe and Errington, 1998). Tagged with a cassette of tandem *lac* operator repeats and visualized through the use of a fusion of the GFP protein to the LacI repressor, origin regions were found to abruptly move apart, travelling an average distance of 1.4 microns in 11 minutes towards the cell poles: representing an average velocity of 0.17 microns.min⁻¹ and reaching a maximum velocity of greater than 0.27 microns.min⁻¹. This movement is also seen in the absence of cell wall growth and in the absence of the product of *spo0J* (Webb *et al.*, 1998).

Chapter 2. Studies on the *ftsK* gene of *E. coli*.

2.1. Identification of a new cell division mutant as an *ftsK* mutant.

2.1.1. Introduction:

A cell division mutant (TOE44), isolated by Dr. Ken Begg, forms long filaments at non-permissive temperature (42°C) in low salt media (NB, or LB without NaCl) and then lyses and dies. This strain can not form colonies under these restrictive conditions, but can do so at lower temperatures (37°C and 30°C) or in higher salt medium (L Broth). The study of this mutant brought about the discovery of a new cell division gene *ftsK* (Begg *et al.*, 1995). The mutated *ftsK* allele in TOE44, named *ftsK44*, carries a single nucleotide mutation at 239 bp and causes the substitution of Gly-80 for Ala-80 (Begg *et al.*, 1995).

It was also found that both the N-terminal (about 200aa) and C-terminal (about 480aa) domains of FtsK are highly conserved among many species of bacteria. One of the homologues for which a function is known is the SpoIIIE protein of *Bacillus subtilis*, which plays an important role in transferring chromosome DNA from mother cell to prespore. Deletion of the *spoIIIE* gene caused transportation of chromosomal DNA to stop after only one third had entered the prespore. This abnormal transfer of DNA happens in only a part of the population. It is proposed that SpoIIIE is a component of the DNA-transportation complex. Deletion of *spoIIIE* has no effect on chromosome segregation during vegetative growth (Wu and Errington, 1994; Wu *et al.*, 1995). In strain TOE44 no abnormal chromosome DNA segregation is observed (Begg *et al.*, 1995). In order to find out if FtsK is involved in chromosomal DNA segregation, new *ftsK* mutants were isolated.

By treating a P1 lysate with hydroxylamine, a new temperature sensitive mutant (named W3531) linked to *lrp*::Tn10 was isolated by Kausalia Vijarayagavan in this laboratory. Its phenotype was identical to TOE44. My work, using recombination with Kohara λ phages, plasmid complementation, and DNA sequencing, localized the W3531 mutation in the 5'-terminus of *ftsK*.

2.1.2. Materials and methods:

Strains:

W3110: F- (*rrnD-rrnE*)1 *rph*⁻.

TOE44: F- Δ (*gpt-pro52*) *argE3 his-4 leu-6 thr-1 ara-14 galK2 lacY1 xyl-5 mtl-1 thi-1 supE44 rpsL31* Str^r *tsx-33 T6^r thyA ftsK44*

W3531 (Tn9): W3110, *zjb274*::Tn9 *ftsK3531*.

Plasmids:

Plasmids pBADK and pBADK' were constructed by C. Draper. In pBADK the whole *ftsK* gene is controlled by the arabinose PBAD promoter. pBADK' carries a 1.7kb fragment ending at *Bsu* 36I, which encodes the N-terminus of FtsK (Fig. 2.1.2).

Bacteriophages:

The chromosome regions carried by bacteriophage λ 213, 214, 215, and DD199 are shown in Figure 2.1.1.

Primers:

Primers T7090 and Bsu-down were used for the amplification of the *ftsK'* by PCR and primers T7090, G2629, G2631, G6689 and Bsu-down were used for sequencing. Their sequences are as follows:

T7090: 5'GGAGAGCCTCATATGAGCCAGGAATAC3'

G2629: 5'TGACGTTGTTACCCG3'

G2631: 5'CACAGCAGTCACAAT3'

G6689: 5'CAGAACAACCGGTGG3'

Bsu-down: 5'CTCGAATTCGGACCAATCCCCTTTGA3'

Bacteriophage λ recombination:

Plate lysates of bacteriophage λ were prepared as described (Sambrook, J *et al.* 1989). Mid-log phase (OD₆₀₀=0.6) cultures of recipients grown in LB+MgCl₂ (10mM) + maltose (0.2%) were used to flood over NB + maltose (0.2%) plates to form the recipient lawn. The plates were dried at 37°C and then 10 μ l of different dilutions of bacteriophage λ were spotted onto the lawn. The plates were incubated at 42°C overnight to allow formation of colonies.

Plasmid complementation:

pBAD18, pBADK' and pBADK were used to transform W3110, W3531 and TOE44 by the "Calcium Chloride method" (Sambrook *et al.* 1989). Transformants were streaked onto NB plates and incubated at 30°C and 42°C overnight to observe colony formation. The transformants were also grown in NB medium at 42°C to test plasmid complementation by measuring growth rate.

PCR:

Preparation of chromosome DNA to be used as template for PCR:

One ml of overnight culture was spun down and washed three times with 1X TE buffer, and then resuspended in 100 μ l TE buffer. After boiling for 10 minutes, cells

were lysed and the suspension was diluted at 10^0 , 10^{-1} and 10^{-2} and used as template for PCR.

PCR reaction:

20 pmol of each primer (Bsu-down and T7090), 1x Vent polymerase buffer, 200 μ M dNTPs, 100 μ g/ml BSA, 10 μ l template (different dilutions) and 2 units Vent or Taq DNA polymerase were mixed to give a 100 μ l mixture. The PCR products were purified by Wizard DNA Clean-up System (Promega company) and used as template DNA for sequencing.

Sequencing:

The reaction was carried out according to the instructions of the DNA Sequencing Kit (PE Applied Biosystems, a division of Perkin-Elmer): 8.0 μ l of Terminator Ready Reaction Mixture, 70ng PCR products as template and 3.2 pmole primer were mixed in a 0.5ml tube (total volume of 20 μ l) and the reaction was run according to the instructions provided by the manufacturer. The sample was sequenced automatically.

2.1.3. Results:

2.1.3.1. Localization and complementation of the cell division mutant W3531.

Bacteriophage λ recombination experiments showed that λ 215, λ 214 and λ DD199 could all give temperature-resistant recombinants with W3531. Therefore, it was possible that the mutation was in *ftsK*.

Transformation of this mutant with pBADK showed that this plasmid could complement W3531. W3531 (pBADK) could form colonies on NB (+ either 0.2% arabinose or glucose) plates at 42°C, whereas W3531 (pBAD18) could not. When

overnight cultures were diluted 1:100 into NB + Ap (50µg/ml) + Thy (10µg/ml) supplied with 0.2% glucose or arabinose and grown to late log phase at 42°C (OD600=0.9), the phenotypes of the cells were as shown in Table 2.1.1. These results suggested that W3531 could be complemented by the pBADK plasmid.

Table 2.1.1.

strain	plasmid	cells in glucose medium	cells in arabinose medium
W3110	pBAD18	all normal	all normal
W3110	pBADK	almost all normal	>80% were long filaments *
W3531	pBAD18	>90% were long filaments	similar to in glucose medium
W3531	pBADK	80% were normal cells	80% were long filaments

* Over-expression of FtsK causes cell filamentation (Draper *et al.*, 1998).

Growth rates were then tested to confirm the complementation tests. Overnight cultures were diluted 1:200 into NB + Ap (50µg/ml) + Thy (10µg/ml) and incubated at 30°C. When OD600=0.1-0.2, the cultures were diluted 1:5 into prewarmed fresh media and grown at 42°C. OD600 was measured at indicated times and the growth curves are shown in Figure 2.1.3.

pBADK' was then used to transform W3110 and W3531. I found that pBADK' could also complement W3531 but only when grown on arabinose plates, not on glucose plates. This was different from pBADK, which could complement W3531 on both arabinose and glucose plates. This might be because FtsK' is less stable than the complete protein.

Therefore the mutation in W3531 which causes cell division to be deficient is probably located within the *ftsK'* region.

2.1.3.2. Sequencing the *ftsK'* region of W3531.

Sequencing the *ftsK'* region showed that there were two nucleotide mutations. One is bp72-G (W3110) to bp72-A (W3531) and the other is bp380-G to bp380-A. The first one is a silent mutation. The second one caused the substitution of Cys-127 by Tyr-127. Topology analysis of FtsK showed that the Cys-127 to Tyr127 substitution was within a possible membrane-spanning α -helix.

2.1.4. Summary and discussion:

By Bacteriophage λ recombination, plasmid complementation and DNA sequencing, the mutation of W3531 was located in the *ftsK* gene. The mutation caused the substitution of Cys-127 by Tyr-127 in the FtsK protein. It is possible that there are other substitutions in the unsequenced *ftsK* region. However, the mutant phenotypes of W3531 and TOE44, appear to be identical, with respect to both cell division and chromosome segregation. Sequencing the whole of *ftsK44* would therefore be unlikely to provide important information about the function of FtsK.

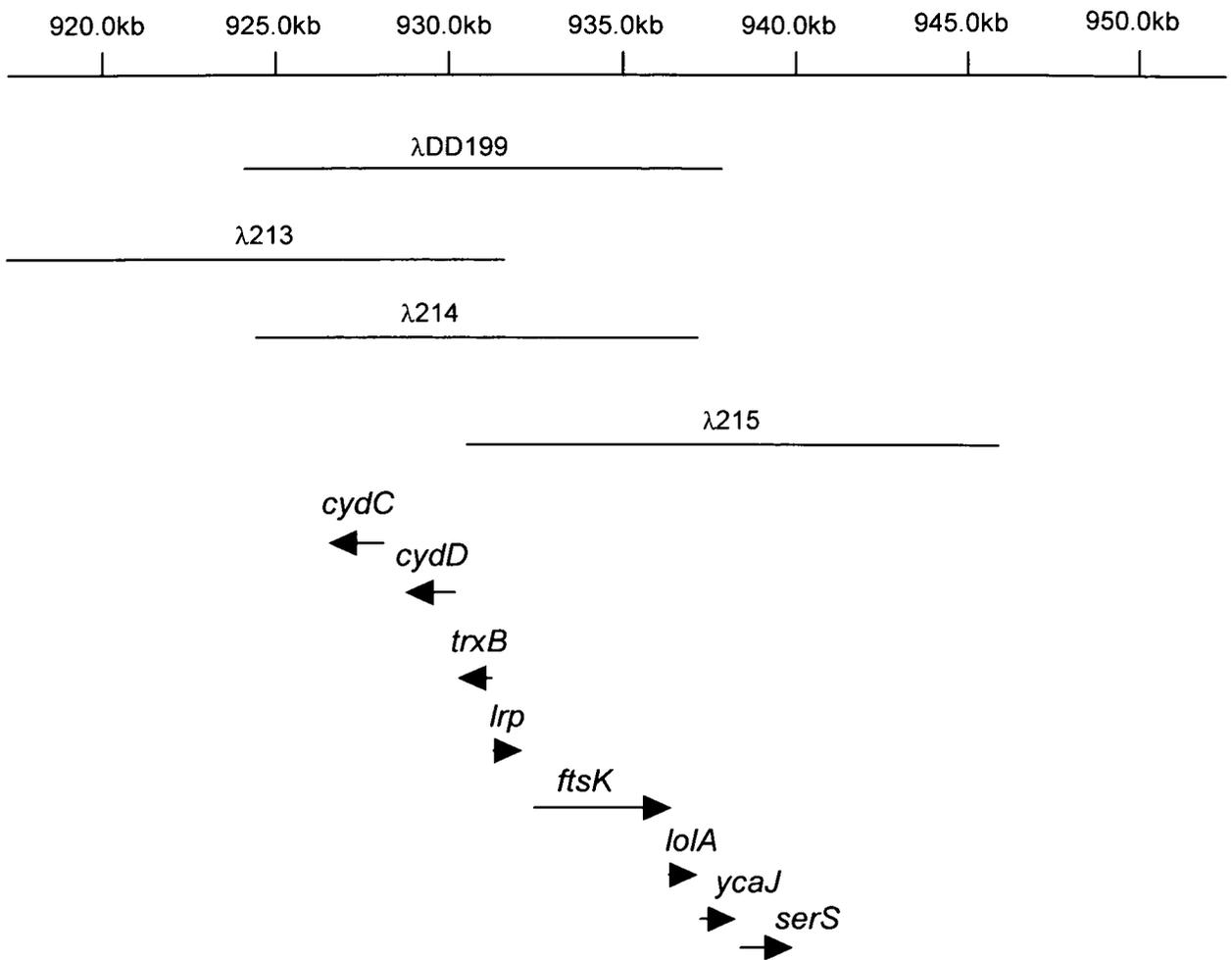


Figure 2.1.1. Chromosome regions carried by λ213, λ214, λ215 and λDD199 and genes around *ftsK*.

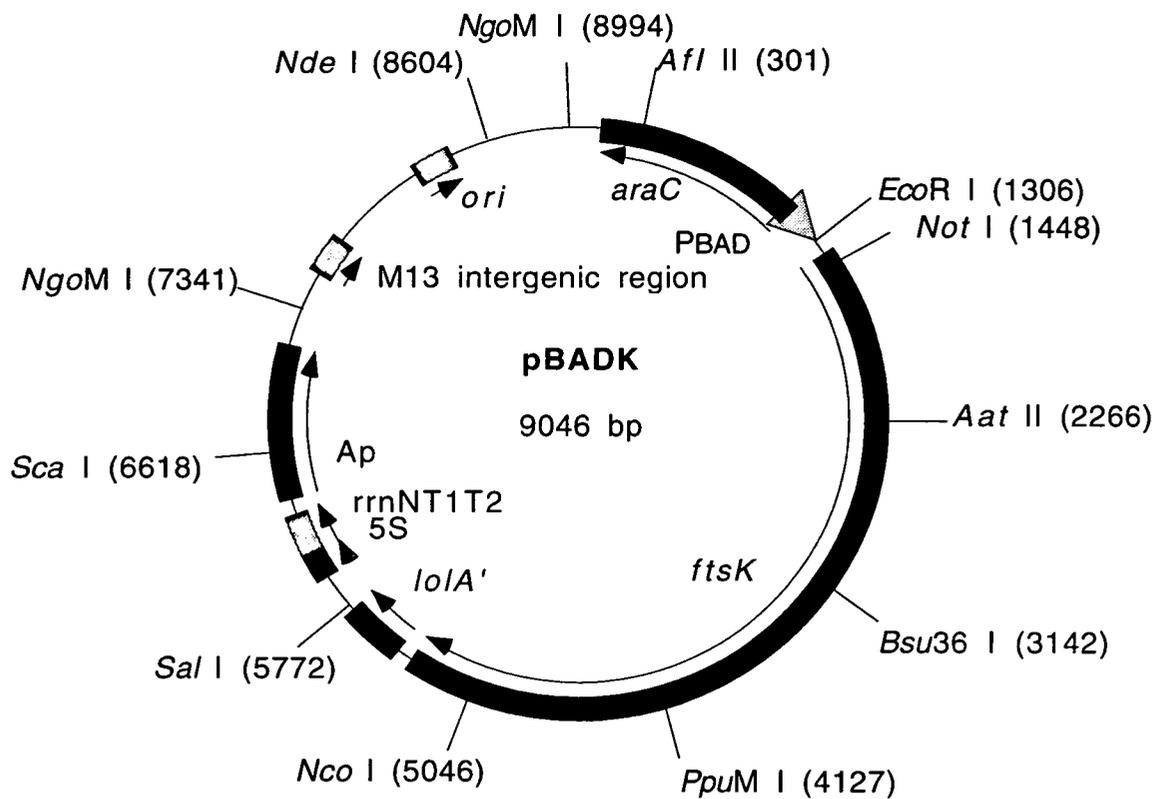


Figure 2.1.2. Map of plasmid pBADK, in which *ftsK* is under the control of the *PBAD* promoter.

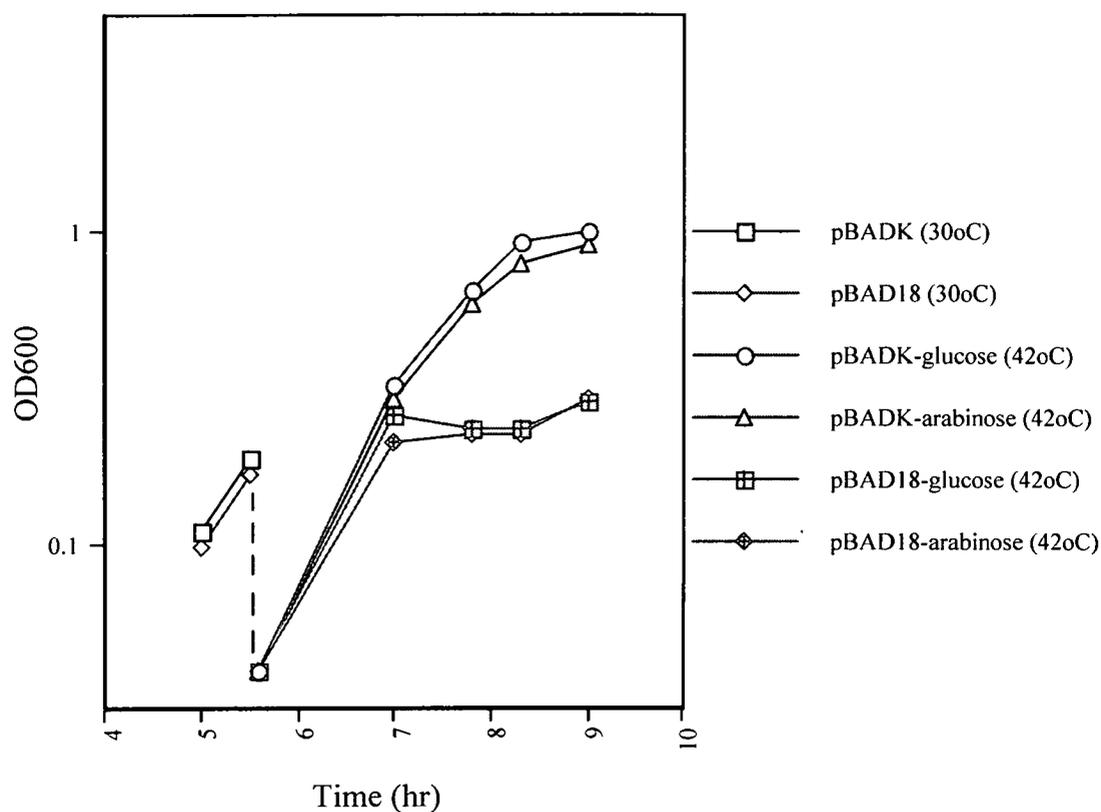


Figure 2.1.3. Complementation of W3531 by the pBADK plasmid.

In this experiment, overnight cultures were 1:100 diluted into Nutrient broth and grown at 30°C. After 5.5 hours cultures were shifted to 42°C. The OD600 of the cultures was measured at the indicated times. Growth curves are shown for W3531 (pBAD18) and W3531 (pBADK) in glucose or arabinose media.

2.2. The C-terminal domain of FtsK is involved in chromosome partition in *Escherichia coli*.

2.2.1. Introduction.

The *ftsK* gene of *E. coli* codes for a 147 kDa peptide, which is one of the largest polypeptides in *E. coli*, and seems to consist of several clearly identifiable domains, *viz.* an amino-terminal trans-membrane domain of about 200 amino acids that is required for the location of FtsK protein (Yu *et al.*, 1998) and completion of cell division (Begg *et al.*, 1995; Wang and Lutkenhaus, 1998; Draper *et al.*, 1998), a proline + glutamine rich domain of about 500 amino acids (found in no other bacterial homologue of FtsK except that of the closely-related *Salmonella* group, although the TraD protein of plasmid R100 has ten Gln-Gln-Pro repeats [Yoshioka *et al.*, 1990]), and about 470 amino acids at the C-terminal region that have two predicted nucleotide-binding pockets and which has extensive sequence similarity or identity with a number of proteins exist in other bacterial species, plasmids and transposons (Begg *et al.*, 1995). A function which described for some of the FtsK homologues is intercellular DNA transfer (some Tra proteins encoded by plasmids and transposons), or DNA transfer between mother cell and prespore (*i.e.* protein SpoIIIE in *B.subtilis* [Wu and Errington, 1994; Wu *et al.*, 1995]).

Two *ftsK* temperature-sensitive mutants with amino acid substitutions in the N-terminal portion of the protein have been obtained previously (Begg *et al.*, 1995; and Chapter 2.1 in this thesis). Both are blocked in cell division and show apparently normal DNA partition within the filamentous cells. A transposon insertion into the middle of *ftsK* has been reported: these mutant cells are mostly normal in appearance, but a proportion form chains of cells, and DNA segregation was reported to be normal

but a proportion form chains of cells, and DNA segregation was reported to be normal (Diez *et al.*, 1997). Therefore, the role of the C-terminal domain of FtsK, which shows similarity with known DNA transferring proteins is deserved for further study.

In an attempt to determine the function of the FtsK protein, especially its C-terminal domain, a strain was constructed in which the arabinose-inducible promoter, P_{BAD} was inserted into the chromosome immediately upstream of the *ftsK* coding sequence. The construction method also left 870 bp of the N-terminal coding region of *ftsK* under the control of its normal promoters. It was shown that growth of this strain under repressing conditions (no arabinose, plus glucose) resulted in partial induction of the SOS regulon and the appearance of cells with abnormally localized DNA.

2.2.2. Methods and Materials.

Plasmids:

(1) pGL1: Plasmid pBADK, created by inserting promoterless *ftsK* gene into pBAD18 (Guzman *et al.*, 1995) (Draper *et al.*, 1998), was digested with *Aat* II and *Sal* I, blunted and religated to generate pBADKa', in which only about 870bp of N-terminal sequence of *ftsK* gene is present. A fragment carrying *Kn* and four tandem T1 terminators (T14) was isolated from pRS551 (Simons *et al.*, 1987) by *Bam*H I and *Sca* I digestion. After blunted, this fragment was inserted into the *Afl* II restriction site in the *araC* gene in pBADKa' to generate a plasmid named pGL1, in which T14 is adjacent to pBAD promoter (Fig. 2.2.1).
(2) Others: The construction of other plasmids carrying part or whole *ftsK* or *lola* is shown in Fig. 2.2.7. Among them pBADK and pGB101 were from C Draper.

Strains.

The following *Escherichia coli* K-12 strains were used.

C600: F⁻ *leuB6 thr-1 lacA? lacY1 thi-1 supE44 fhuA21*; T1^R, Φ80^R; MG1655: F⁻ (λ) *rph*⁻; MG1655 (*aroA*::Tn10);
LCB274: F⁻ *thr-1 leuB86 lacY1 zbj-274::Tn9 rpsL thi-1* (from the National Institute of Genetics, Japan; accession No. ME8434).
Tp8503: F⁻ *Δlac-proB leu thi supE42 fhuA*; T1R, Φ80^R::Tn7 Tmp^R.

The following strains were constructed during this work.

GLC600: GLC600 was derived from C600 as follows. Plasmid pGL1 was digested with *Ngo*MI to remove the *ori* region, and then religated to give circular DNA, named GL1. C600 was transformed with GL1 and selected on LB plates + Amp (30μg/ml) + Kan (50μg/ml) + arabinose (0.2%). A colony that gave normal cell morphology on LB + Arabinose (0.2%) plates but formed a mixture of normal sized cells, chains and filaments on LB+Glucose (0.2%) plates at 37°C was named GLC600.

GLMG: MG1655 was transduced with P1 grown on GLC600 and transductants were selected on LB + amp (30μg/ml) + kan (50μg/ml) + arabinose (0.2%). One of these transductants was named GLMG. This strain was used to show that the phenotype of cells carrying the same insertion as GLC600 is not strain dependent.

TPL and GLTPL: TP8503 was lysogenised with λ*PsfIA-lacZ cInd* (Huisman and D'Ari, 1983) to give strain TPL. TPL was transduced with P1 grown on GLC600 and transductants were selected on LB + Amp (30μg/ml) + Kan (50μg/ml) + Arabinose (0.2%). One of the transductants was named GLTPL and was used to monitor SOS induction as well as growth rate.

GLTPLA: The *ftsA12* mutation was cotransduced with *leu*::Tn10 into GLTPL to give GLTPLA, which grows and divides normally in arabinose at 30°C but is blocked in division at 42°C

Growth conditions:

Cells were grown overnight at 37°C without shaking in L-broth (LB) + ampicillin (30µg/ml)+ kanamycin (50µg/ml) + arabinose (0.1%). For comparison between induced and non-induced cultures, the overnight culture was inoculated into M9 + MgSO₄ (1 mM) + casamino acids (CAA) (5mg/ml) + thiamine HCL (4µg/ml)-- called "M9+CAA" in this thesis, supplied with either arabinose (0.2%) or glucose (0.2%). Cell division was inhibited in strain GLTPLA by a shift from 30°C to 42°C at log phase or by addition of cephalixin or ampicillin (80µg/ml) at 30°C.

PCR and sequencing:

Two pairs of primers were used for PCR:

primers *lrp-up*" (5' ATGTTTCAGGATCCGACAGGAGTA 3') and "*amp-down*" (5' ACGCCTCTAGAGGGCGCGTAAATC 3').

primers "BAD" (5' AACGCTCTAGACATACTTTTCATACTC 3') and "Bsu-down" (5' CTCGAATTCGGACCAATCCCCTCTTTGA 3').

The PCR products were sequenced automatically.

Southern Blotting:

A fragment of about 900bp containing the N-terminal of *ftsK* was isolated by digesting pBADK with *EcoRI* and *AatII* and used as the template of the probe for Southern Blotting. The probe was labelled with dCTP using random oligonucleotides as primers (High Prime Kit from Boehringer Mannheim Biochemicals). The methods for the preparation of chromosome DNA and Southern Blotting have been described previously (Sambrook *et al.*, 1989).

Visualisation of DNA in cells:

The method of Hiraga *et al.* (1989) was used to stain DNA with DAPI and cells were photographed under mixed phase contrast - fluorescence microscopy.

β-galactosidase assay:

The synthesis of β-galactosidase under the control of the *sfiA* promoter was assayed according to Miller (1972) and used as an index of SOS induction.

Glucose starvation:

Overnight cultures of GLC600 or C600 were diluted 1/100 into M9 medium + CAA + glucose or arabinose (0.2%) and incubated with shaking at 37°C. When OD₆₀₀ reached between 0.1 and 0.2, the cultures were diluted 1/10 into fresh medium of the same composition. When the cultures again reached an OD₆₀₀ of 0.1-0.2, cells were centrifuged, washed once and resuspended in M9 medium + CAA without sugar and incubated overnight at 37°C.

2.2.3. Results.

Genetic structure of GLC600:

Strain GLC600 was constructed by integrating a GL1 molecule into the chromosome of C600 at the *ftsK* locus by a single crossover (Fig. 2.2.2). The GL1 molecule contained two selectable markers (Kan^R and Amp^R) and the first 870bp of *ftsK* gene (*ftsKa'*) under the control of the P_{BAD} promoter. In the constructed strain GLC600, the whole *ftsK* gene was totally under the control of the P_{BAD} promoter, because the T14 terminators upstream of the P_{BAD} promoter can prevent any transcription contributed from upstream promoters, while the *ftsKa'* sequence is expressed from the native promoters of *ftsK*. The genetic structure of GLC600 was determined by P1 transduction, Southern blotting, PCR amplification and DNA sequencing.

P1 grown on MG1655 *aroA*::Tn10 or on LCB274 (*zjb274*::Tn9) was used to transduce GLC600 to Tet^R or Cam^R respectively. In both cases close linkage was detected between the selected transposons and the Amp^R and Kan^R markers of GLC600. Co-transduction frequencies were 74% and 67% respectively for *aroA*::Tn10 and

zjb274::Tn9. The *aroA* locus is located at 20.7min, about 0.5 min downstream of *ftsK*, and *zjb274* is at approximately 20 min, upstream of *ftsK* and has been found to be ~ 50-90% cotransducible with *ftsK*⁺ (M. Khattar and C. Draper, pers. comm.). P1 transduction was also used to show that the insertion in GLC600 was not linked to *leuA::Tn10* at 1.8 min, as it would be if it had inserted at the *araC* locus (~1.5 min) (The P1 lysate of *leuA::Tn10* was from Dr. Ken Begg). Therefore, the P1 transduction result suggested that the GL1 molecule were integrated into the *ftsK* gene in GLC600.

To confirm the location and expected order of markers at the *ftsK* locus in GLC600, PCR was used to amplify and confirm the size of critical fragments, as follows. Two pairs of primers were used (Fig. 2.2.2). The 3.0kb PCR product (size just as expected) from primers "*lrp-up*" (5'-ATGTTTCAGGATCCGACAGGAGTA-3') and "*amp-down*" (5'-ACGCCTCTAGAGGGCGCGTAAATC-3') was sequenced and found to confirm the sequence 5'-*lrp-ftsK'-amp-3'*. Primers "BAD" (5'-AACGCTCTAGACATACTTTTCATACTC-3') and "Bsu-down" (5'-CTCGAATTCGGACCAATCCCCTCTTTGA-3') gave a 2.2kb PCR fragment. Sequencing of this product showed that the structure here was 5'-P_{BAD}-*ftsK*-3'.

Then Southern Blotting was applied to confirm the above result as well as show that only one GL1 molecule was integrated into the chromosome. The result of Southern Blotting showed that all the expected bands for one GL1 molecule integration into *ftsK* were obtained and no other bands can be seen (data not shown).

All the above results confirm the predicted structure of *ftsK* region in the GLC600 chromosome as shown in Fig. 2.2.2

Abnormal DNA localization in cells in which *ftsK* is repressed.

In strain GLC600, the initial 870 bp of *ftsK* is expressed from its normal promoters. Downstream from this truncated *ftsK'* gene, a second, complete copy of the *ftsK* gene is under the control of the inducible P_{BAD} promoter (Fig. 2.2.2). Because about 220aa at the N-terminus of FtsK is essential and also sufficient for cell division (Begg *et al.*, 1995; Wang Lutkenhaus, 1998; and Draper *et al.*, 1998), GLC600 is able to divide

no matter whether the complete FtsK protein is expressed or not. Therefore, this strain can be used to investigate the role of the remaining 70-80% of the FtsK protein.

When overnight cultures in LB + arabinose (0.1%) (with kanamycin and ampicillin for GLC600) were diluted 1/100 into M9 + CAA or LB with glucose (0.2%), C600 cells were normal in appearance throughout growth in this medium, but the GLC600 population, initially identical in appearance to C600 cells, soon came to contain short filaments or chains of cells, and then long filaments or chains. When an OD₆₀₀ of between 0.1 and 0.2 was reached, these abnormal cells comprised about 30% of the population. When the cells were allowed to progress to stationary phase, some of the chains or filaments divided into single cells. Examination of DAPI-stained cells showed that, although DNA was localized normally in most cells, a proportion of cells (about 17%) had DNA located abnormally either at constriction sites or at a cell pole (Fig. 2.2.3). In stationary phase about 11% of single cells had all the DNA at one pole (300 cells were counted). In contrast, GLC600 cells grown in arabinose media were all normal and indistinguishable from C600 cells grown in either arabinose or glucose (data not shown).

If the above culture of GLC600 in M9+CAA+glucose was resuspended into M9+CAA without any sugar ("glucose starvation") and incubated at 37°C overnight, cells without DNA appeared (~7%, 49 out of 700 cells scored). It was assumed that these cells resulted from division of filaments in which DNA was abnormally distributed (Hussain *et al.*, 1986). In contrast, GLC600 culture previously grown in arabinose, or C600 culture grown in either arabinose or glucose, produced less than 0.3% DNA-less cells after overnight incubation in M9 + CAA without any sugar.

The altered *ftsK* region was transferred to a different strain (MG1565) by P1 transduction from GLC600, to give strain GLMG. GLMG proved to be similar to GLC600 in cell morphology and DNA distribution when subjected to the same treatments. Therefore, the above phenotype is strain-independent.

Repression of *ftsK* induces the SOS regulon.

TP8503, a *lacZ*⁻ strain, was lysogenised with $\lambda P_{\text{stfA}}\text{-lacZ}$ to give a strain TPL. The altered *ftsK* region from GLC600 was then transduced into TPL to give strain GLTPL (see Materials and Methods). Whereas cells of strain TPL were normal in appearance under all conditions, those of GLTPL were similar to those of GLC600 in both arabinose and glucose media. It was found that TPL cells grew slightly faster in glucose than in arabinose, whereas GLTPL cells grew initially faster in glucose than in arabinose but later slowed down to a slightly lower rate (Fig. 2.2.4). These differences, although small, were consistent.

On X-gal plates with glucose, TPL formed white colonies, but GLTPL colonies were blue. β -galactosidase activities were measured in both TPL and GLTPL under the same condition (Fig.2.2.4). β -galactosidase was produced in similar low amounts in TPL cells grown in arabinose or glucose and in GLTPL cells grown in arabinose (~30 Miller units). When GLTPL cells were grown in M9 + CAA + glucose, however, β -galactosidase synthesis was induced (to ~80 Miller units). For comparison, Fig. 2.2.4 also shows the effect on both strains of SOS induction by naladixic acid. Naladixic acid blocks DNA synthesis and, eventually, cell growth. The specific activity in naladixic acid treated cultures reached about 300 Miller Units before growth stopped.

The SOS induction in *FtsK* depleted cells is cell division-dependent.

It seemed possible that repression of *FtsK* synthesis results in DNA damage, because repression is correlated with the appearance of cells with DNA trapped at cell poles or constriction sites. To test if SOS induction is due to improperly located DNA being broken by septal closure, SOS induction was measured in GLTPLA cells (an *ftsAts* strain). An overnight culture of GLTPLA was 1:100 diluted into either arabinose or glucose medium and grown at 30°C. After 140min half of each of the cultures in both arabinose and glucose media was shifted to 42°C before the onset of SOS induction at about 180min (Fig. 2.2.5). After the shift to 42°C, cell division stopped immediately,

but growth continued for approximately 100min in glucose medium and 160min or more in arabinose medium, so that long filaments were produced. Measurement of β -galactosidase levels in these cultures showed that the shift to 42°C completely prevented SOS induction in *ftsK*-repressed cells (Fig. 2.2.5).

Inhibition of cell division was also achieved by adding ampicillin (80 μ g/ml) or cephalixin (20 μ g/ml) to GLTPLA cultures at 30°C. Treatment with either of the antibiotics caused cells to grow into long filaments and SOS induction was completely absent or greatly reduced. Fig. 2.2.6 shows the effect of cephalixin treatment. Therefore, it was the inhibition of cell division *per se*, rather than the effect of temperature or drugs, that prevented SOS induction in the cells. In another experiment, it was found that SOS induction caused by naladixic acid could not be inhibited with ampicillin (data not shown), showing that not every SOS induction can be inhibited by blocking cell division. Therefore, these results strongly suggest that DNA damage in *FtsK*-deficient cells is caused by cell division.

Complementation of the GLC600 phenotype.

Plasmid pGB101, a derivative of pGB2 carrying the *trxB-lrp-ftsK-lolA* genes, could completely complement the phenotype of GLC600 (both at filamentation and DNA segregation) whereas plasmid pBRlolA, generated by cloning the *lolA* gene into pBR325, could not complement GLC600 (Fig. 2.2.7). This confirms that the abnormal DNA segregation is due to the depletion of the whole *ftsK* gene itself, and not to any possible polar effects on the expression of *lolA*. pGBKp' greatly complements the filamentation phenotype, whereas pBRK κ ' does not. This may be because overexpression of the N-terminus of *ftsK* causes cell chaining (Diez *et al.*, 1997) or filamentation (data not shown). Neither plasmid could correct the abnormal DNA segregation. Plasmid pBRKc' (Fig. 2.2.7) caused wild type cell an abnormal DNA segregation phenotype which is similar to GLC600 in glucose medium (data not shown).

Overexpression of the whole or part of *ftsK* gene causes SOS induction.

Plasmids pBR325, pBRK, pBRKn' and pBRKc' (Fig. 2.2.7) were transformed into TPL. Cells from overnight cultures were diluted 1:100 into LB medium supplied with suitable antibiotics to maintain plasmids and grown at 37°C with shaking. In this experiment, there were no further dilutions. β -galactosidase assay showed that SOS was induced in all three strains that carry whole or part of *ftsK* on a plasmid, while no SOS induction was found in the control strains TPL and TPL(pBR325) (Fig. 2.2.8). When the cells were examined, it was found that pBRK caused cell filamentation, pBRKn' caused cells to grow in chains (as previously reported by Diez *et al.*, 1997) and also to grow very slowly, and pBRKc' caused transient cell filamentation (mainly at early log phase: OD₆₀₀=0.1-0.2) (data not shown).

2.2.4. Summary and Discussion.

It has been found that deletion of the N-terminal part of FtsK, a predicted membrane spanning domain, causes a complete inhibition of cell division, but that this block is reversed by plasmids that contain as little as the first 578 bp of the *ftsK* gene (Draper *et al.*, 1998; Wang and Lutkenhaus 1998). Thus, the first ~200 amino acids of FtsK is necessary for cell division and can be expressed and function as an independent polypeptide. In this study a strain had been constructed in which the whole *ftsK* gene was under the control of the inducible *PBAD* promoter. In this strain the N-terminal trans-membrane domain of FtsK could be expressed from its native promoters. When the full-length FtsK was expressed, cells were normal in size, growth rate and DNA content and distribution, but when the expression of the complete gene was repressed, a proportion of the population showed abnormal DNA localization and the SOS response

was induced. In most of these aberrant cells there was at least one nucleoid located at a cell pole, or at a division constriction site. It was also shown that the SOS induction in *ftsK* deficient cells was cell division-dependent, suggesting that some (if not all) of the improperly located DNA was guillotined by closing septa.

The chromosome localization pattern shown in Fig. 2.2.3A suggested that possibly some of the partition defect might be due to the failure of chromosome dimer resolution. Such a possibility was studied further.

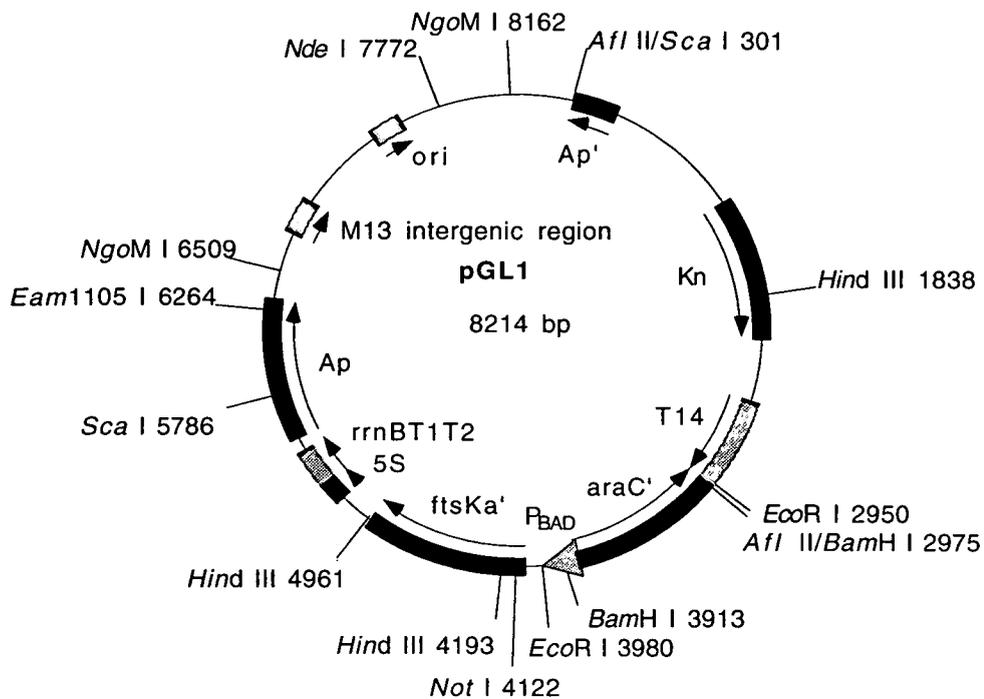


Figure. 2.2.1. Map of plasmid pGL1. pGL1 contains about 870bp of N-terminal coding region of *ftsK* gene under the control of P_{BAD} promoter and four tandem T1 terminators (T14) upstream of P_{BAD} to block transcriptions from other promoters into *ftsK*.

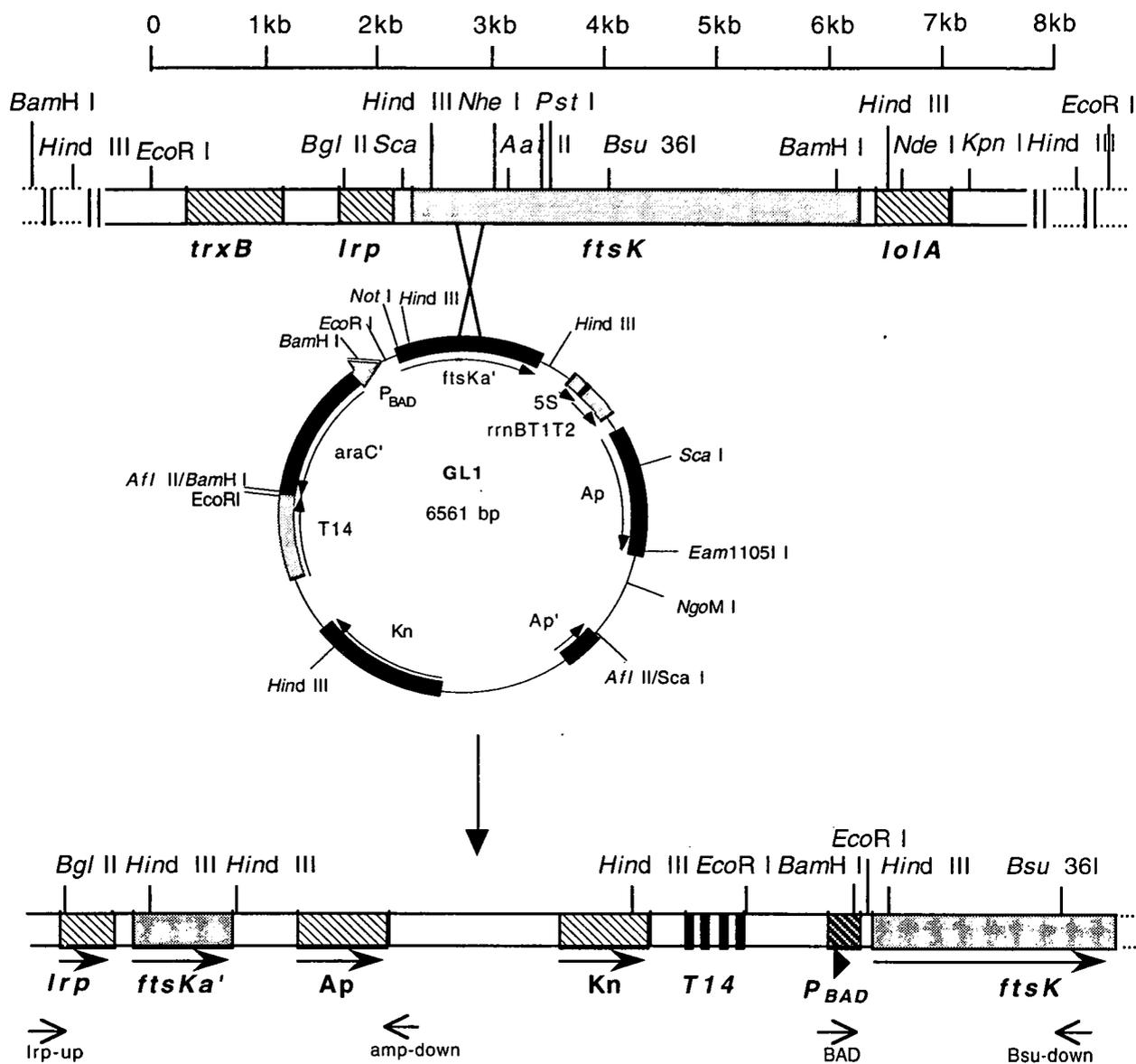


Figure 2.2.2. The construction of strain GLC600. This is to show the integration of DNA GL1 into C600 chromosome and the genomic structures of C600 (upper) and GLC600 (lower) around *ftsK* gene. The positions and directions (shown by arrows) of primers *Irp-up*, *amp-down*, *BAD* and *Bsu-down* used for identification of GLC600 structure are shown below the structure of GLC600.

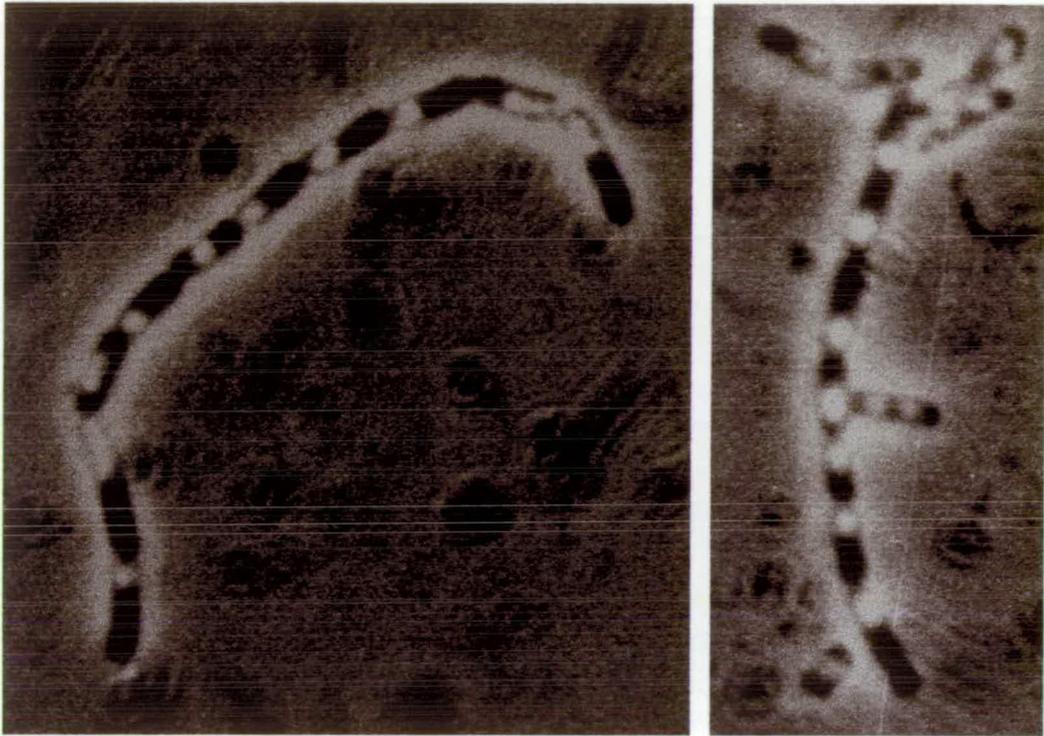


Figure 2.2.3. (A): Chromosome distribution of GLC600 cells. Cells were from log phase culture grown at 30°C in M9+CAA+glucose medium.

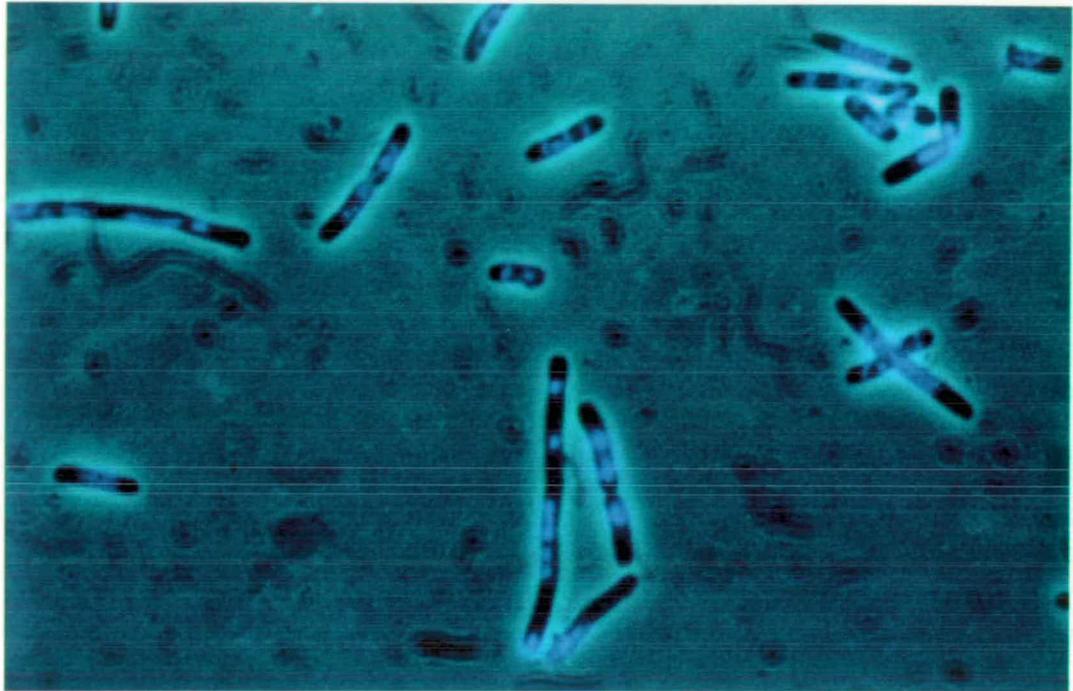


Figure 2.2.3. (B): Chromosome distribution of GLC600 cells. Cells were from log phase culture grown at 37°C in LB+glucose medium.

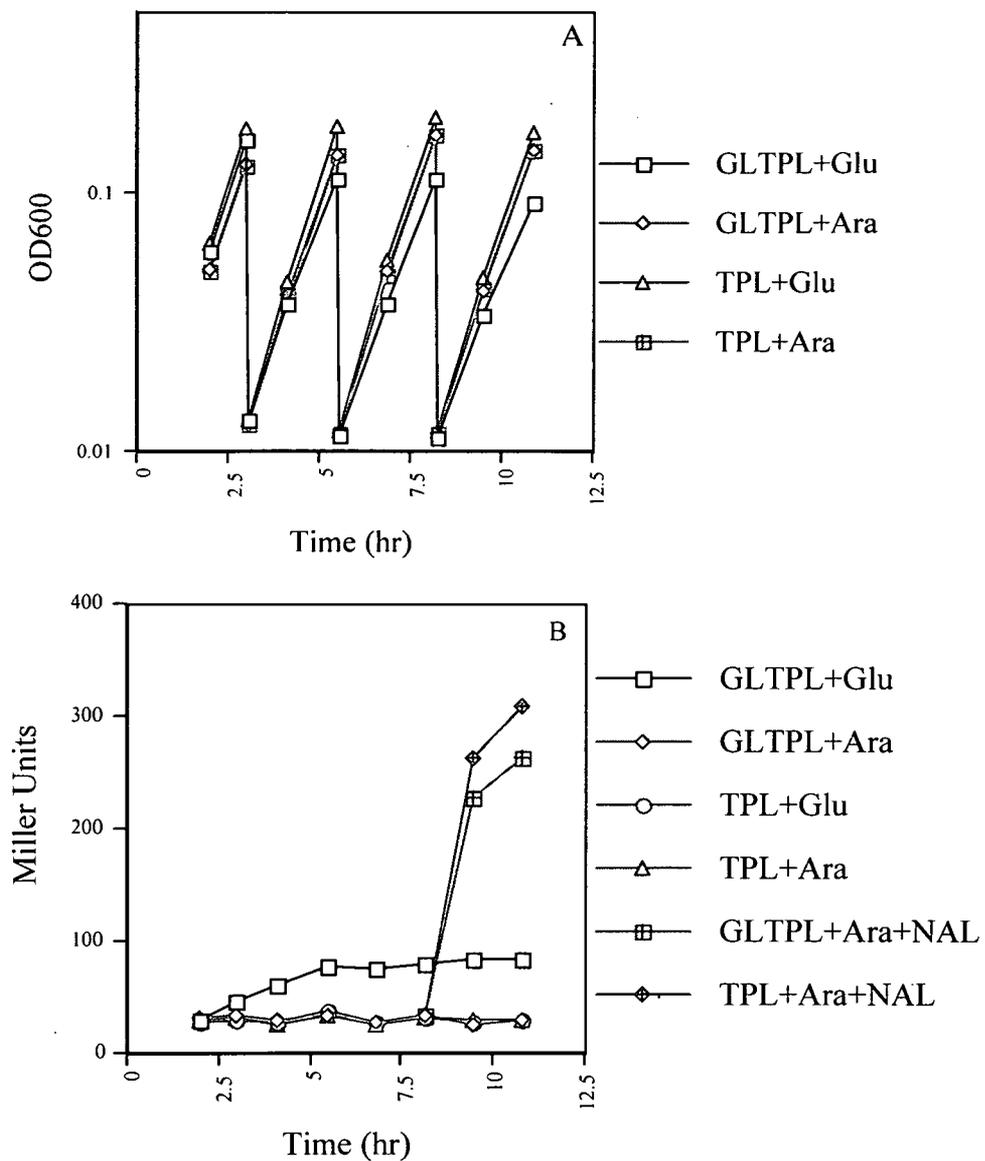


Figure 2.2.4. Depletion of the full-length FtsK protein caused SOS induction. Overnight cultures of TPL and GLPTL in LB+0.1% arabinose with (for GLTPL) or without (for TPL) ampicillin (30 $\mu\text{g}/\text{ml}$) and kanamycin (50 $\mu\text{g}/\text{ml}$) were diluted 1:100 into M9+CAA with 0.2% glucose or arabinose and grown at 37°C with shaking. The cultures were kept OD600<0.2 by dilutions at 1:10 into prewarmed fresh media. OD600 (A) and β -galactosidase (B) were measured at indicated times. Nalixic acid was added at 8.1hr.

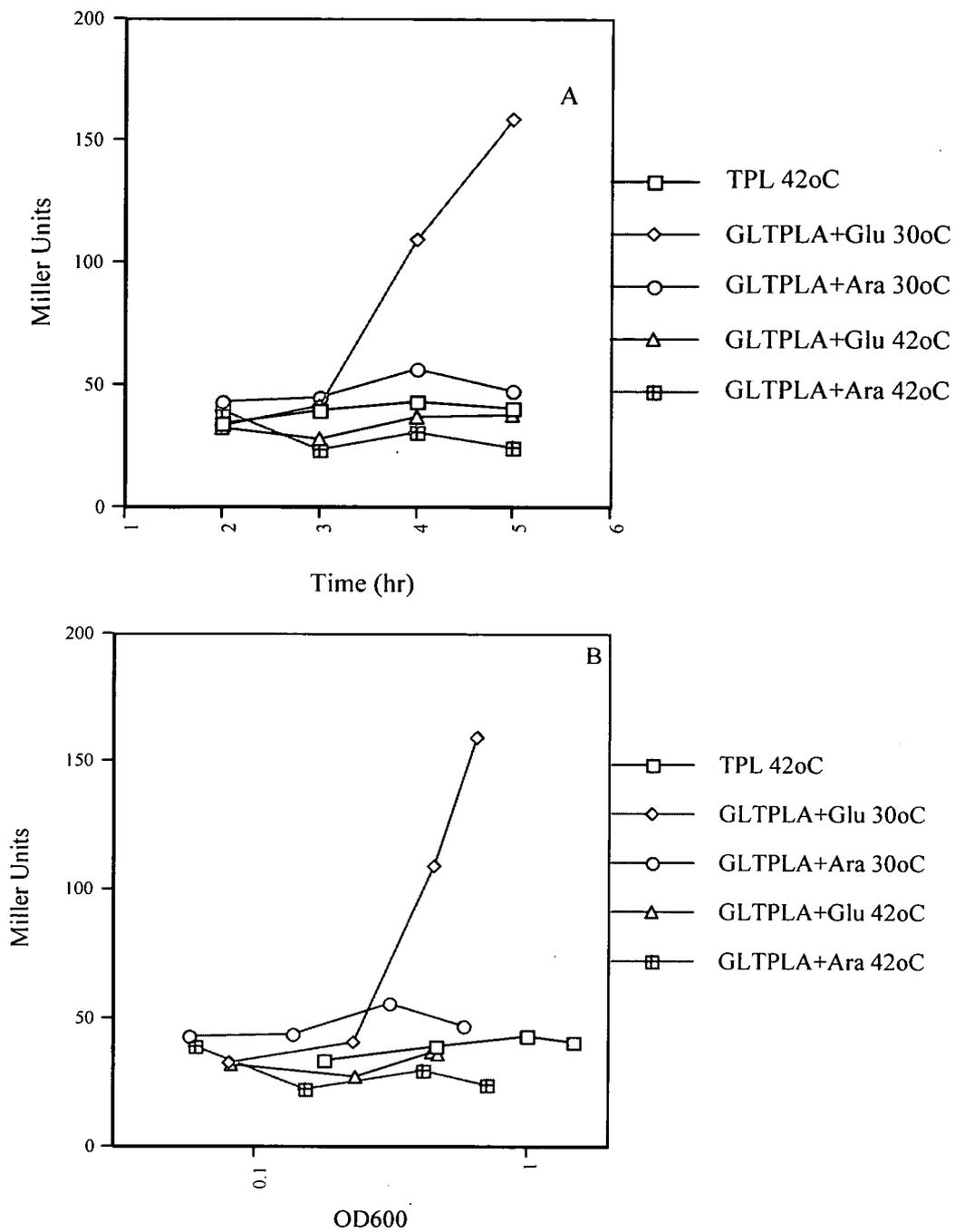


Figure 2.2.5. Cell division-dependent SOS induction in *ftsAts* strain. Overnight culture of GLTPLA was diluted 1:100 into glucose or arabinose medium and kept grown at 30°C in log phase (OD600<0.2) by dilutions. After 140min, part of each culture was shifted to 42°C, while the remainder was still grown at 30°C.

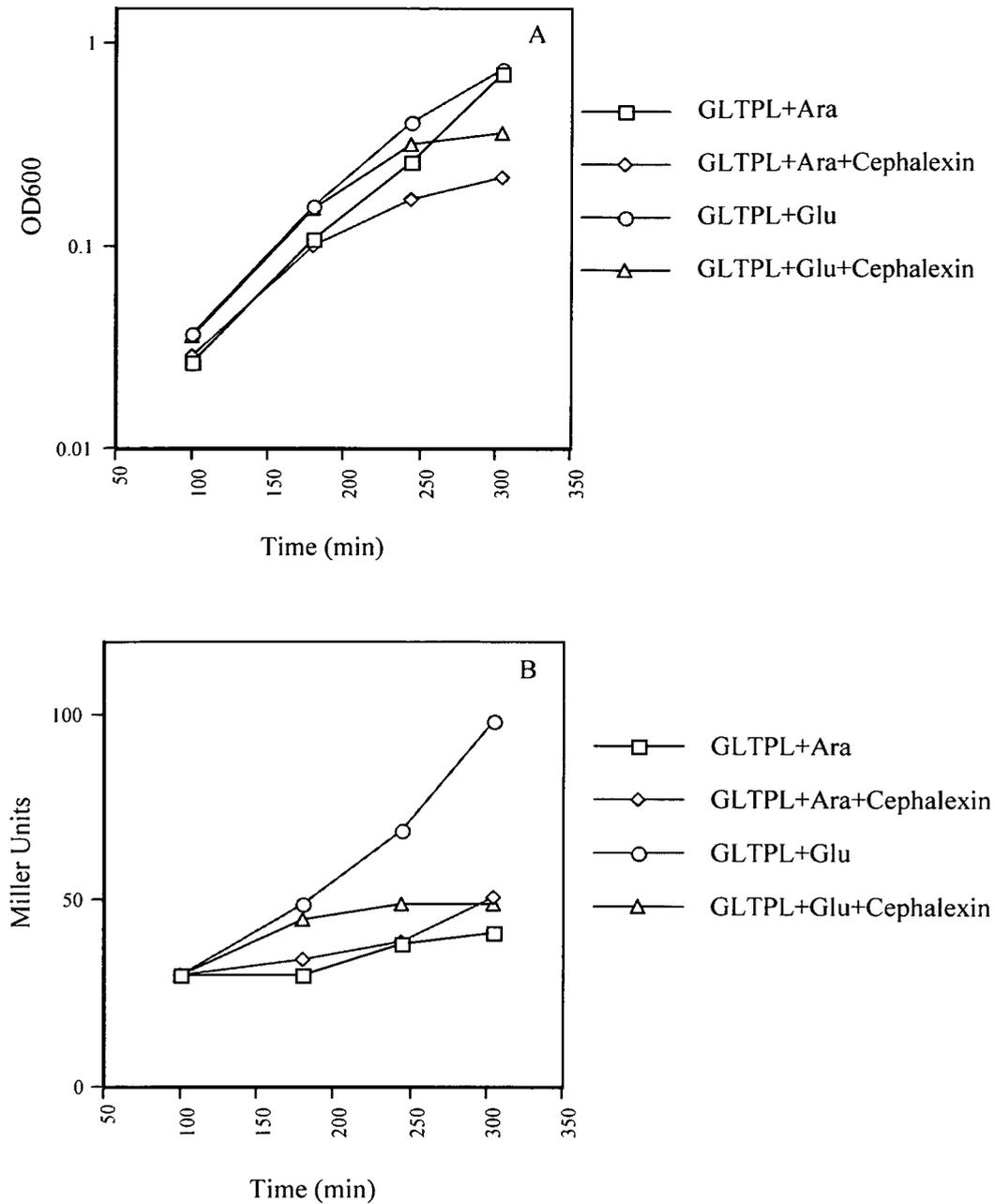


Figure 2.2.6. Cell division-dependent SOS induction —inhibiting cell division with cephalexin. Overnight culture of GLTPLA was diluted 1:100 into glucose or arabinose medium and kept grown at 30°C in log phase (OD600<0.2) by dilutions. After 150min, part of each culture was treated with cephalexin (20µg/ml) to block cell division.

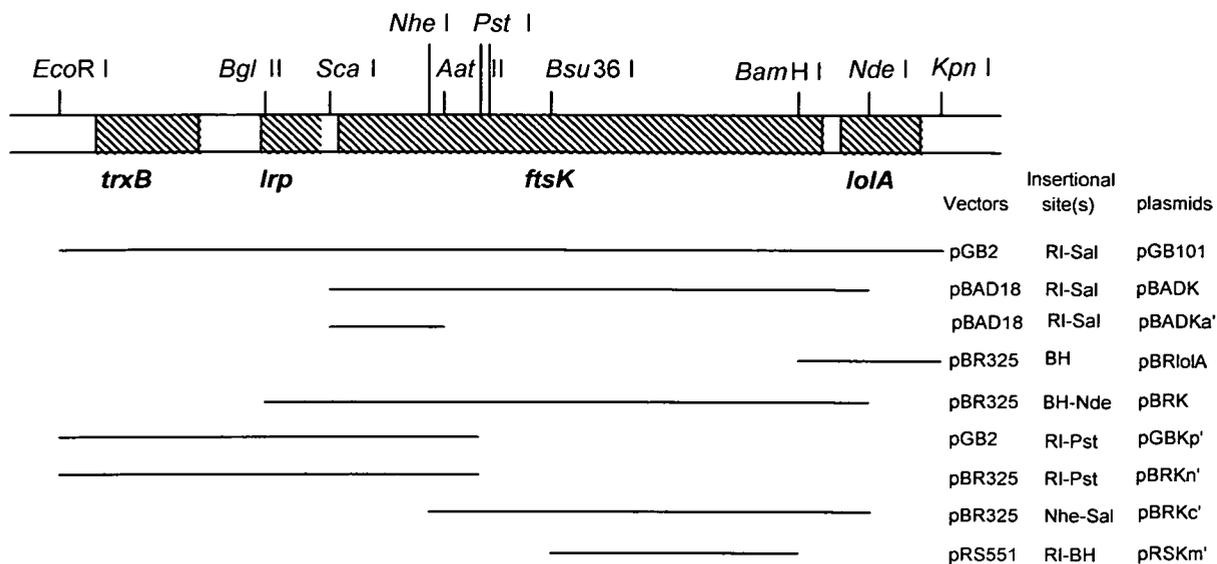


Figure 2.2.7. Plasmids carrying part or whole of *ftsK* or *lolA* gene.

Abbreviations: BH: *Bam*H I, RI: *Eco*R I, Nde: *Nde* I, Nhe: *Nhe* I, Pst: *Pst* I, Sal: *Sal* I.

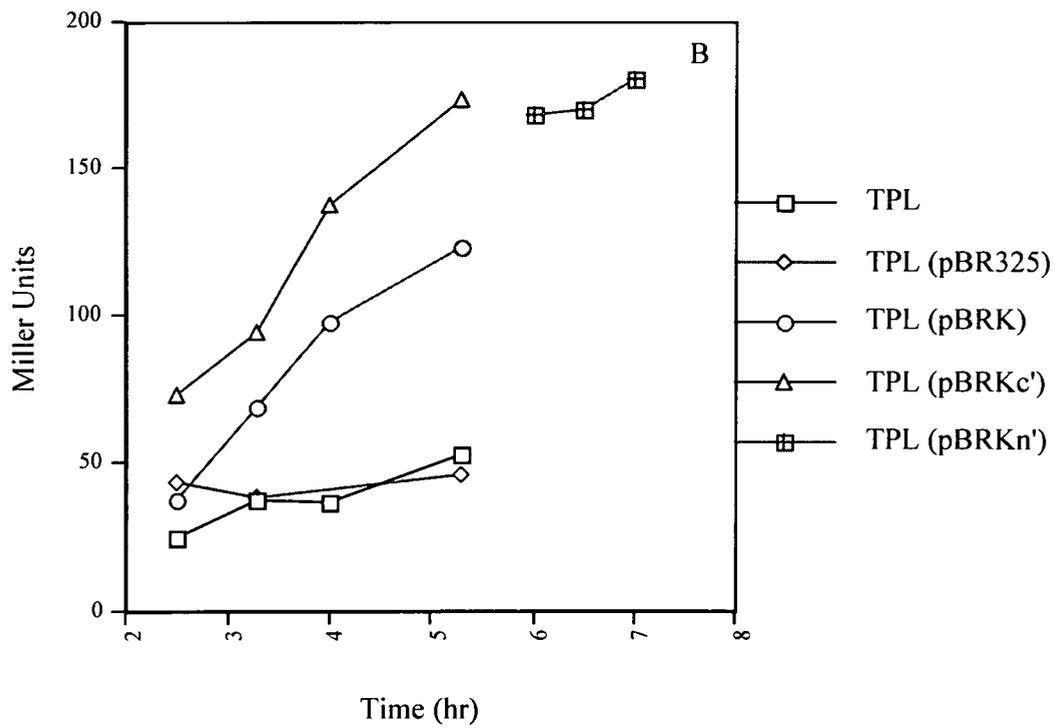
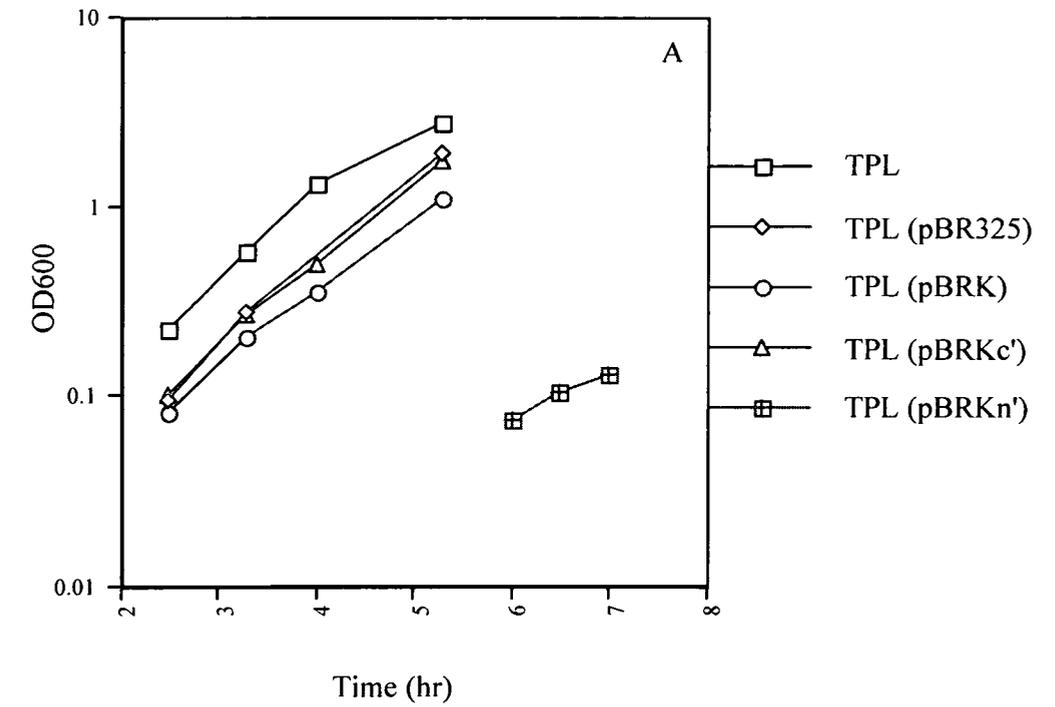


Figure 2.2.8 (to be continued).

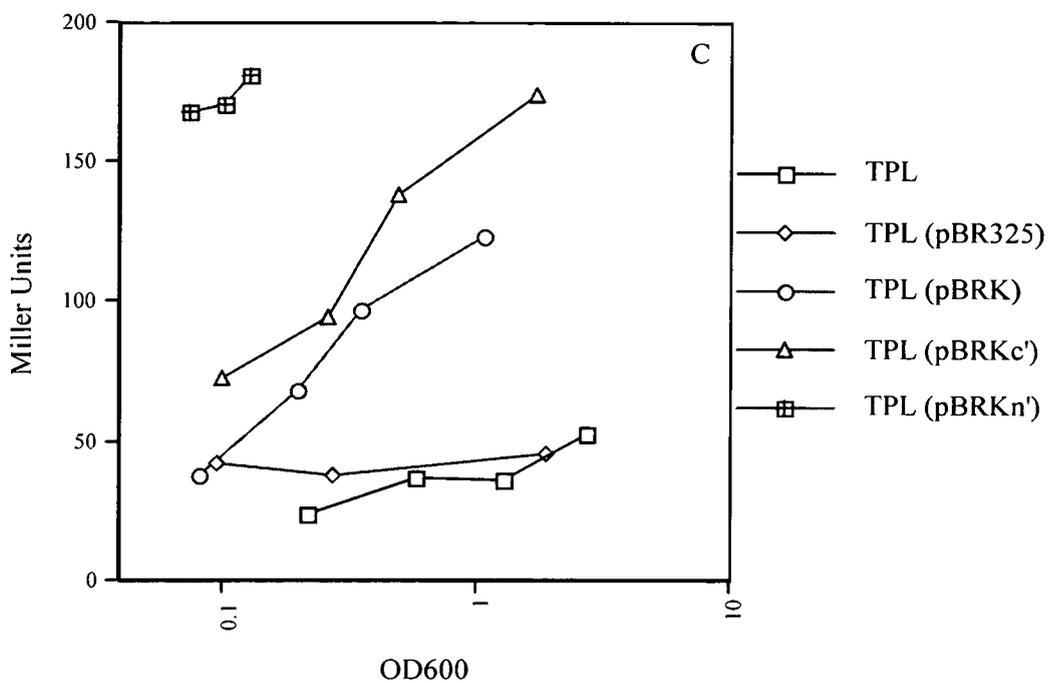


Figure 2.2.8. Growth and SOS induction of TPL cells with different plasmids. Overnight cultures of TPL, TPL(pBR325), TPL(pBRK), TPL(pBRKc') and TPL(pBRKn') were diluted 1:100 into fresh L broth supplied with antibiotics if suitable and grown at 37°C without further dilution. OD600 (A) and β -galactosidase (B and C) were measured at indicated times.

2.3. Localization of the C-terminus of FtsK protein to the cytoplasm.

Based on the analysis of the primary sequence of FtsK protein, it is predicted that there are several potential trans-membrane regions in its N-terminal domain (Begg *et al.*, 1995). However, the distribution and number of predicted hydrophobic sequences make it uncertain whether the C-terminus is located in the cytoplasm or periplasm. Here I show that by fusing β -lactamase to the C-terminus of FtsK it can be determined that the C-terminus is located in the cytoplasm, consistent with its function in chromosome partition and dimer resolution.

Three enzymes have been used for the study of protein topology structure in *E.coli*. β -galactosidase (Silhavy and Beckwith, 1985) and β -lactamase (Broome-Smith and Spratt, 1986) have been successfully used for the analysis of many proteins. The usefulness of alkaline phosphatase (Hoffman and Wright, 1985) is limited, since it can be used only to identify those fusions which translocate the enzyme across the inner membrane. Alkaline phosphatase possesses enzymatic activity only after translocation across the cytoplasmic membrane. In the β -lactamase method, in-frame fusions can be selected easily by growing cells at high density in ampicillin medium. Single cells from clones with in-frame fusions are further characterised by determining their MICs to ampicillin. This can be used to predict the location of the fusions, since single cells with periplasmic fusions can usually grow in high concentration of ampicillin (such as 1600 μ g/ml) while cells with cytoplasmic fusions will be killed even at very low concentrations of ampicillin (5 μ g/ml).

The vector pJBS633 and also the method used here were obtained from Dr J. K. Broome-Smith (Broome-Smith and Spratt 1986). pJBS633 contains the coding region

for the mature form of TEM β -lactamase which has been engineered to introduce a unique blunt end cloning site (*PvuII*) at the start of the coding region (Fig 2.3.1). To make an in-frame fusion to the C-terminus of FtsK, pJBS633 was digested with *Sal I* and *Pvu II* and then religated to produce plasmid pJB1. The plasmid can also be digested with *BamH I* and *Pvu II* and religation will produce pJB2, in which the *BamH I* site is immediately upstream of the β -lactamase gene. In the following study only pJB1 was used. The *ftsK* gene carried by pUC19 (constructed by C. Draper in this lab) was released after the digestion with *EcoR I* and *Sal I* and inserted into the same sites in pJB1. The plasmid produced in this way is pJKap. pJKap was digested with *Sal I* and *BamH I*, treated with *Bal 31I* for a short time, religated, transformed into C600 and selected on LB + kanamycin (50 μ g/ml). The transformants were patched onto L agar + Km (50 μ g/ml) (master plate) and then onto L agar + Ap (200 μ g/ml). Among 150 transformants patched, nine transformants with putative in-frame fusions (*i.e.* those that grew up on Ap in the above test) were obtained and named pJKtop1-9, respectively. To check the level of Ap resistance of these fusions more reliably a “spot test” was used. Cells from the Km master plate were inoculated into L broth + Km to get overnight cultures. 6 μ ls of the overnight cultures were spotted onto L agar + Km, and then onto L agar + Ap (200 μ g/ml). Any recombinants that grew on Ap (200 μ g/ml) should have had in-frame fusions to mature β -lactamase. The plates were first incubated at 30 $^{\circ}$ C for 8hours and then at 37 $^{\circ}$ C overnight. Results showed that cells carrying pJKtop1, 2 or 5 grew well on L agar + Ap, that those carrying 3 or 8 could not grow, and that those carrying 4, 6, 7 or 9 formed 20-50 colonies in the spot. This suggests that 1, 2 and 5 were in-frame fusions, and that 3 and 8 were not: the remaining clones had unclear phenotypes. Rabbit antibody against bacterial β -lactamase was used to detect the production of fused β -lactamase in the transformants. Results showed that no proteins bigger than 32 kDa could be detected in 3, 8 or 9. Others gave major bands at 150 kDa, 70 kDa, 58 kDa and also polypeptides located at the top of the resolving gel, but 1, 2 and 5 produced much more protein than 4, 6 or 7 (Fig 2.3.2). Earlier work in this lab by David Boyle showed that FtsK protein migrates in SDS-PAGE with an apparent

molecular weight greater than 175 kDa (the predicted size is 147 kDa). I suggest that the full length fusion protein was probably the protein at the top of resolving gel and that the other polypeptides were degradation products.

To confirm that the fusions in pJKtop1 and 2 are in-frame, the junctions between *ftsK* and Ap in plasmids pJKtop1, 2 and 3 were sequenced. The sequence showed that pJKtop1 and pJKtop2 were the same and both in-frame. The fusion in pJKtop3 was out-of-frame. The sequencing result showed that β -lactamase was fused at 1265aa of FtsK and the junction sequence of the fusion protein was FDGAEELSRLRHPET (underlined sequence belongs to FtsK and the remaining belongs to β -lactamase). The predicted molecular weight of this fusion protein is 169 kDa, 139 kDa from FtsK and 29 kDa from β -lactamase.

To study the location of the C-terminus of FtsK, overnight cultures of the above transformants, C600 and GLC600 were diluted and spread onto LB, LB+Kan (25 μ g/ml) and LB+Ap (5 μ g/ml) plates. While they could all form 1000-3000 colonies on LB or LB+Kan plate, C600 and C600 (pJKtop1, 2 or 3) could form fewer than two colonies (*i.e.* 0 or 1) and GLC600 could form 1000-3000 colonies on LB+Ap plates. This suggests that the C-terminus of FtsK is located in the cytoplasm, because if the fused β -lactamase is located in the cytoplasm individual cells should not be protected from killing by ampicillin (Broome-Smith and Spratt, 1986). *Cytoplasmic* β -lactamase cannot intervene to prevent ampicillin inactivating the penicillin-binding proteins which are located on the outer surface of the inner membrane. However, if the fusion protein is translocated across the cytoplasmic membrane, then the *periplasmic* β -lactamase will confer significant levels of protection on individual cells. Broome-Smith & Spratt (1986) have reported that numerous fusion proteins in which the β -lactamase part is translocated to the periplasm give single cell MICs of up to 1600 μ g/ml Ap. In contrast, all of the cytoplasmic fusion proteins that they have made confer no protection against Ap and single cells are always killed by plating on 5 μ g/ml Ap, even if a cytoplasmic fusion protein is massively overproduced (Broome-Smith and Spratt, 1986).

According to the above experimental results, the C-terminus of FtsK (tested at the site of 1265aa) is located in the cytoplasm. This is consistent with its function in chromosome partition and/or dimer resolution. The whole topology of FtsK protein needs to be studied further.

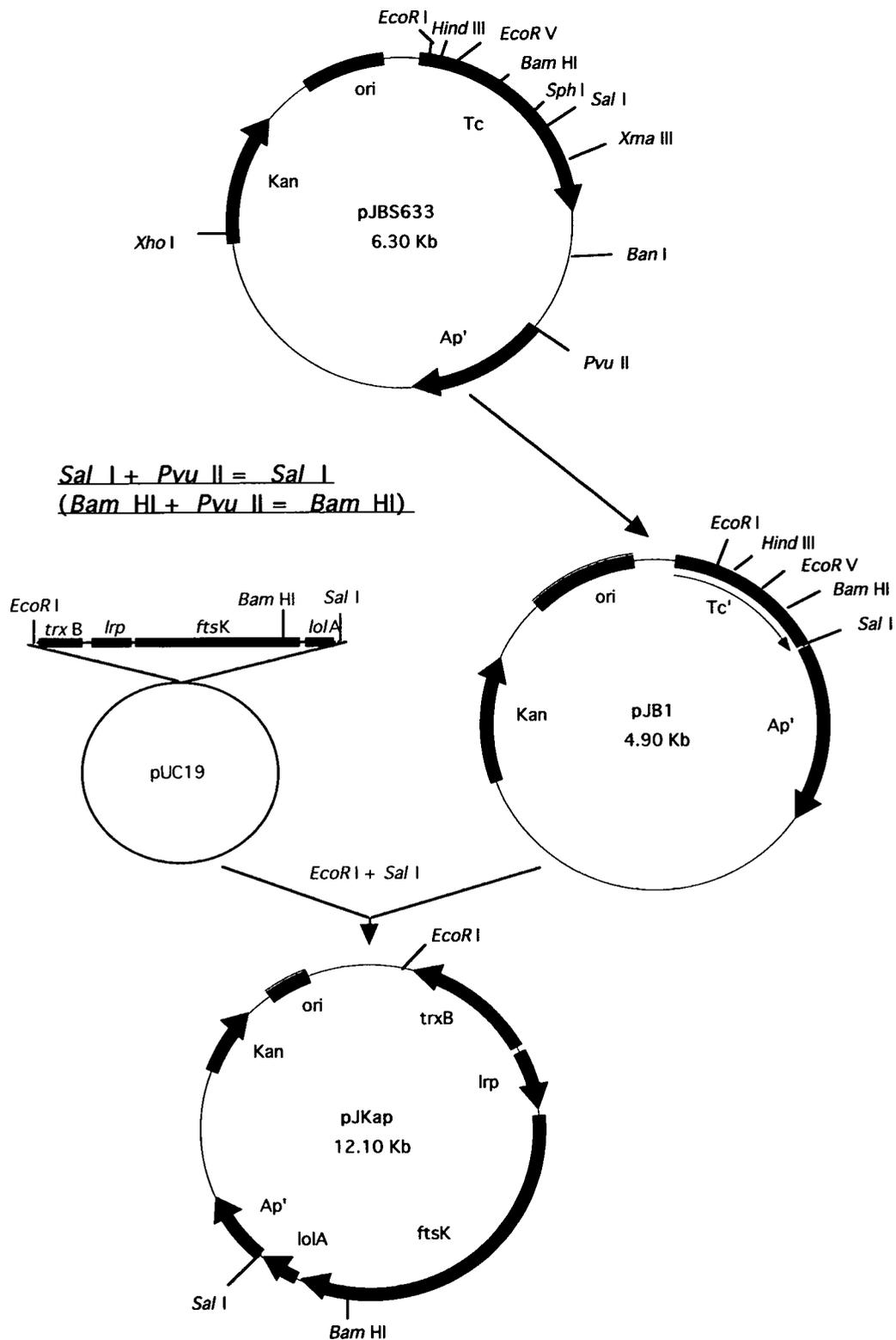


Figure 2.3.1. Construction of plasmids for the study of FtsK topology.

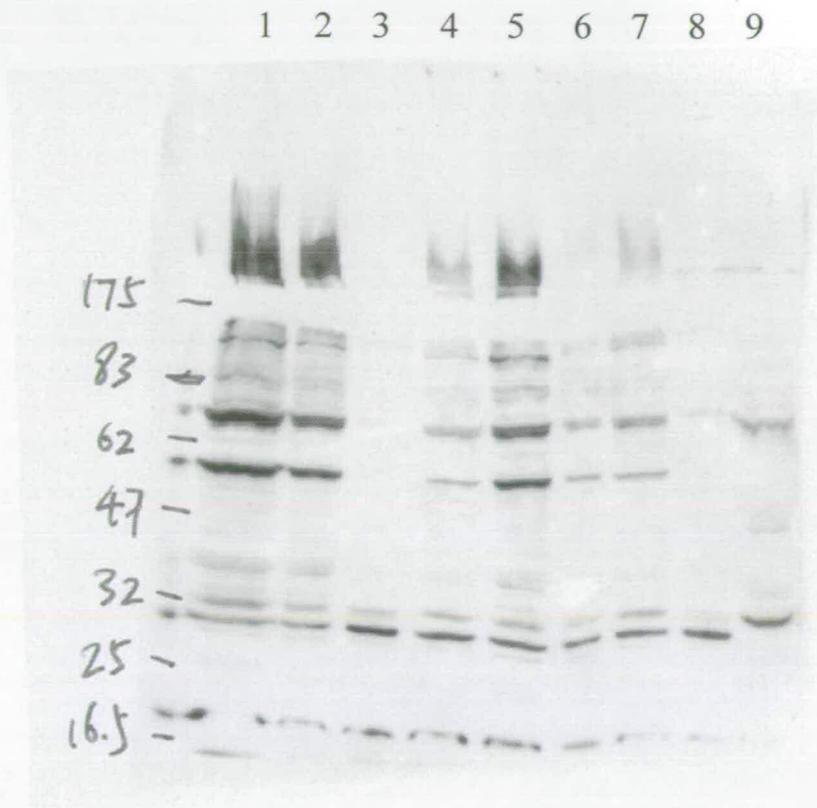


Figure 2.3.2. Detection of β -lactamase from cells with different fusion plasmids by Western blotting. In this experiment, C600 with pJKtops were grown to late log phase (OD600 about 0.8) and then harvested for Western blotting. Same amount of protein was loaded to each lane of the PAGE gel. Lanes 1 to 9 (shown on the top) correspond to C600 with plasmids pJKtop1 to 9, *i.e.* lane 1 correspond to C600 with pJKtop1, and lane2 correspond to C600 with pJKtop2, *ect.* The protein molecular weight marker (in kDa) is shown on the left.

2.4. The C-terminal domain of FtsK is required, and probably required only, for chromosome dimer resolution during cell division.

2.4.1. Introduction:

Any odd number of homologous recombination events between two circular sister chromosomes will create a dimer. Recent studies have shown that hyper-recombinogenic mutations such as *polA*, *dut*, and *uvrD* will increase the frequency of dimer formation. Abolition of homologous recombination by a *recA* mutation will eliminate dimer formation. *RecB* or *recF* mutations can each reduce the frequency by half, and both together will diminish the formation (Steiner and Kuempel, 1998).

After chromosome replication, dimers need to be resolved into monomers before they can be properly segregated into daughter cells. Failure of resolution will cause DNA damage and abnormal DNA segregation (Blakely *et al.*, 1991; Kuempel *et al.*, 1991). These dimers are converted into monomers by site specific recombination at a 33bp site (*dif*) located in the middle of the chromosome terminus region (Clerget, 1991; Kuempel *et al.*, 1991; Tecklenburg, 1995) and requires two recombinases XerC and XerD (Blakely *et al.*, 1991; 1993). The XerC and XerD proteins have 37% identity and bind to separate halves of the recombination site. Both proteins act catalytically in the recombination reaction. Recombination site asymmetry and the requirement of two recombinases ensure that only correctly aligned sites are recombined (Blakely *et al.*, 1993).

A recent study has shown that dimer resolution also requires cell division (Steiner and Kuempel, 1998). If cell division is blocked by Cephalixin or in an *ftsZts* mutant at 42°C, no recombination will take place at the *dif* site.

The resolution of dimers or multimers of ColE1-related plasmids needs not only XerC and XerD but also ArgR and PepA (Stirling *et al.*, 1988, 1989; Summers 1989; Blakely *et al.*, 1993.). The resolution happens at Xer-specific sites (*e.g. cer* in ColE1 and *psi* in pSC101), which includes the 33bp core site and also about 200bp of adjacent accessory sequences. Xer site-specific recombination at natural plasmid recombination sites is preferentially intramolecular. In contrast, Xer recombination at the *Escherichia coli* chromosomal site *dif* can occur both intermolecularly and intramolecularly. It is proposed that these accessory factors (proteins and sequences) ensure that recombination is intramolecular in plasmids (Blakely *et al.*, 1996).

2.4.2. Materials and Methods:

Strains constructed in this work:

TPL1 *ftsK::cat1*: TP8503 λ P*sfiA-lacZ ftsK::cat*.

TPL1 *xerC::cat*: TP8503 λ P*sfiA-lacZ xerC::cat*.

GLTPLX: TP8503 λ P*sfiA-lacZ ftsK::PBAD-ftsKa' xerC::cat*.

MukBL: C600 λ P*sfiA-lacZ mukB::kan*.

GlmukBL: C600 λ P*sfiA-lacZ mukB::kan ftsK::PBAD-ftsKa'*.

CDmukB-L: C600 λ P*sfiA-lacZ mukB::kan ftsK::cat*.

XermukB-L: C600 λ P*sfiA-lacZ mukB::kan xerC::cat*.

Assay of β -galactosidase activity:

The specific activity of β -galactosidase (Miller Units) was assayed according to Miller (1972). The Miller Units was used to measure the level of SOS induction in strains where *lacZ* is under the control of the *sfiA* promoter.

2.4.3. Results:

The C-terminal domain of FtsK is required for chromosome dimer resolution in *E.coli*.

The following evidence suggested that the aberrant chromosome partition phenomenon in GLC600 might be due to the failure of dimer resolution. Firstly, the percentage of cells with abnormally distributed chromosomal DNA in GLC600 is almost the same as that of strains in which dimer resolution fails. Secondly, the pattern of abnormal chromosome localization at septation sites in GLC600 cells grown in M9+CAA+glucose suggested that the abnormality were originated from the failure of dimer resolution. Thirdly, SOS induction is dependent on cell division in both GLC600 and *dif* mutants. In order to test whether chromosome dimers can still be resolved in cells in which the full length FtsK is depleted, we collaborated with Peter Kuempel's lab in the University of Colorado, USA and got the following results (Steiner *et al.*, 1999).

First of all, a density-label assay was used to test for site-specific recombination at the *dif* locus. In this experiment, cells were grown in heavy medium ([¹³C]-glucose and [¹⁵N]-ammonium chloride) and then shifted to light medium for two generations. Chromosomal DNA was prepared and centrifuged in CsCl density gradients. The fractions were then digested with *Bgl* I and probed with [³²P]*dif* DNA to detect a 9.9kb *Bgl* I *dif* fragment. If resolution happened at the *dif* site, semi-heavy, one-quarter heavy and light density *dif* arabinose DNA would be detected. Results showed that in cells transduced with the *ftsK'* (*Ap Kan*) *PBAD::ftsK* construct from GLC600, and grown in the presence of, 13% of the *dif* DNA was located at the semi-hybrid position, as in wild type strains. Without arabinose induction, no semi-hybrid DNA could be observed, suggesting that no dimer resolution happened under these conditions. This experiment

only tests for recombination at *dif* sites over a period of two-generations. It was therefore possible that resolution was only delayed, but not totally prevented. Therefore, a strain with two inverted *dif* sites in the same chromosome was used to show if site-specific recombination between the *dif* repeats requires the C-terminal domain of FtsK.

Recombination between two inverted *dif* sites in the same chromosome will invert the intervening DNA. In this experiment, this inversion will alter the size of an *EcoR* V *spec-dif* fragment, which can be detected by southern blotting with *spec* DNA as a probe. The results showed that without arabinose induction there was no recombination whatever between the two *dif* sites, whereas with arabinose induction recombination happened as in wild-type cells.

Based on the above results and the fact that most of the cells divide normally in the above experimental conditions, it was concluded that the C-terminal domain of FtsK (rather than cell division itself) was required for chromosome dimer resolution.

Considering that failure of chromosome dimer resolution also caused abnormal chromosome partition, the chromosome segregation deficiency in *ftsK* mutants (GLC600 and CDK1, *etc.*) is at least partly due to the failure of chromosome dimer resolution.

It would be very interesting to know if the chromosome dimer resolvase, XerC or XerD, is also located at the septation sites.

C-terminal domain of FtsK is only required for chromosome dimer resolution in *E.coli*.

In order to test whether the C-terminal domain of FtsK (In order to make the expression easier, I will call it FtsKc in this thesis. In *ftsKc*⁻ strains, the N-terminal region of FtsK protein is expressed from its native promoters and functional.) has functions in chromosome partition other than dimer resolution, I compared the behaviour of strains in which FtsKc is unexpressed, with strains in which XerC is

inactivated (*xerC::cat*), or with strains in which both FtsKc is unexpressed and XerC is inactivated. I did not find that depletion of FtsKc could cause more defects than inactivation of XerC. I further found that *ftsKc*⁻ and *mukB*⁻ double mutants were viable, in contrast to a report which claimed that the combination of the truncated *ftsK* gene with a *mukB* null mutation resulted in a synthetic lethal phenotype (Yu *et al.*, 1998), but in accord with the idea that FtsKc is only required for dimer resolution in chromosome partition.

(1). An *ftsK* mutant and an *xerC* mutant cause similar levels of SOS induction and there are no additive or synergistic effects between *ftsKc*⁻ and *xerC* on SOS induction.

Because SOS induction in *ftsKc*⁻ strains is completely dependent upon cell division, it was proposed that FtsKc could not have any significant function in chromosome replication. In strains in which SOS induction is caused solely by a defect in chromosome partition, the level of SOS induction should reflect the extent of the partition defect, provided that cell division is not affected by factor(s) other than those from the SOS pathway. Therefore, comparison of the SOS induction levels in *ftsKc*⁻ and *xerC*⁻ strains should provide some information about the function of FtsKc in chromosome partition. If FtsKc had a function in addition to dimer resolution, we might expect that an *ftsKc*⁻ strain would have higher level of SOS induction than a *xerC*⁻ strain. To test whether this was so, strains TPL1 *ftsK::cat1* and TPL1 *xerC::cat* were constructed by transduction of strain TPL1 with P1 lysate grown on either CDK1(*ftsK::cat*) or C600 *xerC::cat*. To confirm the genotypes of these strains, TPL1 *ftsK::cat1* and TPL1 *xerC::cat* were transduced with P1 lysate grown on *lrp::Tn10* (*lrp* is the gene located immediately upstream of *ftsK* in the *E. coli* chromosome). Transformants were selected on LB+Tc. 22 transductants of TPL1 *ftsK::cat1* were tested and it was found that all were Tc^RCm^S, while amongst 50 transductants of TPL1

xerC::cat , all were Tc^RCm^S. This result suggests that the genotypes of the two strains were as expected. They were therefore used for measuring the levels of SOS induction.

Surprisingly, it was found that the SOS induction level of TPL1 *ftsK::cat1* was at the beginning higher but later lower than that of TPL1 *xerC::cat* (Fig.2.4.1.a). In this experiment the cultures were maintained in early log phase for about 5 hours by serial dilutions and the growth rates of the two strains were almost the same (data not shown). If the cultures were left to grow into stationary phase, their SOS levels both increased except that in TPL1 *ftsK::cat1* it increased for a longer time and to a higher level (Fig. 2.4.1.b).

It is known that there is some cell division deficiency in *ftsKc*⁻ strains, possibly due to the instability of truncated polypeptide consisting only of the N-terminal domain of FtsK. In L broth + arabinose GLC600 could grow and divide well in the presence of 80µg/ml Ap, but could not divide if glucose was supplied (see chapter 2.2). GLC600 was also much more sensitive to nalidixic acid treatment when grown in glucose medium than in arabinose medium (data not shown). Therefore, it is possible that inactivation of either FtsKc or XerC has the same effect on dimer resolution but that a lower number of cell divisions to break DNA in *ftsKc*⁻ strains reduces the level of SOS induction. Alternatively, FtsKc might be responsible for the resolution of only a proportion of dimers.

I propose that inactivation of FtsKc and XerC causes the same chromosome partition defect, but that in *ftsKc*⁻ strains in early log phase the relatively reduced amount of cell division results in relatively less DNA damage, and causes the level of SOS induction to be lower than that observed in *xerC::cat* strains. With the abortion of septa, cells that otherwise would have died because of chromosome damage during division would be able to grow further by forming chains or filaments. When the culture grew into late log and stationary phases, the increase in cell division potential (at least partly due to the expression of FtsA and FtsZ from *ftsQ1p*) would ensure that most of these cells would proceed to division. The survival of the cells with improperly located DNA would leave more DNA to be damaged at late log and stationary phase than would

have been the case if these cells had been killed by septation immediately after dimer formation. This scenario predicts that *ftsKc*⁻ strains should show a higher level of SOS induction than *xerC*⁻ strains at late log or stationary phase.

The above results suggested that FtsKc and XerC worked in the same pathway in chromosome partition. If this were true, then *ftsKc*⁻ and *xerC::cat* mutations should not have additive effects on SOS induction. To confirm this, *xerC::cat* was transduced into GLTPL (TPL *ftsK::PBAD-ftsKa'*) by P1 transduction to produce strain GLTPX (TPL *ftsK::PBAD-ftsKa' xerC::cat*). When shifted from arabinose medium into glucose medium, it was found that β -galactosidase was gradually induced to a stable level in GLTPL, as shown before (chapter 2.2). Interestingly, the SOS induction level of GLTPX was stable in arabinose medium, but when shifted into glucose medium it gradually reduced to the stable level of GLTPL in glucose medium (Fig. 2.4.2). This indicated that there was no additive effect for *ftsKc*⁻ and *xerC*⁻ in SOS induction. An alternative but unlikely explanation for this result is that *ftsKc* mutants might be limited to a lower maximum level of SOS induction. To test this, TPL *xerC::cat* and TPL *ftsK::cat* were treated with nalidixic acid and it was found that Nal could still greatly increase the SOS level in the two strains (data not shown). In contrast, in the experiment described in the next section, I showed that the effect of *mukB*⁻ on SOS induction was additive (or even synergistic) with that of *ftsKc*⁻. Because the level of SOS induction should reflect any significant defects in chromosome replication and partition, it was concluded (based on the above results) that FtsKc is required only for dimer resolution, unless XerC also has more functions than dimer resolution.

(2). *mukB::kan* and *ftsKc*⁻ together are not lethal.

It has been reported that the combination of *mukB::kan* and *ftsK1::cat* is lethal (Yu *et al.*, 1998(b)). This implies that FtsKc should have a role(s) in chromosome

partition other than dimer resolution. This is in contrast to the conclusion of the previous section. Therefore, I decided to see whether I could make an *ftsKc⁻ mukB⁻* double mutant. For this purpose, a *mukB::kan* strain GC7528 (Niki *et al.*, 1991) obtained from Dr. Ken Begg was used. The *ftsK::PBAD-ftsKa'* structure of GLC600 was introduced into the chromosome of GC7528 by P1 transduction. Transformants were selected on LB + Ap (40 µg/ml) + Kan (25 µg/ml) + arabinose (0.2%) and then tested for temperature sensitivity at 42°C to make sure that *mukB::kan* had not been replaced. Strain GlmukB was constructed in this way. This strain could grow both on glucose and arabinose plates and colony sizes on the two kinds of plates were almost the same. However, when shifted from arabinose liquid medium to glucose liquid medium for a period, GlmukB slowed down its growth sharply (see Fig. 2.4.6) although it could still grow to a high density after a long time. GlmukB formed long filaments when grown in LB medium with glucose and could divide better if grown in M9+CAA with glucose, while cells were almost the same as GC7528 when grown in arabinose medium. Observation of chromosome partition in this strain showed that about 40% of cells had abnormally distributed chromosome DNA when grown in glucose media (Fig. 2.4.3 and 2.4.4). In arabinose media this strain behaved similarly to the *mukB::kan* strain GC7528: about 5% of cells without DNA or with abnormally located DNA.

It was possible that the viability of GlmukB in glucose media was due to some possible leakage expression of the whole FtsK protein from the PBAD promoter. Therefore, P1 lysates of CDK1 (W3110 *ftsK::cat1*) and AD12 (MC4100 *ftsK1::cat*) was used to transduce the *mukB::kan* strain. After selection on LB+Cm (20µg/ml) +Kan (25µg/ml) some colonies appeared after two to three days at 30°C. These clones were still temperature sensitive, indicating that *mukB::kan* had not been replaced. To test the possibility that there might be a second mutation in the *mukB::kan* strain suppressing the lethality of the *ftsKc⁻ mukB⁻* double mutant, a P1 lysate of GC7528 was made and used to transduce CDK1. Again, I could get transductants which were Kan^RCm^R and also temperature sensitive. This confirms that *ftsKc⁻* and *mukB⁻* together are not lethal. One of the transductants was picked out and named CDmukB-L. *xerC::cat* was also

transduced into GC7528 to produce a strain named XermukB-L. It was found that XermukB-L grew better than CDmukB-L (Fig.2.4.5), possibly due to the fact that the *ftsKc*⁻ strain was more sensitive to cell division inhibitors such as SOS and ampicillin.

To study the effect of *ftsKc*⁻ on SOS induction in a *mukB::kan* strain, strain GC7528 was first lysogenised with $\lambda P_{sfiA-lacZ}$ to produce strain mukB-L. Then mukB-L was transduced with P1 grown on GLC600 to produce strain GImukBL. SOS induction in this strain was measured and it was found that depletion of FtsKc greatly increased the SOS induction level in the *mukB*⁻ strain (Fig 2.4.6).

Based on all the above results, I conclude that the combination of *ftsKc*⁻ and *mukB*⁻ is not lethal, but does cause cells to grow very slowly. Considering that only some of the cells in a population form dimer chromosomes, combination of a dimer resolution defect with any chromosome partition mutation (if unlethal) should not cause lethal effect, unless in that mutant only cells with dimer chromosome can survive. Therefore, the results are consistent with the conclusion that FtsKc is only required for dimer resolution in chromosome partition.

2.4.4. Summary:

It was found that *ftsKc*⁻ strain and *XerC*⁻ strain caused similar levels SOS induction, and that *ftsKc*⁻ and *XerC*⁻ together could not cause additive SOS induction. This suggests that *ftsKc*⁻ is probably only required for chromosome dimer resolution. It was also found that *ftsKc*⁻ and *mukB*⁻ together were not lethal. This is consistent with its dimer resolution function but different from a former report that claimed *ftsKc*⁻ and *mukB*⁻ double mutant was not viable.

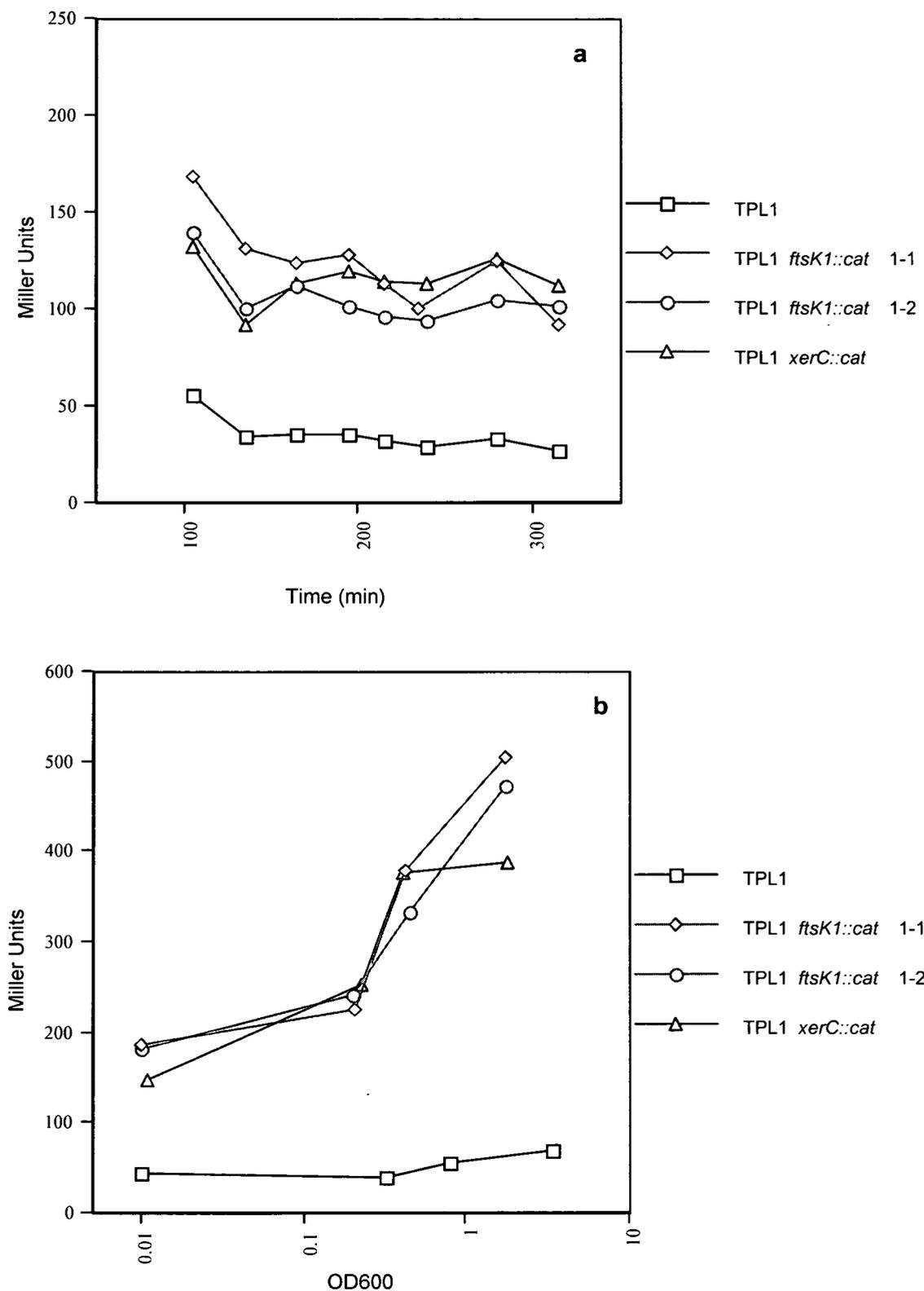
Figure 2.4.1. SOS induction in TPL1, TPL1 *ftsK::cat1* and TPL1 *xerC::cat*.

(a). Overnight cultures of the strains were 1:100 diluted into M9+CAA+glucose (0.2%) medium and grown at 37°C. The cultures were kept in early log phase (OD600 below 0.2) by serial dilutions. Samples were taken at indicated times to measure OD600 and β -galactosidase activity.

(b). Overnight cultures of the strain were first 1:100 diluted into L broth and grown at 37°C. When their OD600 reached about 0.2, the cultures were diluted about 20 times (to OD600 about 0.01) and from then samples were taken at indicated OD600s. The cultures were left for growing into stationary phase without further dilutions.

TPL1 *ftsK::cat1-1* and TPL1 *ftsK::cat1-2* were two random transductants of TPL1 with P1 lysate grown on CDK1.

Figure 2.4.1.



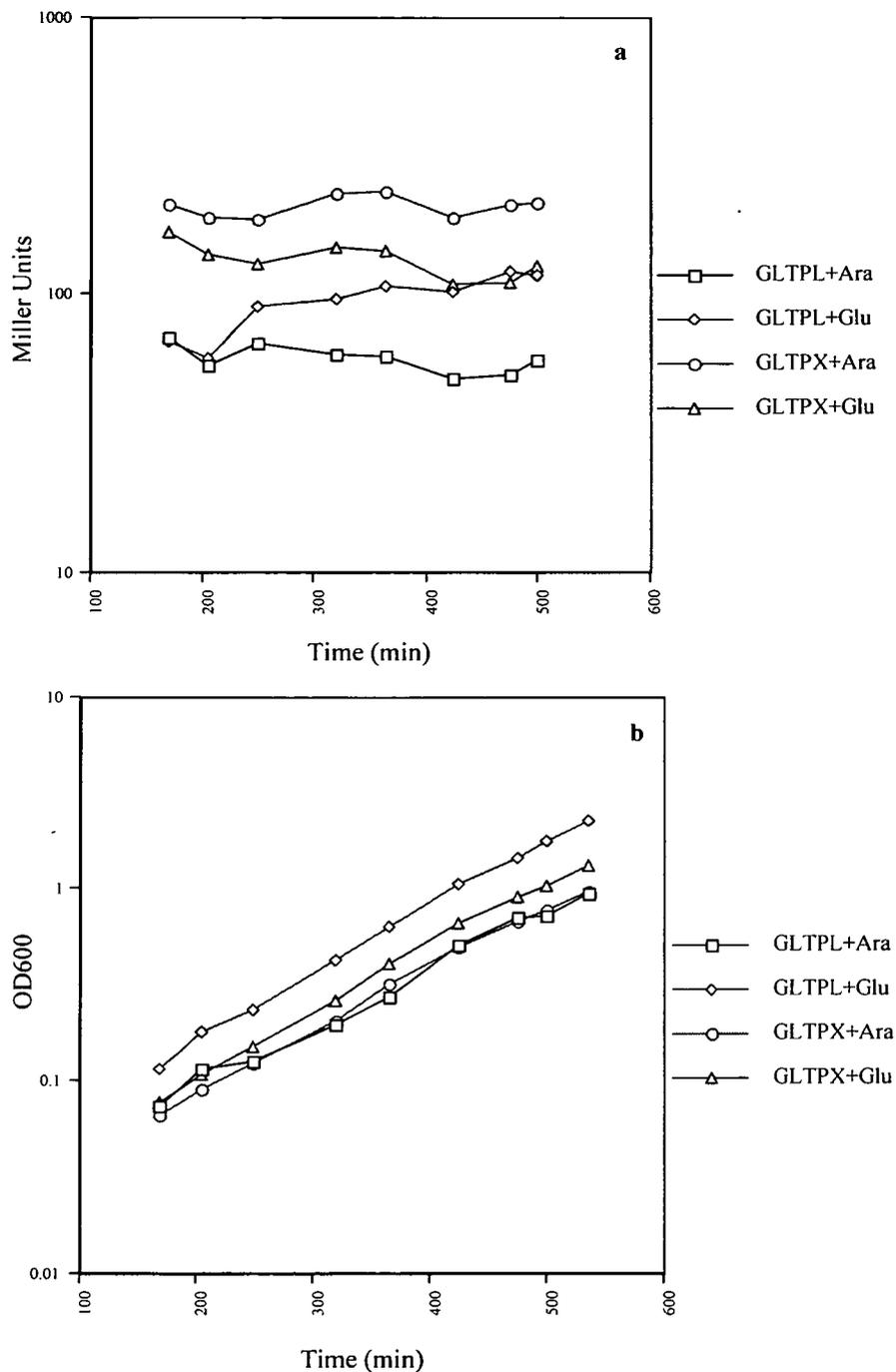


Figure 2.4.2. SOS induction in GLTPL and GLTPX strains. In this experiment, overnight cultures of GLTPL and GLTPX in LB + Ap (40 $\mu\text{g/ml}$) + Kan (25 $\mu\text{g/ml}$) + arabinose (0.2%) (+ Cm at 20 $\mu\text{g/ml}$ for GLTPX) were 1:100 diluted into M9+CAA medium supplied with 0.2% arabinose or glucose. Samples were taken at indicated times and OD600 and β -galactosidase activity were measured.

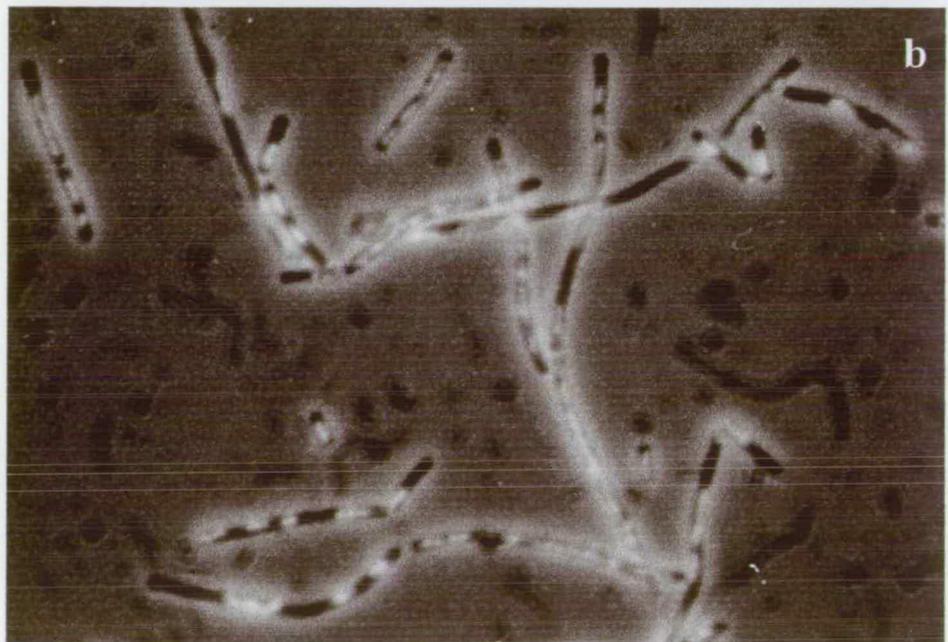
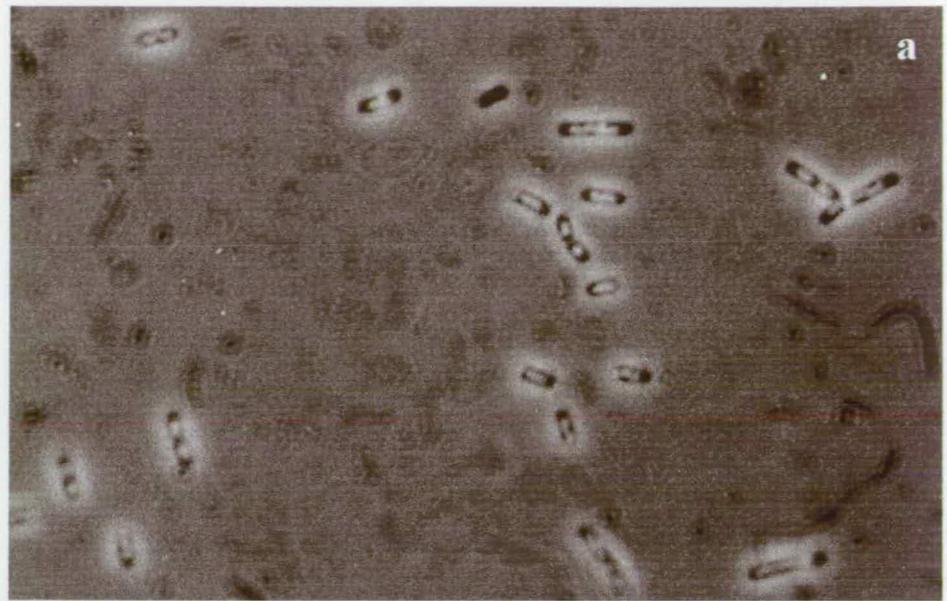
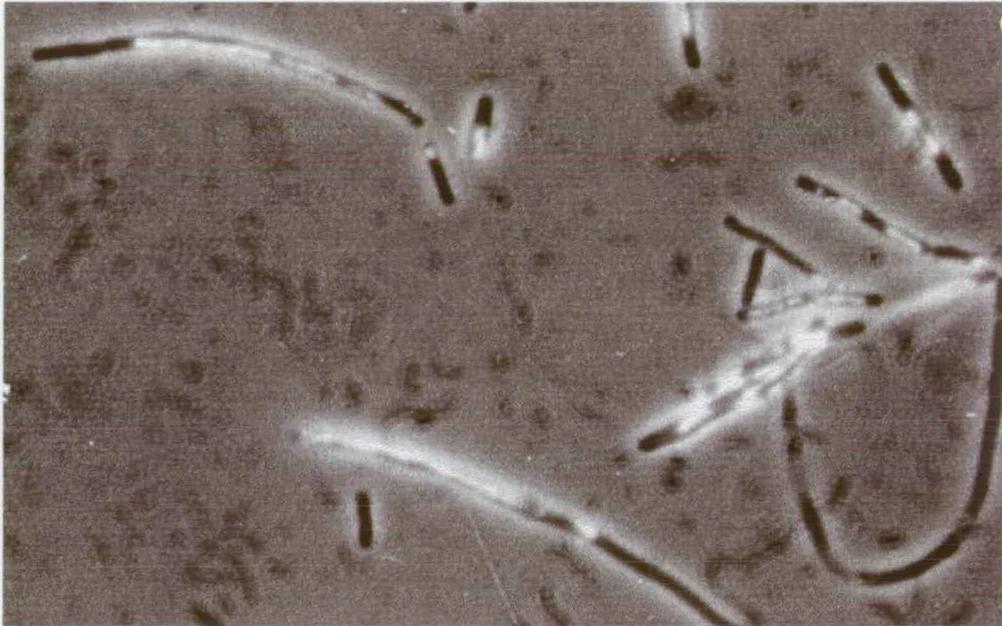


Figure 2.4.3



C

Fig. 2.4.3. Visualization of chromosome DNA in GLmukB cells grown in L broth. Overnight culture of GLmukB in LB+Ap (40 μ g/ml) +Kan (25 μ g/ml) was diluted into L broth with 0.2% arabinose (a) or 0.2% glucose (b and c) and grown at 30°C. When OD600 of the cultures reached 0.2 samples were taken for photographing.

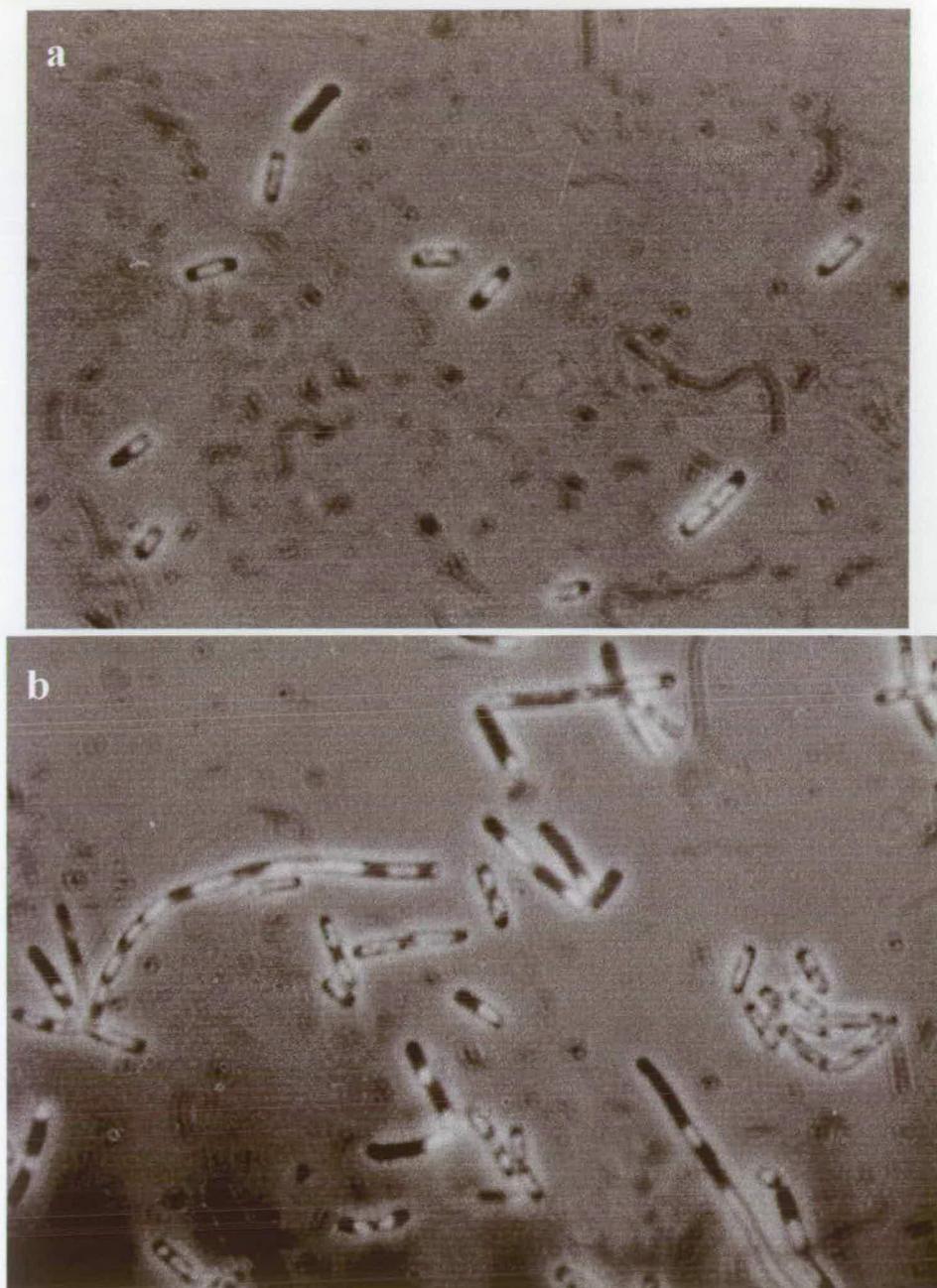


Fig. 2.4.4. Distribution of chromosome DNA in GlmukB cells grown in “M9+CAA” medium. Overnight culture of GlmukB in LB+Ap (40 μ g/ml) +Kan (25 μ g/ml) was diluted into “M9+CAA” medium with 0.2% arabinose (a) or 0.2% glucose (b) and grown at 30°C. When OD600 of the cultures reached 0.2 samples were taken for photographing.

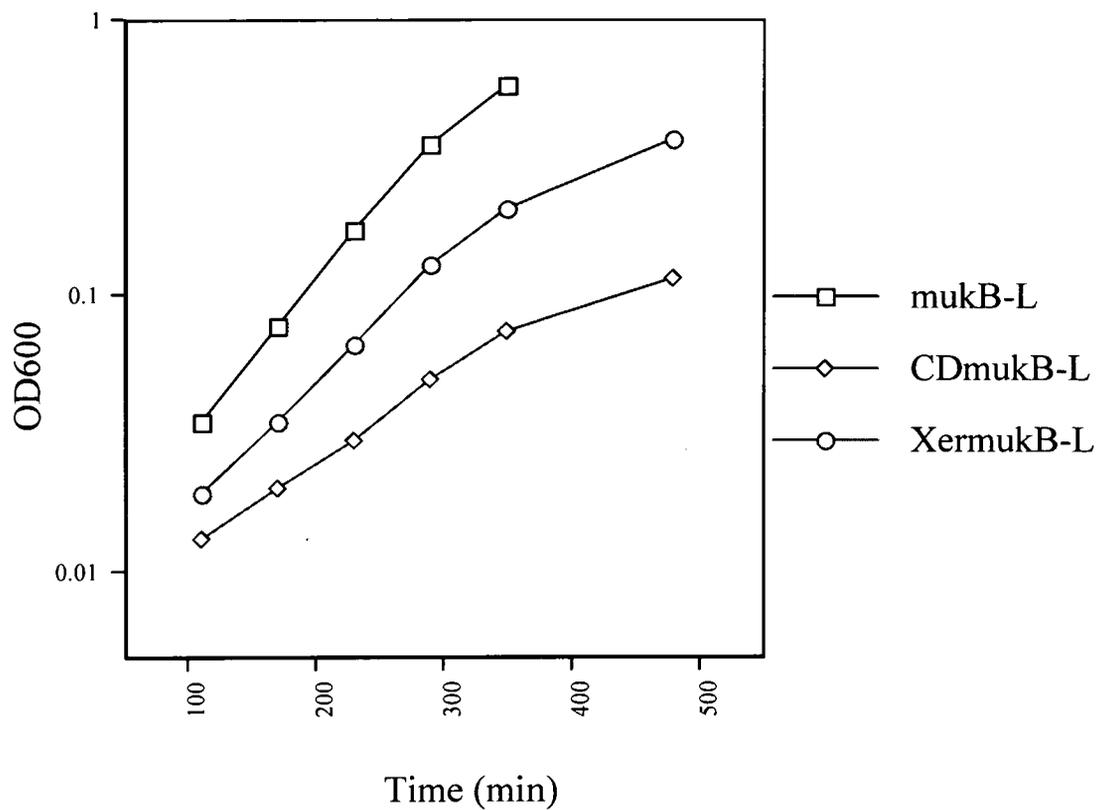


Figure 2.4.5. Growth curves of mukB-L, CDmukB-L and XermukB-L. Overnight cultures of the strains were 1:100 diluted into L broth and then grown at 30°C. OD600s were measured at indicated times.

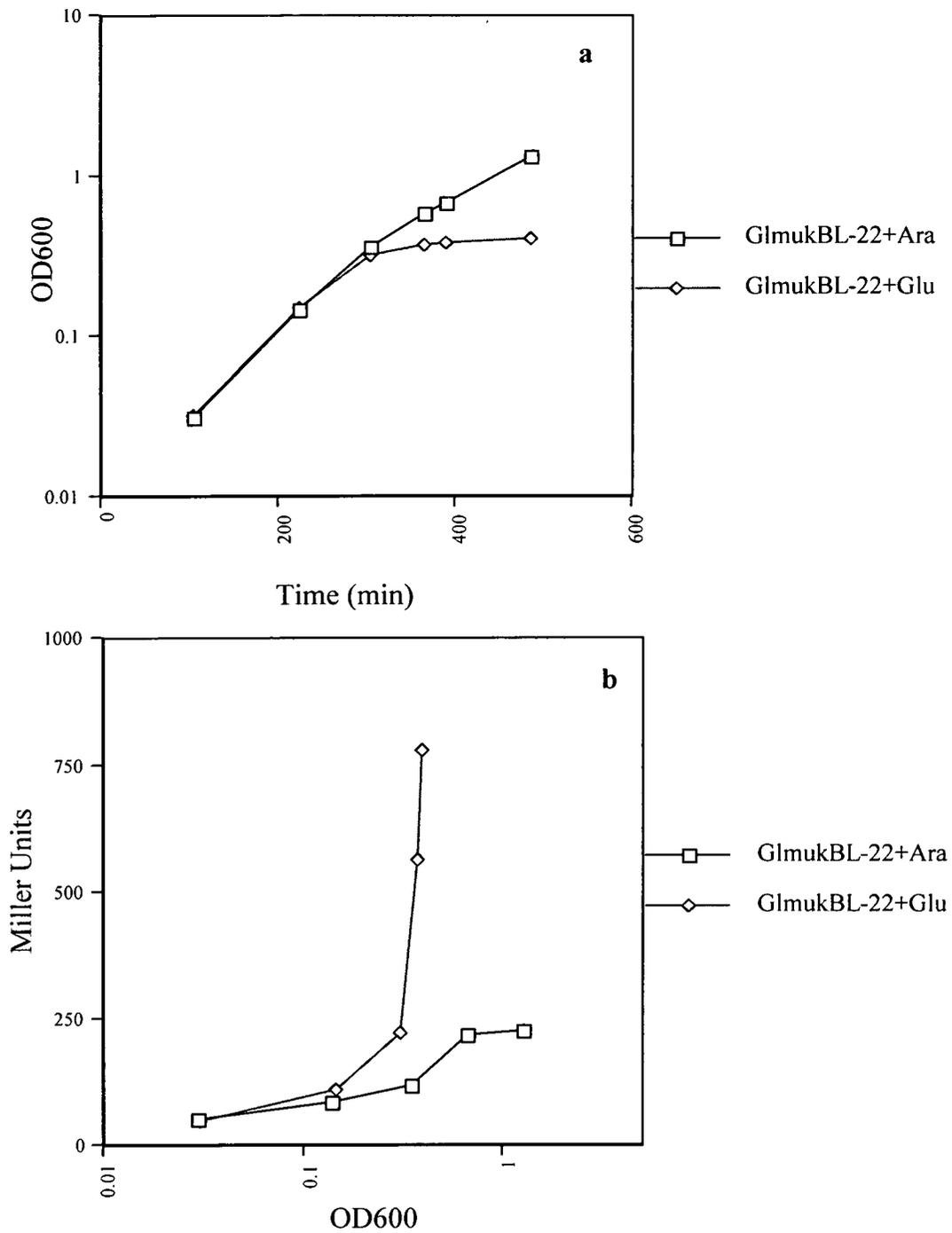


Figure 2.4.6. SOS induction in GlmukBL-22 strain. In this experiment overnight culture of GlmukBL-22 in arabinose medium was 1:100 diluted into L broth with glucose or arabinose and kept growing at 30°C without further dilution.

Chapter 3. Isolation of potential chromosome segregation mutants in *Escherichia coli*

—Establishment of a new method for isolation of genes involved in chromosome partition in *E. coli*

3.1 Introduction.

Chromosome partition is one of the fundamental events in the bacterial cell cycle. However, so far our knowledge about its mechanism is very limited (see chapter 1). Three genetic methods of isolating chromosome segregation mutants have been published (for review see Neidhardt *et al.*, 1996).

Method 1. Microscopic screening of conditional lethal mutants.

Most of the conditional lethal cell division mutants in *E. coli* form long filaments at non-permissive temperature, but chromosome replication can continue and chromosome partition mutants are rare in this collection. Under nonpermissive conditions cell division mutants that are also defective in chromosome partition would be expected to continue DNA replication, but to leave the newly replicated chromosomes in the centers of the filamentous cells. Therefore, partition mutants can be searched for by microscopy in the collection of conditional lethal mutants. Genes identified by this method are *parC* and *parE*, encoding topoisomerase IV, which is required for nucleoid decatenation (Kato *et al.*, 1988; 1990).

Method 2. DNA-less cell producers.

In this method the starting strain cannot express the plasmid-encoded *lacZ* gene because of the existence of the chromosome-encoded repressor LacI. However, if mutations causing a loss of fidelity in chromosome segregation occurred in this strain, DNA-less cells or anucleate cells might be produced. In these DNA-less or "anucleate" cells loss of chromosomal *lacI* allows the expression of the plasmid *lacZ* gene. Thus, mutants that frequently produce DNA-less cells will form blue colonies on X-gal plates. The important potential chromosome segregation genes *mukB*, *mukE* and *mukF* were isolated by this method (Niki *et al.*, 1991; Yamanaka *et al.*, 1996).

Method 3. Camphor resistant mutants.

Most of the mutants resistant to camphor vapors have lost the ability to maintain one copy of chromosome per cell. By screening camphor resistant mutants, several *mbr* genes were isolated (Trun and Gottesman, 1990; 1991)

Here I describe a new method for isolating chromosome partition mutants. This method is based on the phenomenon observed in *ftsK* mutants. In FtsK depleted cells, if chromosomes cannot be properly segregated, the closing septum may guillotine the trapped chromosome during cell division and then induce the SOS response (Fig 3.1). Therefore, chromosome partition mutants should also have continuous SOS induction caused by cell division.

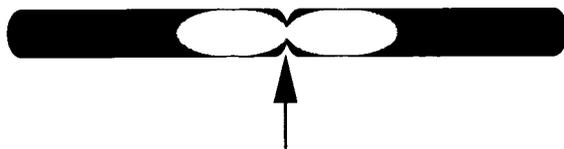


Figure 3.1. Guillotining of unsegregated chromosomal DNA by the closing septum. Arrow indicates the position of septation.

The new method consists of three selection steps.

Step I. Isolation of SOS induction mutants.

The starting strain has a reporter gene (*lacZ*) under the control of an SOS inducible promoter (*e.g.* P_{sfiA}). Mutants in which SOS is induced should form blue colonies on LB+X-gal plates.

However, blue colonies might arise for several different reasons, such as mutations in the SOS inducible *sfiA* promoter, mutations in *lexA* or *recA* genes, mutations in some DNA replication genes, *etc.* Therefore, further screening is needed.

Step II. Microscopic screening of partition mutants.

Chromosome partition mutants should show the effects of abnormal chromosome segregation, such as producing DNA-less cells, unsegregated chromosomes in filaments or DNA abnormally located in cell pole(s).

Step III. Selection of mutants with cell division-dependent SOS induction.

If the SOS is due to septation-caused DNA damage, blocking cell division should inhibit the SOS induction in the mutants. Only the mutants with cell division-dependent SOS induction are regarded as partition mutants and worthy of further study.

3.2. Materials and Methods.

Bacterial strains.

Two similar reporter strains TPL1 and CL4 were constructed by lysogenizing TP8503 and C600 with $\lambda P_{sfiA-lacZ}$, respectively. The *lacZ* gene in $\lambda P_{sfiA-lacZ}$ is under

the control of the *sfiA* promoter, which is SOS inducible. These two strains were then mutagenized by UV irradiation.

The genotype of the donor strains used for Hfr mating was listed in table 3.1.

Construction of an *E. coli* genome library.

In order to locate the putative mutations, an *E. coli* genome library was constructed for selecting complementary DNA fragments or genes. Chromosomal DNA of C600 was prepared on a large scale and partially digested with *Taq* I and *Sau*3AI. Then the digested DNA was loaded onto agarose gel and the DNA fragments in the 8-10kb region were purified. Ligation mixtures of these purified fragments and *Bam*HI+*Cla*I-digested pACYC177 were transformed into DH5 α . Transformants were pooled in groups of 50, and the plasmids extracted from such a mixture by the midi-preparation method were stored as one preparation. In total, 47 preparations were made and used as an *E. coli* genome library. This library contains about 1.8×10^4 kb of DNA, which is equivalent in amount to about 4 complete *E. coli* chromosomes.

The process of ultraviolet light mutagenesis:

An overnight culture of TPL1 or CL4 was inoculated 1:100 into L broth and grown at 37°C with shaking until the OD600 was about 0.5. 10ml of the culture were centrifuged and resuspended in 7ml 10mM MgSO₄. The suspension was poured into a glass petri dish and exposed to UV for 100 seconds with shaking. Before this exposure the UV light had been pre-warmed for at least 20min so that the light would deliver UV at about 10 ergs/mm²/second. The exposed cells were then spun down and resuspended in 10ml L broth. After growth at 37°C for 3-5 hours with shaking, the cells were spread onto LB+X-gal (30 μ g/ml) plates. The plates were left at 30°C for about 30 hours and then moved to 4°C until dark blue colonies could be easily distinguished from the remaining pale blue or white majority. The dark blue colonies were purified and their chromosome segregation behaviours were studied by phase-contrast fluorescence microscopy after DAPI staining. The UV mutagenesis procedure is based on Miller (1972).

microscopy after DAPI staining. The UV mutagenesis procedure is based on Miller (1972).

Many colonies have been isolated in this way and those clones with interesting chromosome segregation phenotypes have been studied further. The selected mutations were mapped by Hfr mating, plasmid complementation and/or P1 transduction.

Hfr matings.

The donor strains used for Hfr matings are listed in table 3.1 and their DNA transfer properties are shown in figure 3.2. These strains were all from Dr. Ken Begg in this lab.

Table 3.1. Genotypes of the Hfr mating donor strains.

Strain name (No.)	Chromosome	locus of selectable marker
A1 (5051)	Hfr H: P01; <i>relA1 thi-1 spoT supQ80 nadA57</i> /Tn10	Tn10 (16.75)
A3 (5052)	KL227: P03; <i>metB1 relA1 btuB3191</i> /Tn10	Tn10 (88.75)
A5 (5053)	KL208: P031; <i>relA1 zbc-280</i> /Tn10	Tn10 (12.0)
A7 (5054)	KL96: P044; <i>relA1 thi-1 trpB83</i> /Tn10	Tn10 (28.0)
A9 (5055)	KL16: P062; <i>relA1 thi-1 zed-3069</i> /Tn10	Tn10 (43)
A11 (18209)	KL228: P085; <i>thi-1 leu-6 gal6 lacY1 zgh-3075</i> /Tn10	Tn10 (67.0)
C2 (18160)	KL14: P068; <i>relA1 leu thi-39</i> /Tn10	Tn10 (90.25)

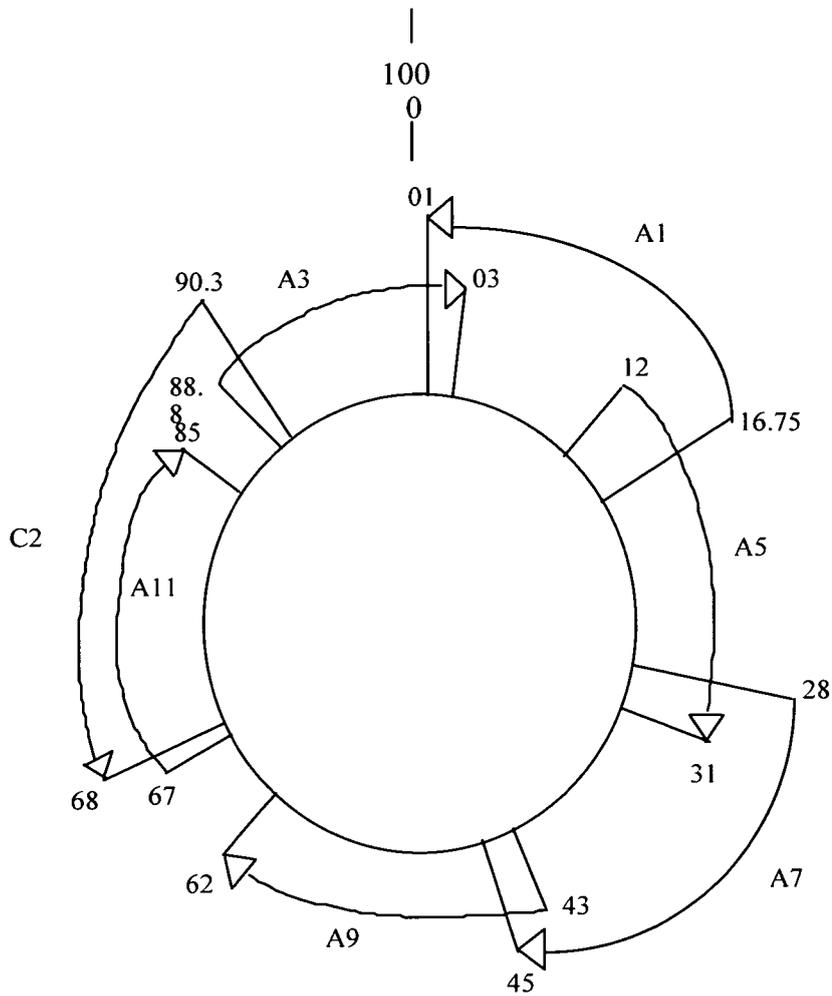


Figure 3.2. Locations of selectable Tn10(tet^R) markers (straight lines), F-factors (straight lines with triangles connected) and direction of DNA transfer in the seven Hfr mating donor strains used in this work.

For Hfr matings with donor strains, the mutants were first transformed with pUCAT (see chapter 1) to introduce a Cm^r maker for selection. I also tried using Ap^r in pUC19 for selection, but found that Ap^r can not be used for Hfr mating selection, because the degradation of Ap by β -lactamase secreted by the recipient cells will allow the Ap^r donor cells to grow up and form a lawn. The information about the seven donor strains is shown in figure 3.2, according to Dr. Ken Begg's drawing in this lab and their genotypes (see table 3.1).

Plasmid complementation:

For plasmid complementation, *E. coli* genomic library DNA was transformed into mutants and selected on LB+Ap (50 μ g/ml)+X-gal (30 μ g/ml). In transformants carrying complementing plasmids the SOS induction should be suppressed and the colonies should be pale blue. Therefore, after transformation pale blue colonies among the majority of dark blue colonies were picked up and purified. The morphology of the cells in these colonies was observed. Complementing plasmids should also be able to convert filamentous mutant cells to normal-sized cells. The putative complementing plasmids were then extracted and transformed into the corresponding mutants to confirm the complementation result. Finally the inserted fragments carried by these plasmids were sequenced and the complementing genes identified.

3.3. Results.

During this work the following mutants with chromosome segregation defect were isolated by the new method. Their locations in *E. coli* chromosome were further studied by Hfr mating, P1 transduction, and/or plasmid complementation.

Mutant G22:

Most of the cells in the culture of this mutant are normal-sized, but about 20-30% of cells are long cells or middle-length filaments. Visualisation of chromosome DNA by DAPI staining showed that anucleate cells could be seen frequently and that there were also cells with polar DNA.

Hfr mating showed that G22 is linked to the Tc markers in A3 (~5-10% among 450 colonies) and A5 (~7% among 150 colonies). This suggests that the mutation is located around 0 min on the *E.coli* chromosome. P1 transduction showed no linkage to *leu::Tn9* at 1.4 min.

Then G22 was transformed with *E.coli* DNA library DNA. Three plasmids named D3, D6 and GN2 could complement G22. Among them the degree of complementation was in the order D3>D6>GN2.

Sequencing of these complementary plasmids showed that the complementary fragments in the plasmids are:

In D3: 3000bp in AE000130 to 880bp in AE131.

In D6: AE000130 2.58kb-10.8kb.

In GN2: AE000130 2.0-10.0kb.

Note: The DNA sequences carried by AE000xxxxs can be found at ECDC web site (<http://susi.bio.uni-giessen.de/ecdc/ecmapov.htm>).

This means that it is probably the same gene in each of the plasmids that is complementing mutant G22. D3 was then used to define the gene by subcloning.

D3 plasmid (2.5µg in 20µl buffered by NEB4) was partially digested by *TaqI* (4u at 65°C for 3.5min) and then *Sau3A I* (0.8µl at 37°C for 4 min). The reaction mixture was loaded onto agarose gel and 0.5kb-1.0kb restriction fragments were isolated and inserted into pACYC177/*BamH I*-*Cla I*. The ligation mixture was transformed into G22 and three complementary plasmids were isolated. Sequencing of these plasmids showed that the complementary fragment is 4100bp-5100bp in AE000130. Genes in this region of AE130 are *o246*: 3827-4567, *rnhA* 4564-5031, and *dnaQ* 5096-5827.

Therefore, the complementing gene for G22 is *rnhA*. In the subcloned complementary plasmid the *kan* promoter can transcribe the *rnhA* gene.

The gene product of *rnhA* is RNase HI, which is a major RNase H in *E. coli* (Carl *et al.*, 1980). RNase HI participates in the removal of the RNA primer from Okazaki fragments during the synthesis of the lagging DNA strand (Ogawa and Okazaki, 1984). It also functions as an endonuclease cleaving an RNA transcript in a specific RNA-DNA hybrid in ColE1-type plasmids and the processed RNA is then used as a primer for the initiation of plasmid replication by DNA polymerase I (Itoh and Tomizawa, 1980). By removing the R-loop, consisting of an RNA-DNA hybrid and the replaced single strand DNA, it can prevent the possible initiation of chromosome replication there (Kogoma *et al.*, 1985; von Meyenburg *et al.*, 1987). In *rnhA*⁻ strains, constitutive stable DNA replication (cSDR) is activated. In this condition R-loops become origins (*oriKs*) of chromosome replication (for a review see Asai and Kogoma 1994a). Although initiation of chromosome replication from *oriKs* can make the normal origin *oriC* dispensable, the cell growth is perturbed, possibly because this kind of initiation is not well regulated (von Meyenburg *et al.*, 1987). cSDR needs RecA, but does not need RecB, RecC, RecF or RecJ (Kogoma *et al.*, 1994).

Another abnormal initiation mechanism is inducible stable DNA replication (iSDR), which is activated in SOS induced cells (for a review see Asai and Kogoma, 1994a). It occurs at D-loops generated by RecBCD and RecA (Asai *et al.*, 1993). Major origins (*oriM1* and *oriM2*) of iSDR are located in the *oriC* and *terC* regions (Magee *et al.*, 1992).

Since in an *rnhA* mutant chromosome replication can be initiated at several *oriKs*, in addition to *oriC*, the co-ordination of cell division and chromosome replication is disturbed. Therefore, it is possible that an *rnhA* mutant could show a cell division defect and produce anucleate cells or cells with polar DNA. The reason for SOS induction in the *rnhA* mutant is not clear so far (Kogoma *et al.*, 1993). It would be interesting to see whether the SOS induction is caused by cell division.

In conclusion, the mutation in G22 was roughly located by Hfr mating near the 0 min region of the *E. coli* chromosome. Screening complementing plasmids showed that the *rnhA* gene could complement G22. The *rnhA* gene is at 5.1 min on the *E. coli* chromosome. Therefore, the mutation is probably in *rnhA*.

Mutant G27:

The population is a mixture of normal-sized cells and filaments (about 1:1). DAPI staining showed the existence of anucleate cells and cells with polar or unsegregated DNA. By Hfr mating it was found that the mutation in G27 was linked to the Tc^r in A9. However, screening of complementing plasmids from the DNA library failed because the colour of the colonies after transformation was not sufficiently distinctive to allow complementation to be easily scored.

Mutant G28:

About 10-20% of the population were long filaments and others were normal-sized cells. DAPI staining showed the existence of anucleate cells and cells with polar DNA.

Hfr mating showed the mutation in G28 was linked to Tc^r in A3(~95%) and C2 (~50%). Therefore, the mutation in G28 is probably at 86-90min in *E. coli* chromosome. It was also found that this mutant could produce mini-cells.

Interesting genes in this region, in which mutations cause or might cause chromosome partition defects, are *xerC* (86.07min), *priA* (88.8) and *hupA* (90.5). Amongst these, the *priA* locus is very close to the Tc^r marker (88.75min) in A3.

The function of XerC has been described in chapter 1.

PriA is a single-stranded DNA-dependent ATPase, DNA translocase, and DNA helicase (for review see Marians, 1999). PriA is the primosome component that initially binds to the DNA template-primosome assembly site (PAS) (Shlomai and Kornberg, 1980; Lee *et al.*, 1990), followed by PriB. Then DnaT, in association with PriC, transfers DnaB from the DnaB-DnaC complex to the DNA-protein complex, now termed the preprimosome. Finally, binding of DnaG primase to the preprimosome completes the assembly of the primosome, after which the synthesis of the first RNA primer for the DNA polymerase III holoenzyme (replisome) starts (Allen and Kornberg, 1993). It is known that inactivation of *priA* can cause SOS induction (Nurse *et al.*,

1991) and affect cell growth and cell division (Lee and Kornberg, 1991). Recent studies show that PriA is required for homologous recombination and double strand break repair (Kogoma *et al.*, 1996) and is essential for inducible and constitutive stable DNA replication (Masai *et al.*, 1994). It has been found that the DnaA-dependent primosome supports normal chromosomal replication from *oriC*, while the PriA-dependent primosome functions in *oriC*-independent chromosomal replication observed in DNA-damaged cells or cells lacking Rnase H activity (for a review see Masai and Arai, 1996). The latest studies show that PriA can direct the loading and assembly of replication forks at a D loop, an intermediate that forms during homologous recombination, double-strand break-repair, and stable DNA replication (Liu *et al.*, 1999; Liu and Marians, 1999; Masai *et al.*, 1999) Thus, PriA plays an important role in the connection between recombination and replication. Since PriA is involved in the resolution of chromosome recombination intermediates, it is possible that mutations in *priA* can cause abnormal chromosome localisation (see chapter 4 about *ruvABC* for reasons).

hupA encodes the HU α subunit of HU protein and *hupB*, located at 9.9min, encodes the other subunit (HU β) of HU protein (Rouvière-Yaniv and Kjeldgaard, 1979). It is known that HU protein is required for the maintenance of chromosome structure and involved in the initiation of DNA replication and also chromosome segregation (Yamazaki *et al.*, 1984; Dixon and Kornberg, 1984; Broyles and Pettijohn 1986; Wada *et al.*, 1988; Ogura *et al.*, 1990). The double mutant *hupAhupB* is filamentous and also produces anucleate cells, but *hupA* or *hupB* single mutants do not have any differences in phenotype from the wild type parent (Wada *et al.*, 1988). Therefore, it seems probable that the mutation in G28 is not located in the *hupA* gene. However, since the *hupA* insertion mutant in that report may really behave differently from some other *hupA* mutants, this phenotypic difference between mutant G28 and known *hupA* mutants cannot absolutely exclude the possibility that the mutation in G28 is in *hupA*.

In conclusion, the mutation in G28 is approximately located at 86-90min on the *E. coli* chromosome. Genes *xerC*, *priA* and *hupA* in which mutations cause or might cause chromosome segregation deficiencies are also located in this region. The exact location of the mutation in E8 needs to be studied further.

E8:

Most of the cells were normal-sized. DAPI staining showed that normal-sized anucleate cells could be seen frequently, while most of the filaments had unsegregated and irregularly or centrally located DNA or polar DNA.

Hfr mating showed this mutation is linked to Tc^r in A11 (60-70%) only, appearing to be located around 67 min. Screening of complementing plasmids showed that it can be complemented by EN12, EC2-3/4 and ED41. EN12 can perfectly complement E8, while ED41 can only partly complement it, *i.e.* there were still some filamentous cells in E8(ED4-1/3). Plasmids EC2-3/4 caused a strange phenomenon (see below). The plasmids were then sequenced.

Sequencing of EN12 and ED4-1/3 showed that the chromosome fragment carried by N12 is from (Cla) 0.00kb to 7.8kb (Bam) in AE000373, and that by D4-1/3 is from (Bam) 11.2kb in AE000372 to 8.6kb in AE000373. Therefore, the complementary sequence should be 0.00kb-7.8kb in AE000373. The genes in this region are (in bp) 76-1809 *recJ*, 1815-2525 *dsbC*, 2550-3446 *xerD*, 3558-4079 *fldB*, 4119-4526 b2896, 4507-4773 *ygfY*, 5016-5996 *ygfZ*, 6192-6851 b2899, 7015-7326 *yqfB* and 7365-8804 *bglA*. The location of this fragment in the *E. coli* chromosome is 65.5min. Therefore, considering the location suggested by the Hfr mating, the mutation of E8 should be in one of these genes. It is possible that the mutation might be in *recJ* or *xerD*.

The function of *xerD* has been described in chapter2.

RecJ is a 5' to 3' single-stranded DNA exonuclease (Lovett and Kolodner, 1989). RecJ is involved in the RecF pathway of recombination (Lovett and Clark, 1984; Lloyd *et al.*, 1987) and also in the DNA base excision-repair pathway (Sargentini and Smith, 1986; Lloyd *et al.*, 1988; Dianov and Lindahl, 1994; Viswanathan and Lovett, 1998). RecJ stimulates the rate of heteroduplex product formation in RecA-mediated strand-transfer reactions. RecJ also dramatically enhances the efficiency with which RecA is able to transverse regions of non-homology in the substrates, possibly by degrading the displaced strand produced by strand exchange which competes for pairing with the transferred strand, thus driving forward the unidirectional branch migration mediated by

RecA protein (Corrette-Bennett and Lovett, 1995). Recent study also shows that the RecJ protein preferentially stimulates illegitimate recombination at the hotspot in the formation of specialized transducing lambda phage (Ukita and Ikeda, 1996).

It is shown that deficiency in the resolution of recombination intermediates could cause abnormal chromosome segregation phenomenon (see chapter 4 about *ruvABC* in this thesis). Therefore, it is possible that a mutation in the *recJ* gene could cause a chromosome partition problem.

Plasmids EC2-3/4 caused E8 to form white colonies on LB+X-gal plates. However, EC2-3 and 2-4 also caused over-division: all the cells with this plasmid were very small, and E8(EC2-3/4) grew very slowly at temperatures above 30°C and quite quickly at room temperature, compared to the wild type strain or to E8 without this plasmid. When transformed into wild type strain CL4, EC2-3/4 still caused over division and slow growth at temperatures above 30°C.

Sequencing of plasmids C2-3/4 showed that the insertion fragment is (Cla)-0.00 in J04039 or 4.1kb in AE000362 to 2.7kb in J04039 or 1.4kb in AE000362-(Bam). Genes in this region are *relA* (280-2514bp) and *mazE* (2592-2840) in J04039. Therefore, the only intact gene in this fragment is *relA*.

It is known that the stringent response of *E. coli* to amino acid starvation leads to the activation of *relA* and consequent accumulation of ppGpp (for a review see Cashel *et al.*, 1996). ppGpp can regulate the transcription activity of many genes, possibly by binding to the β -subunit of *E. coli* RNA polymerase (Chatterji *et al.*, 1998). RelA is a ppGpp synthase. It can convert pppGpp to ppGpp. It is known that high ppGpp pools confer mecillinam resistance and make the *pbpA* gene dispensable (Vinella *et al.*, 1992; Joseleau-Petit *et al.*, 1994). Overproduction of ppGpp can also induce resistance to novobiocin, which targets the B subunit (GyrB) of DNA gyrase (Gellert *et al.*, 1976; Contreras and Maxwell 1992; Maxwell 1993; Milija *et al.*, 1999.). Recent study also shows that over-expression of *relA* (or *dnaN*, *pcnB* or *cyaA*) can suppress the lethal phenotype resulting from the induction of a mutant DnaA protein (DnaA E204Q) with decreased intrinsic ATPase activity (Makise *et al.*, 1999). The effect of the expression

of *relA* on cell growth rate has been very well studied. It has been found that when the ppGpp pool is increased by expressing hyperactive RelA' product or in other ways, then growth is slowed down (Schreiber *et al.*, 1991; Joseleau-Petit *et al.*, 1994), and in natural growth conditions growth rate is an inverse function of ppGpp concentration (Joseleau-Petit *et al.*, 1994). *relA* can also affect the expression of some stationary phase genes through ppGpp-engendered stimulation of the synthesis of the alternative sigma factor, RpoS (Gentry *et al.*, 1993; Eichel *et al.*, 1999).

The observation that plasmids EC2-3/4 caused E8 and CL4 to grow slowly as very small cells may be explained by the described functions of *relA*. However, it is not clear why they caused the mutant to form white colonies on X-gal plates. One possibility is that the high expression of *relA* induced the stringent response and this caused the expression of a suppressor of the mutation in E8.

In order to test the possibility that the white colony, *i.e.* the inhibition of SOS induction in E8 by *relA*, is due to RelA inhibiting SOS induction, CL4 was transformed with plasmids pACYC177 and EC23 separately. Two transformants from each transformation were picked up and SOS induction by nalidixic acid was tested. Overnight cultures of these cells were diluted 1:100 into LB or LB+Nal (20µg/ml) and grown at 30°C with shaking. Samples were taken after 3 and 4 hours incubation. The result (Fig. 3.3) showed that the Nal-induced SOS induction level of C14 (EC23) was even higher than that of C14 (pACYC177). Therefore, the inhibition of SOS induction by *relA* in E8 is possibly due to the correction of a DNA segregation deficiency.

DAPI staining of E8 (EC2-3/4) showed that there were almost no anucleate cells in the population. This suggests that the chromosome segregation defect of E8 could be suppressed by *relA*.

In sum, by Hfr mating the mutation in E8 was roughly located at the 67min region in the *E. coli* chromosome. A DNA fragment including the *recJ* and *xerD* region, located at 65.5min in the chromosome, could complement this mutant perfectly. Strangely, this mutant could also be complemented (suppressed) by *relA* but the reason

for this is not clear. The suppression possibly involves a RelA-mediated stringent response.

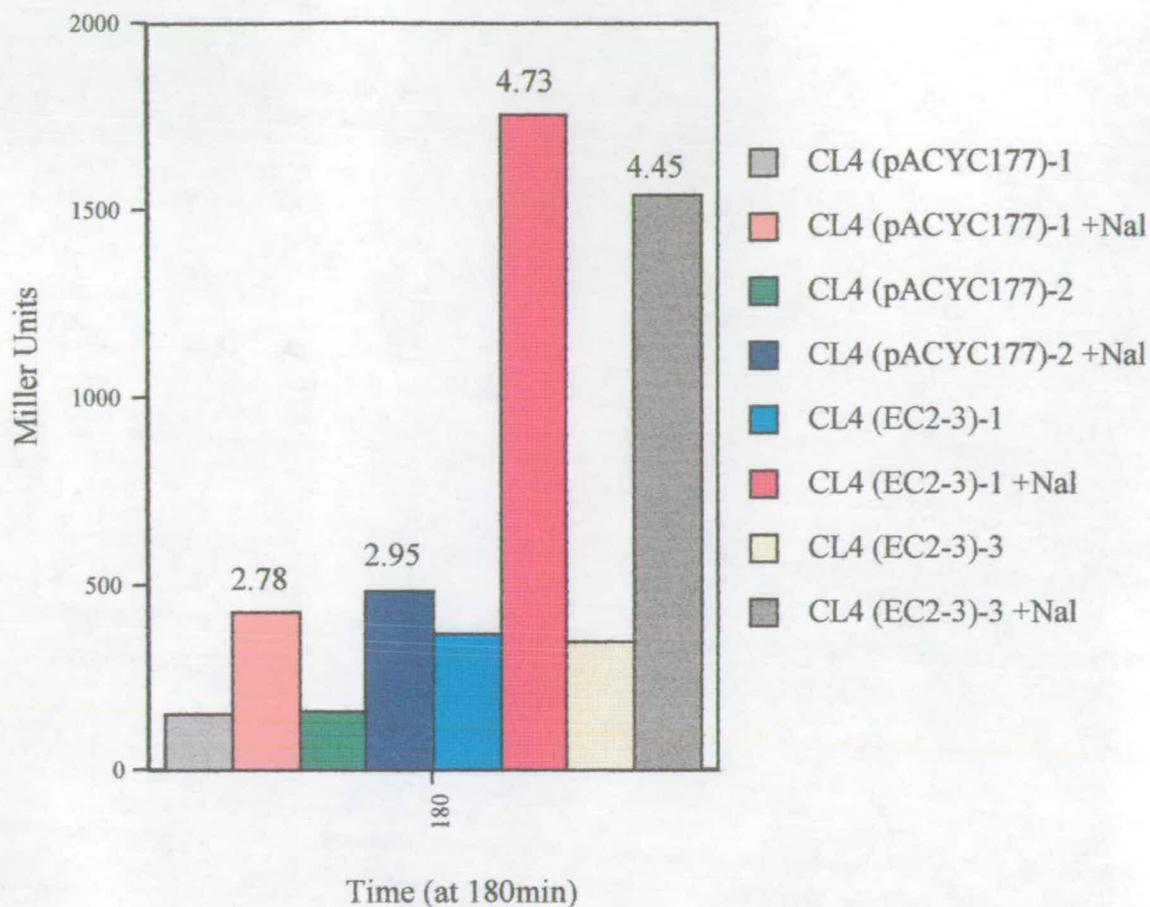


Figure 3.3. The effect of overexpression of *relA* on SOS induction.

In this experiment, overnight cultures of CL4 (pACYC177)-1/2 and CL4 (EC2-3)-1/3 were inoculated 1:100 into LB or LB+Nal (20 μ g/ml) and grown at 37°C for three hours. Samples were then taken and the activity of β -galactosidase was measured. The ratios of MUs of Nal-treated cultures to that of their corresponding uninduced controls (left) are indicated above the columns.

C2-2:

It is a temperature sensitive mutant. It could not form colonies at 42°C on LB plates. Colonies grown at 30°C on LB plates consisted of a mixture of filaments and normal-sized cells. If grown in liquid LB medium, the cells were normal-sized at early log phase at 30°C or 37°C, but started to elongate without division when OD600 reached 0.5. DAPI staining showed chromosome replication stopped when cells started to filament. At 42°C, the mutant cells did not grow into filaments, but just stopped growing.

Hfr mating showed that the C2-2 mutation was linked to the Tc^r markers of A9 and A11, but closer to A9's (at 43min) than to A11's (at 67min). This suggests that the mutation is roughly located at 50min on the *E. coli* chromosome.

It was found that five plasmids from the library could complement the C2-2 mutant. Each of these plasmids could reverse the temperature-sensitive phenotype of C2-2, while the control plasmid pACYC177 could not. Among them, C2-2 (pN9) still produced some filaments and C2-2 (pF6) only produced a very low proportion of filaments, while C2-2 (pN6/pF9/pE12) was perfectly normal.

Sequencing of these plasmids showed the complementary fragments in the plasmids were:

In E12: from 4650bp in AE000320 to 287bp in AE000321.

In N9: from 11089bp in AE000319 to 8328bp in AE000320.

In N6: from 1732bp in AE000320 to 621bp in AE000321.

The shared sequence of the three plasmids are 4650bp to 8328bp in AE320. Genes in this region are *dedD*, *folC* and *accD*, located at 52.4min in the chromosome.

To define the complementary gene, the E12 plasmid was digested with *EcoR* I and *Stu* I. It was found that the RI fragment could complement C2-2, while the *Stu* I fragment could not. This plasmid was named E12/RI. Therefore, the complementary gene is *dedD* or *folC*, but not *accD*. Then *Msc* I or *Msc* I+*Nde* I were used to destroy *dedD* and *Sal* I+*EcoR* I, or *Mlu* I+*Sal* I to destroy *folC* in E12/RI plasmid. Result

showed that C2-2 (E12/RI//RS OR MS) cells still filament, while C2-2 (E12/RI//M) cells were normal. Therefore the complementary gene of C2-2 is *folC*.

The *E. coli folC* gene product, folylpolyglutamate synthetase-dihydrofolate synthetase, is a bifunctional enzyme catalyzing two distinct reactions in the folate biosynthetic pathway. Folylpolyglutamate synthetase (FPGS) catalyzes the conversion of folates to polyglutamates (Bognar *et al.*, 1985; 1987) and the dihydrofolate synthetase adds L-glutamates to dihydropteroate to form dihydrofolate (Bognar *et al.*, 1985; Bognar *et al.*, 1989 and the references therein). It is reported that *folC* is an essential gene in *E. coli*. It is not sufficient to support the growth of a *folC* deletion strain by including the end products of folate dependent biosynthetic pathways, possibly because such a supplement can not satisfy the folate requirement for the synthesis of formyl-methionyl-tRNA, which is required for the initiation of protein synthesis (Pyne and Bognar, 1992).

The temperature sensitive phenotype of the C2-2 mutants suggests that the mutagenised gene is possibly an essential gene, which is consistent with the function of *folC* gene. It is known that the initiation of chromosome replication at *oriC* requires concomitant protein synthesis and transcription. It is possible that the *folC* mutation may block the initiation of chromosome replication by a deficiency in the initiation of protein synthesis. However, it is not clear why chromosome replication and cell division were blocked only after the culture grew to OD600 above 0.5. There are two possibilities. One is that the expression of *folC* is growth phase dependent so that when the culture reaches mid-log phase the expression is lowered and the residual activity of the mutant enzyme is no longer sufficient to support protein synthesis. Another possibility is that when the cells start to enter late-log phase or early stationary phase the initiation of chromosome replication needs other factors.

In conclusion, by Hfr mating the mutation in C2-2 was located at approximately 50min on the *E. coli* chromosome. Screening of the genomic library showed that *folC*, an essential gene located at 52.4min, could complement the mutant. These suggest that

the mutation is probably located in *folC*. Since the deficiency in cell division and chromosome replication seems not directly linked to the function of FolC, some published *folCts* mutants showed be obtained and their cell division and chromosome replication behaviours should be studied and compared to those of C2-2.

T1-6:

It is a Ts mutant. Cells filamented and then lysed when grown at 42°C in L broth. Its chromosome partition pattern at 42°C is very similar to that of a *mukB* mutant (Niki *et al.*, 1991). It also produced a low proportion of anucleate cells at 30°C.

Transformation of T1-6 with plasmids carrying *mukB*, *mukE* or *mukF* (Yamanaka *et al.*, 1996) (from Ken Begg) and ED51 (carrying *rus* gene, see below in “E10, H and N24” mutants section) showed that this mutant can only be complemented by the plasmid carrying *mukB* gene. P1 transduction of this mutant with P1 *aroA::Tn10* showed that the mutation in this strain has about 50% linkage to *aroA*. *aroA* is at 20.65 min and *mukB* is at 21.03 min on the *E.coli* chromosome. Therefore, this confirms that the mutation is in the *mukB* gene.

T2-7:

It is a typical partition mutant. It produced many anucleate cells (~40%) and most of the other cells had chromosomal DNA congregated in the middle of filaments. Screening of the library for complementing plasmids showed that C1, C5, N6 and N8 could complement T2-7. N8 and N6 could complement T2-7 perfectly: no filaments, no anucleate cells. C5 and C1 could partly but largely complement it. T2-7 (C1) produced about only 2% anucleate cells and 5% filaments, but DNA in all of the filaments was unsegregated. T2-7 (C5) is very similar to T2-7 (C1), except it gave about 10% filaments.

Sequencing of these plasmids showed that the complementary sequence in C1 is bp4660—8907 in AE000402. Genes in AE000402 around this region are bp4169-4807 *sspA* (regulator), 5202--6038 *rpsI* (50s ribosomal subunit protein L9?), 5610--6038

rplM (50s ribosomal subunit protein L13), 6257-7384 *yhcM*, 7572-7976 *yhcB*, and 8130-9497 *degQ* (serine endoprotease).

The complementary sequence in C5 is bp1937—10098 in AE000167. Genes in AE167 around this region are bp1803-2483 *citB*, 2524-3909 *dcnC*, 4498-5058 *crcA*, 5233-5442 *cspE* (cold shock protein), 5496-8876 *crcB*, 5972-6199 *ybeH*, 6196-6759 *ybeM*, 7192-8157 *lipA*, 8366-9166 *ybeF*, and 9578-10153 *lipB*.

The complementary sequence in N8 is bp2723—8203 in AE000271. Genes in AE000271 around this region are bp1608-2771 *b1762*, 2776-4737 *topB* (DNA Top III), 4742-5785 *selD/fdhB* (Aminoacyl tRNA synthetases), 5902-6453 *ydjA*, and 6614-8470 *sppA* (protease IV, a signal peptide peptidase). The sequence in N6 is bp891-9919 in AE000271, which is overlapped with N8.

In order to test if the mutation is located in 72.5-73.0, which is the location of the *E. coli* DNA fragment carried by the C1 plasmid, P1 CAG12153 was obtained from Dr. Ken Begg. This P1 lysate was made on a strain with a Tn10 at 72.1min. P1 transduction showed that there is no linkage between the mutation in T2-7 and the Tn10 at 72.1min. Therefore, C1 plasmid is only a multiple suppressor of the mutation in T2-7. Further study with Hfr mating showed that it is located in the *parCE* region.

It is important to identify the suppressing genes in C1 and C5 plasmids, because genes can suppress chromosome partition mutant should also has a role in chromosome partition, replication or topology structure maintenance. The suppressing gene in N8 plasmid is probably *topB*.

It is known that a *topA* mutation defective in topoisomerase I could be compensated by increasing both the *parC* and the *parE* gene dosage (Kato *et al.*, 1990), indicating the overlap of functions of DNA topoisomerases. Therefore, the suppressing gene carried by plasmid N8 is probably *topB*. It might be worth to directly test if *parC* or *parE* mutant can be suppressed by a plasmid carrying *topB* gene.

E10, H and No24:

Mutants E10, H and No24 were independently isolated at a different time. The three mutants looked very similar. They all produced anucleate cells (5-10%) and about 20-30% cells had irregularly located DNA. They all could be complemented by plasmid ED51, carrying the *rusA* gene, and F91, carrying the *ruvABC* genes. The mutations were located in the *ruvABC* region by Hfr mating and P1 transduction. Further study showed that E10 and H were located in *ruvC* and No24 was located in *ruvB*. More details can be found in chapter 4 in this thesis.

3.4 Summary.

The identification of several genes, including *mukB*, *parCE* and *ruvABC*, which when mutagenised affected chromosome partition suggests that the new method is applicable. To understand the mechanism of chromosome partition in bacteria, more mutants need to be isolated to identify new partition genes.

Chapter 4. RuvAB is required for both chromosome replication and segregation in *E. coli*.

4.1 Introduction.

In *E. coli* the RuvA, RuvB and RuvC proteins play an important role in the resolution of recombination intermediates. RuvA and RuvB promote branch migration and RuvC catalyzes the resolution of Holliday junctions (for reviews see West, 1997; Shinagawa and Iwasaki, 1996).

The *ruv* mutants were first isolated as mutants sensitive to UV light, ionizing irradiation and mitomycin C (Otsuji *et al.*, 1974; Sharples *et al.*, 1990; Stacey and Lloyd, 1976). The involvement of *ruvABC* in homologous recombination was established by finding that although *ruv* mutants are only mildly defective in recombination, *recBC sbcA*, *recBC sbcBC* and *recG* strains become severely defective in recombination when a mutation in *ruv* is introduced (Lloyd *et al.*, 1984, 1987; Lloyd, 1991). By studying conjugative recombination between F' donors and F' recipients with *recBC sbcBC ruv* mutations, it was proposed that without the *ruv* function recombination intermediates could not be processed into mature recombinants (Benson *et al.*, 1991).

The *ruv* locus contains three genes (*ruvABC*) and is mapped at 41min on the *E. coli* chromosome (Sharples *et al.*, 1990). The *ruvA* and *ruvB* genes form an SOS-inducible operon (Shurvinton and Lloyd, 1982; Benson *et al.*, 1988; Shinagawa *et al.*, 1988). The *ruvC* gene is separated from *ruvAB* by an open reading frame and its expression is not regulated by SOS induction (Takahagi *et al.*, 1991; Sharples and Lloyd, 1991).

The *ruvA* gene product, RuvA, is a structure-specific DNA-binding protein which has a high affinity for Holliday junctions (Iwasaki *et al.*, 1992; Parsons *et al.*, 1992; Tsaneva *et al.*, 1992). RuvB is a DNA-dependent ATPase (Iwasaki *et al.*, 1989; Shiba *et al.*, 1991). It can bind to DNA with a low affinity (Muller *et al.*, 1993b) and at high protein concentration can promote branch migration (Tsaneva *et al.*, 1992; Muller, 1993a). In the presence of RuvA, RuvB can bind to Holliday junctions at very low concentrations, suggesting that RuvA may direct the assembly of RuvB *in vivo* (Tsaneva *et al.*, 1992b). In solution RuvA forms tetramers (Tsaneva *et al.*, 1992) and RuvB forms hexamers or dodecamers (Mitchell and West, 1994). RuvA and RuvB form specific complexes with Holliday junctions, in which DNA is sandwiched between two RuvA tetramers, and the hexameric ring of RuvB assembles on opposing arms of the junction via contacts with RuvA (Parsons *et al.*, 1995; Rafferty *et al.*, 1996). RuvAB complex possesses DNA helicase activity, attributed to RuvB, that promotes branch migration with 5'→3' polarity (Tsaneva *et al.*, 1993; Adams and West, 1995).

RuvC is an endonuclease specific for Holliday junctions and forms dimers (Iwasaki *et al.*, 1991; Dunderdale *et al.*, 1991). It resolves Holliday junctions into duplex products by introducing symmetric nicks in two of the four DNA strands at the crossover (Iwasaki *et al.*, 1991; Dunderdale *et al.*, 1991; Bennett and West, 1996). The resolution occurs preferentially at sequences that exhibit the consensus 5'-(A/T)TT↓(G/C)-3' (where ↓ indicates the site of incision) (Bennett *et al.*, 1993, 1996; Shah *et al.*, 1997).

It is found that RuvC acts in concert with RuvAB and that RuvABC forms a complex during branch migration and resolution (Eggleston *et al.*, 1997; Davies and West, 1998; Zerbib *et al.*, 1998; van Gool *et al.*, 1998, 1999).

It is known that mutations in *ruvABC* causes SOS induction, presumably because replication forks are arrested by the unresolved recombination intermediates (Asai and Kogoma, 1994). However, recent study shows that in addition to homologue recombination and DNA repair, *ruvAB* is also involved in restoring arrested replication forks. It is proposed that a Holliday junction can arise at a stalled replication fork by annealing the newly synthesized strands, which creates a double-stranded end adjacent

to a Holliday junction. The junction is moved away from the DNA end and this provides an entry point for RecBCD to restore the blocked replication fork (Seigneur *et al* 1998). Therefore, it is not clear what causes SOS induction in the *ruv* mutants, block of replication fork by by unresolved recombination intermediates or failure of restoring the arrested replication fork.

The resolution of Holliday junctions can occur in either of two orientations, according to which DNA strands are cut, and the orientation preference of resolution is directed by RuvAB, possibly by the preferential binding of RuvB and the consequential unidirectional binding of RuvC dimers (Eggleston *et al.*, 1997; van Gool *et al.*, 1999).

It has been shown that overexpression of *rusA* can suppress the *ruv* mutants and the suppression is dependent on *recG* (Mandal *et al.*, 1993; Sharples *et al.*, 1994). The *rusA* gene is located on the defective lambdoid prophage, DLP12 (Mahdi *et al.*, 1996). RusA protein is a homodimeric Holliday junction-specific endonuclease (Sharples *et al.*, 1994; Chan *et al.*, 1997, 1998) RusA-mediated resolution occurs by dual strand incision predominantly at 5' of CC dinucleotides located symmetrically (Chan *et al.*, 1997).

An alternative route for Holliday junction resolution is RecG-mediated. RecG is a 76 kDa DNA-dependent ATPase and junction-specific DNA helicase (Lloyd and Sharples, 1993). In contrast to RuvAB, RecG unwinds DNA 3'→5' with respect to the single strand bound by RecG (Whitby *et al.*, 1994) and in the presence of RecA promotes branch migration in the opposite direction from that driven by RecA (Whitby *et al.*, 1993). Once RecA is removed, RecG can promote branch migration in both directions, like RuvAB (Whitby and Lloyd, 1995). It is proposed that RecG resolves recombination intermediates into original molecules by promoting reverse branch migration, which acts counter to the 5'→3' polarity of RecA binding and strand exchange (Whitby and Lloyd, 1995). Consistent with this proposal, genetic study shows that RuvAB and RecG have opposite effects: RuvAB is required for RecA-dependent adaptive mutations, whereas RecG inhibits them (Foster *et al.*, 1996).

4.2. Materials and Methods.

(1). Cloning of *ruvA*, *ruvB*, *ruvC*, *ruvAB* and *ruvABn*.

Primers for PCR:

Primers for *ruvA* cloning:

AH3 (Aend) (*Hind* III): 5' TTTCAAGCTTCCGGCAAAGTGGTAC3'

ARI (Astart) (*Eco*R I): 5' TTTAGAATTCATTACGCAGGAGC3'

Primers for *ruvB* cloning:

BH3 (Bend) (*Hind* III): 5' TTTCAAGCTTATCTATCGCTGAACAC3'

BRI (Bstart) (*Eco*R I): 5' TTTAGAATTCCCAAGCCGCATGGTGAGC3'

Primers for *ruvC* cloning:

VC1 (Cend) (*Bam*H I): 5' TTTAGGATCCTGGGTCTATTCCGTGG3'

VC2 (Ctop) (*Hind* III): 5' TTTCAAGCTTACCATAACGGTGAAATCAC3'

The PCR condition for *ruvC* was: 95°C 1min, 44°C 1min and 72°C 1min.

Primers for *ruvAB* cloning:

Bend (*Bam*H I): 5' TTTAGGATCCAGCTAATCTATCGCTGAACAC3'

Atop (*Hind* III): 5' TTTCAAGCTTCGCTGGATATCTATCCAG3'

The PCR condition for *ruvAB* was: 95°C 1min, 54°C 1min and 72°C 2min.

Primers for *ruvABn* cloning:

Bend (*Bam*H I): 5' TTTAGGATCCAGCTAATCTATCGCTGAACAC3'

Anew (*Hind* III): 5' TTTTAAGCTTACGCAGGAGCGTCATGTG3'

The *ruvAB* cloned by this pair of primers is promoterless.

C600 chromosomal DNA was used as template to amplify the *ruvABC* genes.

Construction of plasmids carrying *ruvA*, *ruvB*, *ruvC*, *ruvAB* and *ruvABn*.

(a). Construction of pBAD-*ruvA* and pBAD-*ruvB*.

The PCR products of *ruvA* and *ruvB* genes were digested with *EcoR* I and *Hind* III and then ligated with *EcoR* I and *Hind* III-digested pBAD18. The ligation mixtures were transformed into C600 and selected on LB+Ap plates. Transformants were then purified and plasmids were extracted and named pBAD-*ruvA* and pBAD-*ruvB*, respectively.

(b). Construction of pACYC-*ruvC*.

The PCR product of *ruvC* was digested with *BamH* I and *Hind* III and then inserted into pACYC177 at *BamH* I and *Hind* III to produce plasmid pACYC-*ruvC*.

(c). Construction of pUC-*ruvAB*.

The PCR product of *ruvAB* was purified and digested with *BamH* I and *Hind* III and then inserted into pUC19 digested with *BamH* I and *Hind* III. The constructed plasmid was named pUCAB, in which the *ruvAB* genes were expected to be under the control of *PlacZ*. However, this plasmid always caused DH5a and C600 to form filaments, even on glucose plates. This is possibly due to the high copy number of pUC19, the leakage of the *PlacZ* promoter and/or the inclusion of native promoters of *ruvAB* in the cloned fragment.

(d). Construction of pUCABns and pUCABns-Cm.

With new primers the *ruvAB* genes without their native promoter were cloned and named *ruvABn* to distinguish it from the previously prepared *ruvAB* DNA. The *ruvABn* fragment was digested with *BamH* I and *Hind* III and inserted into pUC19 digested with the same enzymes. Several clones were picked up and patched onto LB+Ap+glucose (0.2%) and LB+Ap+IPTG (20µg/ml). It was found that all the colonies could grow up on glucose plate, but not on IPTG plate at 37°C. Observation showed that cells on this IPTG plate were filamentous and lysed. Transformants were then purified

and plasmids were extracted and named pUCABn1, 2, 3, 7, 9, and 11. These plasmids caused similar phenotypes when transformed into C600. Plasmids pUCABn3 and pUCABn11 were used for further studies to disclose the effect of the overexpression of *ruvAB* on cell cycle.

In order to use Cm as a marker for overexpression of *ruvAB* in strain VIP407, which has an Ap marker in its chromosome, the *cat* gene was isolated from pUCAT18 (Draper *et al.*, 1998) after the plasmid was digested with *Hinc* II and *Ecl*136 II. The *cat* gene was then inserted into the *Sca* I site in the Ap gene in pUCABn3 and pUCABn11 to give plasmids pUCABn3-Cm and pUCABn11-Cm, respectively.

(2). Preparation of probes corresponding to *oriC* and *ter* regions of the *E. coli* chromosome for Southern blotting.

C83 probe DNA:

A 916bp DNA fragment at 82.26min in *E. coli* chromosome was amplified to detect the DNA around *oriC*. The primers for this PCR are 83U: 5'ATTTCCCGCCTGCAACAGC3' and 83D: 5'TCCAGCATGACAGGTGCGAG'.

C30 probe DNA:

A 911bp DNA fragment at 29.66min in *E. coli* chromosome was amplified to detect the DNA in the replication terminus region of the chromosome. The primers for this amplification reaction are 30U: 5'TCAACGAACTGGTGAACCTG3' and 30D: 5'CGCGCCTGGCAAGTTGTG3'.

The template of the PCR reaction in preparing C83 and C30 probes was chromosomal DNA of C600. For southern blotting, the probe DNA fragments were labeled by P³²-dCTP with a High Prime kit.

4.3. Results.

4.3.1. Chromosome segregation defect in *ruv* mutants.

4.3.1.1. Identification of three mutants as *ruv* mutants.

During the isolation of new chromosome partition mutants, three mutants E10, H and No24 were isolated at different time. The three mutants looked very similar. They all produced anucleate cells (5-10%) and about 20-30% cells have unsegregated DNA (Fig. 4.1A and B).

Hfr mating showed they had the same location: the mutations were linked to the Tn10 marker inserted in A9 (at 43.0min) (80-90%), A7 (at 28.0min) (~11%) and A11 (at 67.0min) (~1-2%). Therefore, the mutations were around 43min on the genetic map.

Screening of complementing plasmids from the constructed genomic library showed they all could be complemented perfectly by either of two plasmids, ED51 and F91.

Sequencing of ED51 showed the complementary sequence was (Cla)-6608bp to 7461bp-(Bam) in AE000160. The genes or *orfs* in AE000160 around this region are: *ybcO* (6432bp-6722bp), *rusA* (6719bp-7081bp) and *ybcQ* (7304-7687). Therefore, the complementing (suppressing) gene was *rusA*, located at 12.4min. In plasmid ED51, the *rusA* gene could be expressed from Kan promoters. It is known that overexpression of *rusA* can suppress *ruv* (located at 41.9min) mutants. Hfr mating also showed that the mutations were at about 43min. Thus, the mutations are probably located in *ruvABC*, which encode Holliday junction specific endonuclease and motors.

Sequencing of plasmid F91 showed the complementary fragment was (Cla)-0.4kb to 9.2kb-(Bam) in AE280, in which *ruvB* is at 2765bp←3775bp, *ruvA* is at 3784bp←4395bp and *ruvC* is at 5274bp←5795bp.

To further confirm that the mutations were in *ruv* locus, their linkage to *eda-51::Tn10* was tested by P1 transduction. P1 grown on DL1075 (*ruvC53 eda-51::Tn10*)

was used to construct strain W3110 *eda-51::Tn10*. Then the P1 lysate of W3110 *eda-51::Tn10* was used to transduce mutants E10, H and No24. It was found that the mutations in these mutants were all linked to *eda-51::Tn10*. This confirmed that the mutations were located in *ruvABC* genes.

To further locate the mutations into genes, *ruvA*, *ruvB*, and *ruvC* were separately cloned by PCR (see methods and materials). After transformation, it was found that mutants E10 and H could be complemented by pACYC-*ruvC* and mutant No24 could be complemented by pBAD-*ruvB*. Therefore, It was concluded that the mutations in E10 and H were located in *ruvC* and the mutation in No24 was located in *ruvB*.

4.3.1.2. Chromosome partition defect in *ruv* mutants.

It is reported that after exposure to UV light, the chromosome in the filamentats of *ruv* mutants can not be distributed regularly (Ishioka *et al.*, 1998). Here I show that even at normal growth conditions the isolated *ruv* mutants still had partition defect (Fig. 4.1). They all produced cells without DNA (about 5%), with DNA at poles or irregularly distributed (about 20%-30%) (E10 was similar to H, data not shown). In order to confirm that *ruv* mutants have chromosome partition defect even under normal growth conditions, three mutants DL1074 (*ruvA60::Tn10*), DL1075 (*ruvC53 eda-51::Tn10*) and DL1076 (Δ *ruvAC65 eda-51::Tn10*) from Dr. David Leach were obtained for comparison. Visualization of their chromosomes by DAPI staining showed they had similar chromosome partition defects to E10, H and No24 (Fig. 4.2). The mutations in these strains were also transduced into Cl4 to construct *ruv* mutants with the same genetic background as E10, H and No24. Study of chromosome partition pattern in the constructed Cl4 *ruvA60::Tn10*, Cl4 *ruvC53 eda-51::Tn10* and Cl4 Δ *ruvAC65 eda-51::Tn10* further confirmed the above result.

The chromosome partition defects in the *ruv* mutants is probably directly due to the failure of resolution of chromosome recombination intermediates. Unresolved Holliday junction can hold two chromosomes together and make their partition impossible.

4.3.1.3. The filamentation of *ruvABC* mutants is probably caused by SOS.

Since *ruvABC* mutants caused cell to become filamentous, it is proposed that they may have function in cell division. In order to test this, *recA::cat* was transduced into Cl4 *ruvA::Tn10* by P1 transduction. It was found that it took three days to get transductants. When cells in the colonies were observed, it was found that most of the cells were long filaments. Then the transductants were streaked out. However, observation of the purified transductants showed that there was no long filament any more. The growth rate of the streaked cells according to the size of the colonies was almost the same as wild type cells. When grown in liquid medium (L broth), again the cells were normal sized and the growth rate of the culture was quite close to that of wild types. It is not clear why cells in the original transductant colonies were filaments. According to the morphology of the purified transducts in LB agar plate or liquid LB medium, the cell division defect of these mutants was due to SOS induction and *ruvABC* does not have a direct role in cell division. However, to confirm this conclusion *ruv* mutations should be introduced into strains with a controllable *recA* gene. If in such a constructed strain depletion of RecA could diminish the formation of filaments, then we could definitely conclude that *ruvABC* had no effect on cell division.

During the screening of complementing plasmids, I found that in addition to ED51 and F91, E56 and F78 could also partly or largely complement H and E10 (*ruvC* mutants).

Sequencing of E56 did not give a clear result. Sequencing from one primer showed the complementary fragment was 2078bp→lower in M30198 (or AE458: 2676bp→lower), while that from the other showed it was 60566bp→lower in M87049. The plasmid and also F78 should be further sequenced to find out the new suppressing gene.

I also found that a plasmid (named E34) could partly complement N024, a *ruvB* mutant. Sequencing of the E34 showed the complementing gene was located at 4853bp-lower in AE210. This new complementing gene should also be identified.

4.3.2 The effect of overexpression of RuvAB on cell division and chromosome replication.

4.3.2.1. Overexpression of RuvAB causes SOS- and recombination-independent inhibition of cell division.

It was found that overexpression of RuvAB from plasmid pUC-ruvAB and pUCABn (data not shown) could cause cells to form long filaments (Fig. 4.3).

Since the well-known function of RuvAB is for Holliday junction resolution, it was possible that the filamentation was due to the failure of Holliday junction resolution or SOS induction caused by such a failure. Therefore, plasmid pUC-ruvAB was introduced into *recA*⁻ strains (DH5 α and C600 *recA::cat*) to see if cells can still be caused to filament by overexpression of RuvAB. It was found that the two *recA*⁻ strains with pUC-ruvAB were also become filamentous when grown in LB+IPTG (20 μ g/ml) at 37°C (Fig. 4.4). This indicates that the inhibition of cell division by RuvAB is SOS independent and also unrelated to Holliday junction formation and resolution.

MM38 *sfiA*⁻ *lexA*ind was also tested. This strain could not be made filamentous by mitomycin C: MC just stopped its growth quickly. It was resistant to Nal and grew up in LB medium with Nal, as did DH5 α . PUC-ruvAB could make MM38 cells filamentous, but the filaments were much shorter than C600 (pUC-ruvAB) or DH5 α (pUC-ruvAB).

A literature search showed that a recent study has proposed that RuvAB also functions in chromosome replication (see 4.1 Introduction). According to this model, it is possible that an arrested replication fork may be converted into *pseudo*-Holliday structure and overexpression of RuvAB may cause over-pulling back of the arrested replication fork existed in a Holliday junction structure and thus affect replication. If the filamentation is due to this, it means that replication is coupled with cell division. And

this idea brought about my studies of the coordination of cell division and chromosome replication (see chapter 6).

4.3.2.2. Overexpression of RuvAB delays the completion of chromosome replication.

Slowing down or stopping chromosome elongation should increase the ratio of the copy number of *ori* to the copy number of *ter* (called the ratio of *ori/ter* in this thesis) because continuing cell growth should be accompanied by successive reinitiations of replication at *oriC*. Here I show that detected by Southern blotting the ratio of *ori/ter* was increased significantly after RuvAB was overexpressed.

For Southern blotting, chromosomal DNA was first prepared in the following way. Overnight cultures of C600 in LB+Glu (0.2%) and C600 (pUCABn3) in LB+Ap (50µg/ml)+Glu (0.2%) were diluted 1:100 into fresh media and grown at 30°C. When OD600 reached 0.2-0.3, the cultures were spun down and resuspended in the same volume of LB medium. One ml of each of the resuspensions was inoculated into 23ml of LB+Glu (0.2%) or LB+IPTG (100µg/ml) and grown at 37°C. C600 cultures were further diluted 1:3 or 1:4 after this. When OD600s of the cultures were around 0.4 (OD600=0.397 for C600 (pUCABn3) in LB+IPTG, OD600=0.399 for C600 in LB+Glu and OD600=0.475 for C600 in LB+IPTG), 20ml of each of the cultures was centrifuged and resuspended in 1.2ml 1XTE. Then 576µl of each was used and mini-preps of chromosome DNA were made as described in Appendix III. The concentration of the preparations was measured by spectrophotometric method. The same amount of chromosome DNA from each of the samples was taken and digested with *EcoR* V, *Sal* I and *Sca* I, separately. Southern blotting was then carried out and at last the filter was exposed to Phosphor Screen for 3 hours. The screen was then scanned. The image of the southern blotting result is shown in figure 4.5.

The image was analysed by a software named ImageQuant V1.1. For quantitation of the bands the background was corrected by “average local” and the

“volume” was used to represent the strength of blotting signal. The result of this experiment is shown in table 4.1.

Table 4.1. Overexpression of RuvAB increases the ratio of *ori/ter* in *E. coli* cells.

Culture	C600 in IPTG	C600 in glucose	C600 (pUCABn3) in IPTG
Doubling time (T)	25.6min	23.5min	>60min
Ratio of <i>ori/ter</i> detected by digestion with <i>Sal</i> I	2.41	2.65	6.79
Ratio of <i>ori/ter</i> detected by digestion with <i>Sca</i> I	4.77	5.15	8.12
Ratio of <i>ori/ter</i> detected by digestion with <i>EcoR</i> V	3.66	4.90	6.83

Note:

(1). The doubling time (T) of a culture was calculated as the time taken by the culture to double its OD600. Therefore, if at time t_1 a culture has an optical density of OD_{t_1} and after a period at time t_2 its density reaches OD_{t_2} , then the doubling time of this culture in this period is calculated as $T = [(t_2 - t_1) \times \ln 2] / (\ln OD_{t_2} - \ln OD_{t_1})$.

(2). The ratio of *ori/ter* is calculated by dividing the “volume” of the band probed with C83 (corresponding to the *oriC* region) by the “volume” of the band probed with C30 (corresponding to the *ter* region).

It is known that in wild type *E. coli* strains the elongation speed of chromosome replication is almost constant under all growth rates (about 40min for replicating the whole chromosome) and the ratio of *oriC*/cell mass is also constant. Therefore, in wild type cells, fast growing cultures have high ratio of *ori/ter* and slow growing cultures have low ratio of *ori/ter*. Here it is shown that the data for C600 cultures is consistent with this rule. In this experiment, C600 grew faster in LB+glucose medium (doubling time was about 23.5min) than in LB+IPTG medium (doubling time was about 25.6min). Consistently, the ratio of *ori/ter* of cells in the glucose medium (e.g., about 4.90, by *EcoR* V digestion) is higher than that in IPTG medium (about 3.66, by *EcoR* V

digestion). This confirms that the method of determining the ratio of *ori/ter* by Southern blotting is applicable and indicates that the obtained data is reliable. After shifted from LB+glucose medium to LB+IPTG medium, the growth rate of C600 (pUCABn3) slowed down quickly (for an example see Fig. 4.6). Compared to C600 cultures, the very slow growing culture of C600 (pUCABn3) in LB+IPTG (doubling time was more than 60min during the last 50min before sample was taken), however, had a very high ratio of *ori/ter* (about 6.83, by *EcoR* V digestion). This indicates that the process of chromosome replication elongation was slowed down or the completion of chromosome replication (through *ter* region) was significantly delayed when RuvAB was overexpressed, while the alternative explanation that the initiation rate of replication was increased by the overexpression of RuvAB seems unlikely.

4.3.2.3. Overexpression of RuvAB also inhibits the transcription of cell division genes clustered at 2min region in *E. coli* chromosome.

In order to test if the inhibition of cell division by overexpression of RuvAB is due to the repression of transcription of cell division genes, a strain named VIP407 (for details see chapter 6) was used to show the transcriptional level of the cell division genes at 2min region (see chapter 1). Since VIP407 has an Ap marker in its chromosome, to introduce the plasmid carrying controllable *ruvAB* to this strain, *cat* gene was inserted into the Ap gene of pUCABn3 to produce plasmid pUCABn3-Cm. Result showed that when *ruvAB* were induced by growing VIP407 (pUCABn3-Cm) in IPTG medium, the transcriptional level (measured by β -galactosidase activity) of the cell division genes was repressed significantly (Fig. 4.6).

Considering its effect on chromosome replication, overexpression of RuvAB possibly indirectly caused the inhibition of cell division. It is proposed that overexpression of RuvAB directly caused the delay of the completion of chromosome replication, which then caused the inhibition of cell division by repressing the transcription of cell division genes. This suggests the existence of a mechanism coupling cell division with chromosome replication. Such a possibility was further studied in chapter 6.

4.4. Summary.

This study showed that RuvABC, which are required for the resolution of recombination intermediates, were involved in chromosome partition in wild type cells under normal growth conditions. Over-expression of RuvAB caused the inhibition of cell division in both wild type cells and *recA*⁻ cells where recombination intermediates can not form. It was also found that over-expression of RuvAB could slow down the speed of elongation of chromosome replication and also caused the repression of transcription of cell division genes at 2min region in *E. coli* chromosome. It was proposed that it was the chromosome replication defect caused the inhibition of cell division and there might be a mechanism coupling cell division with chromosome replication.

A



B

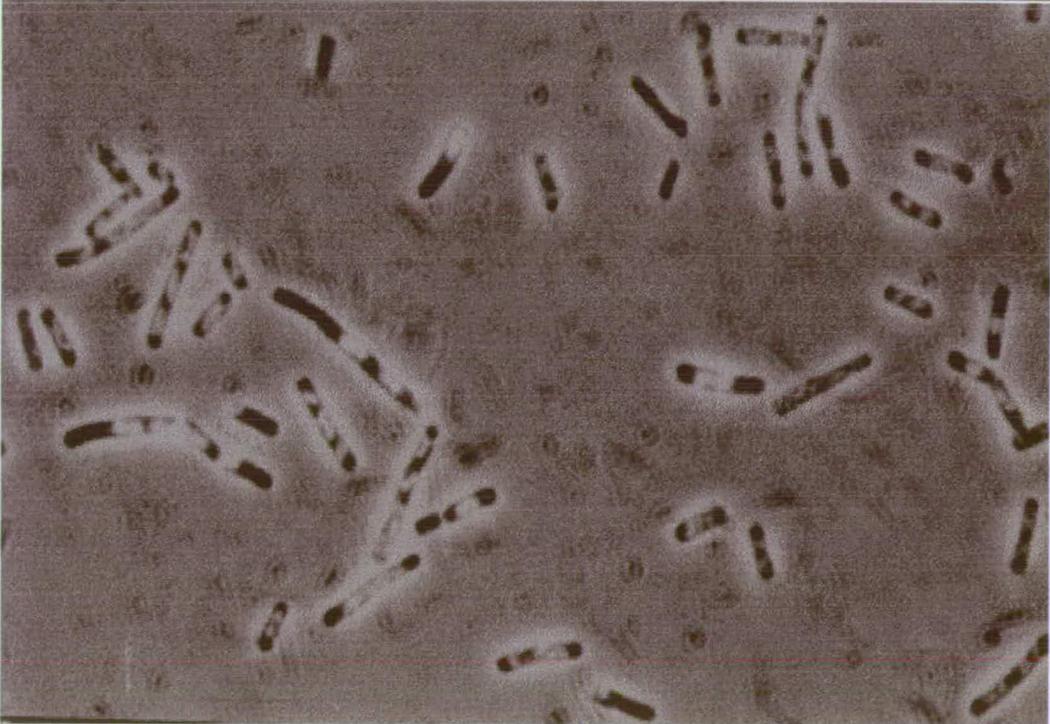
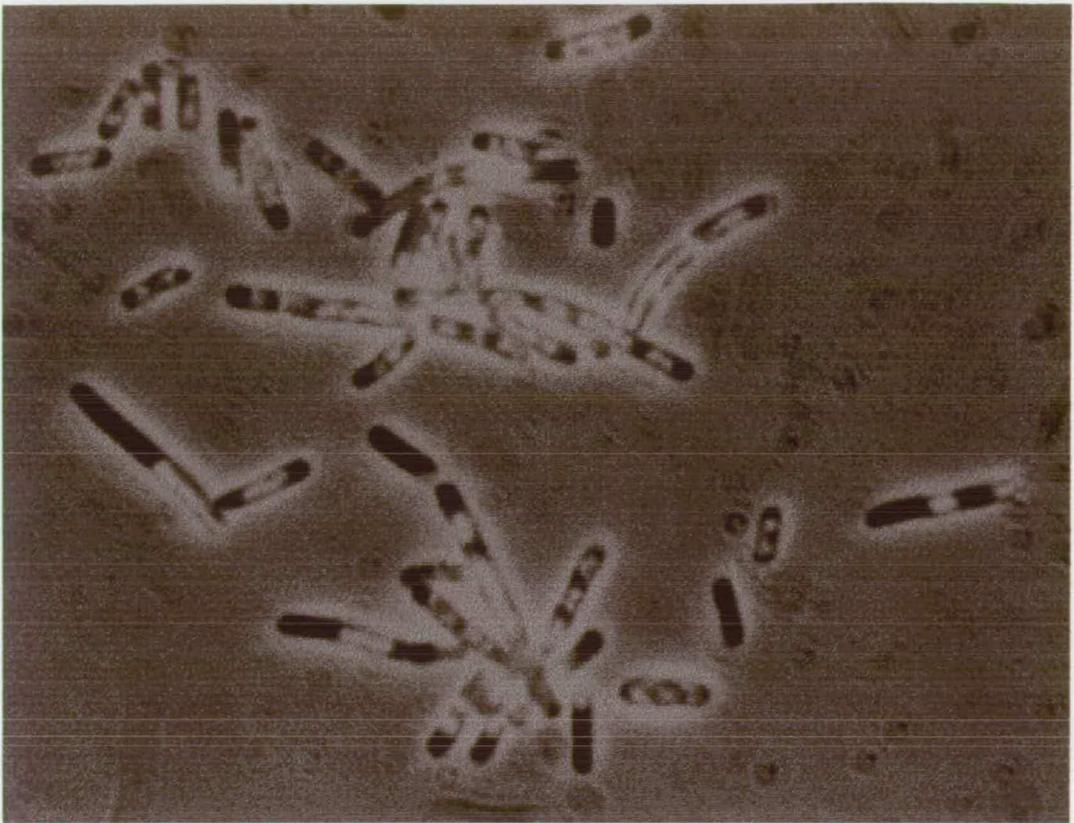


Figure 4.1. Chromosome partition in (A) mutant No24 (a *ruvB* mutant) and (B) mutant H (a *ruvC* mutant). In this experiment, overnight cultures were diluted 1:100 into L broth and grown at 37°C. When OD600 reached 0.2, samples were taken for DAPI staining and then photographing.



A



B

Figure 4.2 (to be continued).

C

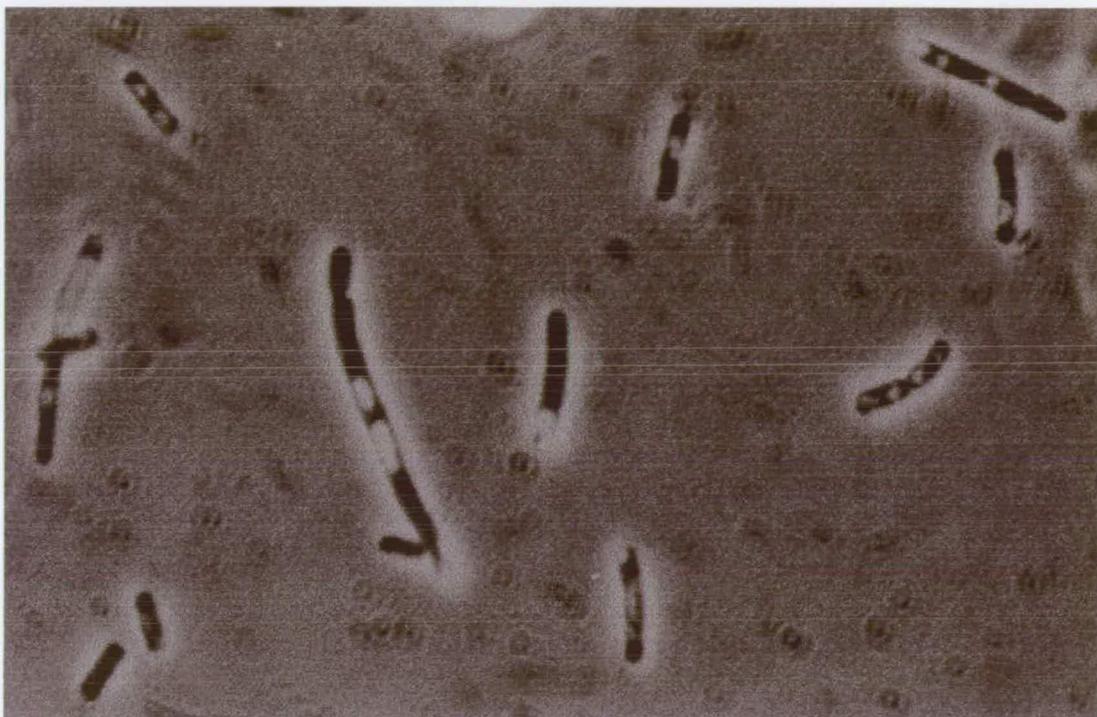


Fig. 4.2. Visualization of chromosome DNA distribution in *ruv* mutants.

The cells of DL1074 (*ruvA60::Tn10*) (A), DL1075 (*ruvC53 eda-51::Tn10*) (B) and DL1076 (Δ *ruvAC65 eda-51::Tn10*) (C) were from early log phase cultures (OD600 around 0.2).



Figure 4.3. Inhibition of cell division by overexpression of RuvAB. In this experiment, overnight culture of C600 (pUC-*ruvAB*) in LB + Ap (50 μ g/ml) + glucose (0.2%) at 30°C was diluted 1:100 into fresh LB+IPTG (20 μ g/ml) and grown at 37°C for 2-3 hours (OD600 about 0.3). Samples were then taken for photographing.



Figure 4.4. Inhibition of cell division by overexpression of RuvAB in strain C600 *recA::cat*, a *recA*⁻ strain.

In this experiment, overnight culture of C600 *recA::cat* (pUC-*ruvAB*) in LB + Ap (50µg/ml) + Cm (20µg/ml) + glucose (0.2%) at 30°C was diluted 1:100 into fresh LB+IPTG (20µg/ml) and grown at 37°C for 3 hours (OD600 about 0.3). Samples were then taken for photographing.

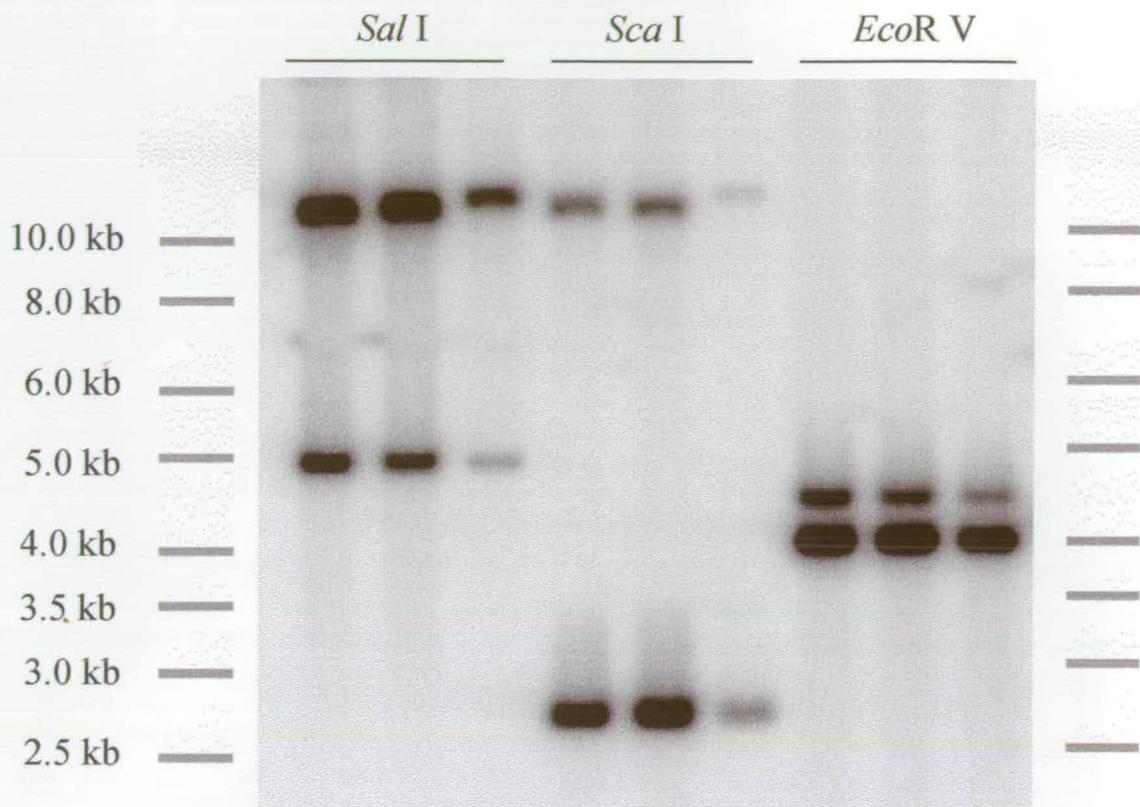


Figure 4.5. Southern blotting of chromosomal DNA from C600 and C600 (pUCABn3) to detect the *oriC* (~83min) and *ter* (~30min) regions.

Lane (from left to right)	Chromosome DNA/restriction enzyme	Expected bands
1	C600 in IPTG/ <i>Sal I</i>	>12 kb (83min), 6.1 kb (30min)
2	C600 in glucose/ <i>Sal I</i>	>12 kb (83min), 6.1 kb (30min)
3	C600 (pUCABn3) in IPTG/ <i>Sal I</i>	>12 kb (83min), 6.1 kb (30min)
4	C600 in IPTG/ <i>Sca I</i>	2.6 kb (83min), >12 kb (30min)
5	C600 in glucose/ <i>Sca I</i>	2.6 kb (83min), >12 kb (30min)
6	C600 (pUCABn3) in IPTG/ <i>Sca I</i>	2.6 kb (83min), >12 kb (30min)
7	C600 in IPTG/ <i>EcoR V</i>	4.0 kb (83min), 4.5 kb (30min)
8	C600 in glucose/ <i>EcoR V</i>	4.0 kb (83min), 4.5 kb (30min)
9	C600 (pUCABn3) in IPTG/ <i>EcoR V</i>	4.0 kb (83min), 4.5 kb (30min)

Figure 4.6. Repression of transcription of cell division genes at 2min region in *E. coli* chromosome by the overexpression of RuvAB.

In this experiment, overnight cultures of VIP407 and VIP407 (pUCABn3-Cm) in LB+Ap (50µg/ml)+IPTG (8µg/ml) [also with Cm (20µg/ml) for VIP407 (pUCABn3-Cm) to maintain plasmid] were diluted 1:100 into fresh media and grown at 37°C with shaking. The cultures were kept at early log phase (OD600 lower than 0.2) by serial dilutions. At 185min the cultures were split and to half of each of them IPTG were added to a concentration of 100µg/ml to induce the expression of RuvAB. OD600 (a) and β-galactosidase activity (b) were measured at indicated times.

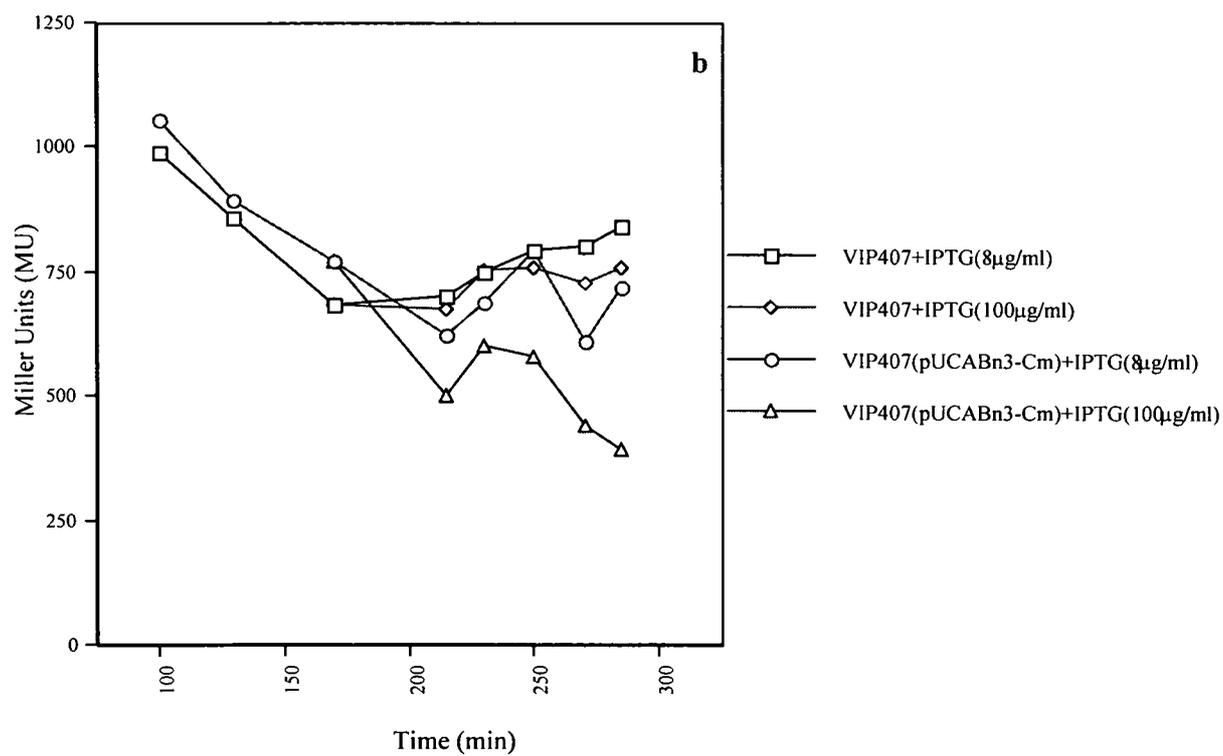
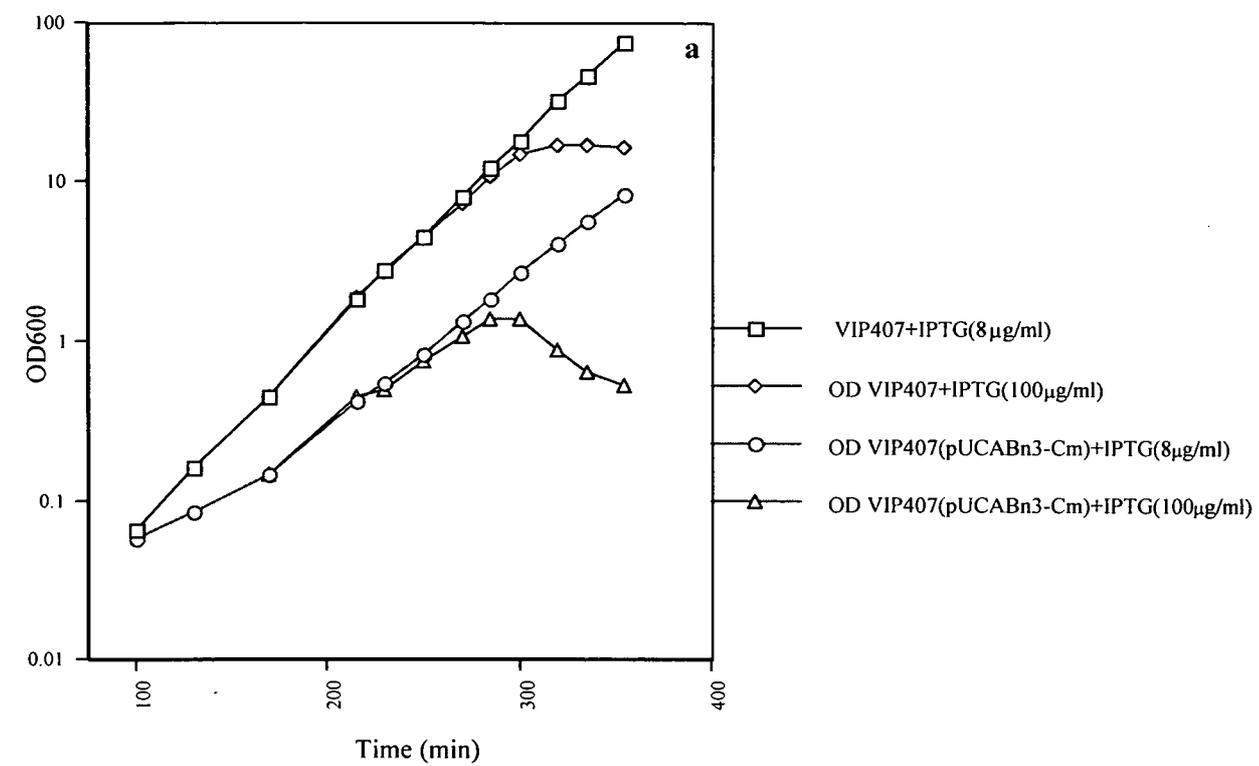


Figure. 4.6.

Chapter 5. Overexpression of *dsbB* and *friL* genes

5.1. Overexpression of *dsbB* causes cells to become spherical.

5.1.1. Introduction.

DsbB was first isolated as a helper for protein disulfide bond formation in *E. coli* (Bardwell *et al.*, 1993). So far six Dsb proteins forming a family of thiol-disulfide bond oxidoreductases have been isolated in *E. coli* (see a review by Raina and Missiakas 1997; and an article on *dsbG* by Andersen *et al.*, 1997). In their primary structure they all carry an active site containing two vicinal cysteines, Cys-X-X-Cys. This site is essential for their dsb function. Among them, DsbA and DsbB are central to the well studied thiol oxidation reaction that takes place in the periplasm. Periplasmic protein DsbA is the direct donor of disulfide bonds in secreted proteins and integral membrane protein DsbB reoxidizes DsbA to restore its activity. Recent study shows that disulfide bond formation is coupled with the electron transport chain in *E. coli*. DsbB uses quinones as electron accepters, allowing various choices for electron transport to support disulfide bond formation (Bader *et al.*, 1999). What I found here is that overexpression of *dsbB* can cause cells to become spherical, possibly by affecting cell division or peptidoglycan synthesis protein(s).

5.1.2. Results.

During the construction of the *E. coli* chromosomal DNA library, I found that fifteen of the transformants grew very slowly. In some of them this is probably due to the toxicity of the cloned fragment. The morphology of the cells in each of the fifteen colonies when grown at 30°C on LB+Ap (50µg/ml) plates is listed below.

No1: most of the cells are filamentous.

No3, 6: Some big and swollen cells.

No4: only a few short filaments.

No5: a few long filaments

No7: some filaments.

No8: Most of the cells are clearly longer than normal cells.

No12: many long cells or short filaments.

No13: a few filaments.

No2, 9, 10, 11, 14, 15: normal-sized cells.

Plasmids from 1, 3, 6, 8 and 12 were extracted separately.

5.1.2.1. Sequencing of the pSWO3 plasmid.

The plasmids from No3 and 6 were further sequenced. These two plasmids were named pSWO3 and pSWO6, respectively. Sequencing results show that the inserted fragments in the two plasmids overlap. The insertional fragment in pSWO3 carries *umuD'-umuC-dsbB-nhaB-fadR-ycgB-dsdA-dadX'* genes (Fig. 5.1).

At 30°C C600 (pSWO3) cells were long and swollen (Fig. 5.2). pSWO3 caused C600 cells to form big spheres (at 42°C) or lemon-shaped cells (at 37°C) and then lyse (Fig. 5.3).

Because ampicillin can cause wild type *E. coli* cells to become swollen, it is possible that the above phenomenon is in some way due to the existence of Ap in the media. To test this possibility, an overnight culture of C600 (pSWO3) in

LB+Ap(50µg/ml) was diluted 1:100 into LB without ampicillin and grown at 37°C with shaking. Three hours later when the culture's OD600 reached 0.3, the cells were still swollen. Therefore, the cell's round shape is not due to the presence of ampicillin.

5.1.2.2. Dissection of plasmid pSWO3.

In order to identify the gene causing cell swelling (temporarily named *swo*), pSWO3 was partially digested by *Taq* I and *Sau* 3A. The fragments between 0.7 and 1.0kb, 1.0kb and 2.5kb were separately purified from agarose gel and then ligated to pACYC177/*Cla* I-*Bam*H I. Finally, six colonies from the 0.7-1.0kb ligation were isolated: these all consisted of spherical cells. The six plasmids from the colonies were purified and named pS1, 2, 3, 4, 5 and 6. These plasmids caused cells to become spherical, but were not lethal at any tested temperature. The morphology of C600 (pS2) at 37°C is shown in figure 5.4. At 42°C C600 (pS2) is round and bigger than at 37°C. Four colonies were obtained from the 1.0-2.5kb ligation. One of the four was as temperature sensitive as pSWO3-containing cells: it formed big swollen cells and then lysed at 42°C. The corresponding plasmid was named pSB (B means big spherical cells). As estimated by electrophoretic mobility on agarose gel, pSB is about 1kb bigger than plasmids pS1-6. Therefore, it is proposed that the lethality of pSB in C600 at 42°C is due to the existence of *swo*'s native promotor(s).

Another colony from the above ligation consisted of filaments (Fig. 5.5) and the corresponding plasmid was named pF.

It seems that the long swollen shape (at 30°C) or lemon shape (at 37°C) of C600 (pSWO3) is caused by the combination of the spherical shape of C600 (pSs) and the long filamentous shape of C600 (pF).

5.1.2.3. Identification of the *swo* gene as the *dsbB* gene.

Sequencing of the above pSs plasmids showed that it is the *dsbB* gene that caused the spherical cell shape.

The genes in the D90753 fragment of *E.coli* are: bp2513-2932: *umuD*, bp2932-4200: *umuC*, bp4246-4782: *dsbB*, bp4922-6463: *nhaB*, bp6684-7403: *fadR*, bp7455-

8987: *ycgB*, bp9317-10615: *dada*, bp10625-11695: *dadB*. A Blast search of the sequences of the inserted fragment in pSs showed that in D90753 their locations are bp4122-4967 in pS2 and bp4126-4960 in pS5, while that in pSB is up to bp6030.

This clearly shows that the *swo* gene is indeed a known gene, *dsbB*.

In order to confirm the above result, plasmid pSWO3 was digested by *Bam*H I, *Hind* III and *Sgf* I separately, and a *cat* gene from pUCAT18 (digested with *Hinc* II and *Ecl*136II) was inserted into the sites. The ligation mix was then used to transform C600. The plasmids produced were called pSWO-cat/*Bam*HI, pSWO-cat/*Hind*III and pSWO-cat/*Sgf*I, respectively (Fig. 5.1). It was found that C600 (pSWO-cat/*Sgf*I) and C600 (pSWO-cat/*Hind*III) filamented only, while C600 (pSWO-cat/*Bam*HI) became swollen and filamented, just as C600 (pSWO3).

These results confirm that it is the overexpression of *dsbB* that caused cells to become spherical. It also suggests that the gene which caused cells to become filamentous is not *umuC* or *nhaB*, which flank *dsbB*, but probably the *ycgB* gene of unknown function.

5.1.2.4. Knock-out of *dsbB*.

In order to see what would happen to cell shape if *dsbB* is deleted, I tried to knock out this gene from the *E. coli* chromosome. Plasmid pSWO-cat/*sgf*I was used for this purpose. pSWO-cat/*sgf*I was digested with *Hind* III and the fragment containing the *dsbB*::*cat*/*sgf*I structure without any vectoral sequence was isolated and used to transform C600. Six Cm^R colonies (C600 *dsbB*::*cat*1, 2, 3, 4, 5 and 6) were obtained. To make sure that they were not transformants with plasmid pSWO-cat/*sgf*I, I streaked them on LB+Ap (50µg/ml) and LB+Cm (20µg/ml) plates and grown overnight at 30°C. It was found that all the six colonies were Cm^r Ap^s.

Strains C600 *dsbB::cat1* and 2 were further studied. They could grow up at 30, 37 and 42°C on LB+Cm (20µg/ml) plates and cell shape was quite normal, except that at 30°C cells were very short and small, and at 42°C a little bit long.

To confirm the above deletion result, *dsbB::kan* mutants were obtained from Dr. JC Bardwell (Bardwell *et al.*, 1993). Observation of the cell shapes of these mutants showed that they are similar to those of *dsbB::cat1* and 2.

The conclusion is that deletion of *dsbB* does not have a significant effect on cell shape.

5.1.2.5. Over-expression of *rodA* and *pbpA* does not suppress the morphological effect caused by the *dsbB* gene.

Because DsbB is required for the folding of some periplasmic proteins by promoting proper disulphide bond formation, the spherical shape is probably due to the inactivation of a cell envelope synthesis-related protein(s). The known genes that can cause cells to become spherical are *rodA*, *pbpA*, *dacA*, *bolA* and *mreB*. Inactivation of *rodA*, *pbpA* or *mreB* or overexpression of *dacA* or *bolA* can cause *E. coli* cells to lose their rod shape and become spherical. Therefore, I decided to see if overexpression of *rodA* and *pbpA* could reverse *dsbB*'s effect.

For this purpose plasmid pBS44 was used (Begg *et al.*, 1986). pBS44 is a pSC101-based kan^R plasmid with a 7kb insertion of a *Sal* I fragment containing *rodA* and *pbpA*. This plasmid is compatible with pACYC177-based pSWO3 and pSB plasmids. Therefore, if pBS44 co-exists with one of them in C600, the genes on these two plasmids should be able to be overexpressed at the same time.

First of all, pBS44 was transformed into C600. Then C600 (pBS44) was transformed with pS2, pSB and pSWO3, separately. The transformants were then grown overnight at different temperatures on plates and their shapes are listed in Table 5.1. The results suggest that over-expression of *rodA* and *pbpA* could not reverse the effect on cell shape caused by overproduction of *dsbB*.

Table 5.1.

Strains	Temperature sensitivity at 42°C	Morphology at 30°C	Morphology at 42°C
C600 (pBS44, pSWO3)	Ts	Spheres	Big spheres and lysis
C600 (pBS44, pSB)	Ts	Spheres	Big spheres and lysis
C600 (pBS44, pS2)	Not Ts	Small round cells	Spheres as C600 (pS2) at 42°C
C600 (pBS44)	Not Ts	Normal	Normal

5.1.3. Discussion:

It is not clear why over-expression of *dsbB* causes cells to become spherical. Perhaps overexpression of its function in disulfide bond formation and its location in the cytoplasmic membrane inactivates (or even activates) a cell shape maintenance protein, or blocks such a protein's transport to the periplasm.

5.2. Over-expression of *friL*.

The gene causing cell filamentation in plasmid pSWO3 is named *friL* (filamentation of *recA*-independent).

According to the observations described in 5.1, the gene that causes cell filamentation when over-expressed is *fadR*, *ycgB* or *dadA*. Then plasmid pF causing C600 to become filamentous (Fig. 5.5) was sequenced by Auste Geddes in this lab showed that it again contains the *fadR*, *ycgB* and *dadA* genes.

Because the SOS response, which can be directly or indirectly induced by many factors, can inhibit cell division, the plasmid pF was transformed into *recA*- strains to test whether or not the filamentation is caused by SOS. It was found that both C600 *recA::cm* (pF) and DH5 α (pF) filamentated at 37°C. The morphologies of C600 (pF) and C600 *recA::cm* (pF) were similar. Their cultures at 37°C were mixtures of normal sized cells and filaments (Fig. 5.5). However, almost all DH5 α (pF) cells at 37°C were undivided long filaments (Fig. 5.6). The reason for the difference is unclear. It may be because DH5 α grows slower than C600 and C600 *recA::cm*. Because its effect on cell division is *recA* independent, this gene was named *friL*. Then, which of the three genes is *friL* ?

FadR is a global transcription regulator of fatty acid metabolism in *E. coli*. It acts both as a repressor of fatty acid degradation and as an activator of fatty acid biosynthesis. The FadR regulon is also interconnected to the universal stress response of *Escherichia coli*. FadR, long-chain fatty acyl-CoA, long-chain acyl-ACP, ppGpp and cAMP together are key players in regulating the activities of enzymes and expression of genes involved in fatty acid and phospholipid metabolism in dividing and ageing *E. coli* cells (for reviews see DiRusso and Nystrom, 1998; Cronan and Subrahmanyam, 1998).

dadA and *dadX* form the D-amino acid dehydrogenase (*dad*) operon (Wild *et al.*, 1985). This operon is required by *E. coli* for growth on L- or D-alanine as its sole

carbon and energy source (Wild and Klopotoski, 1981). *dadA* codes for one subunit of D-amino acid dehydrogenase and *dadX* for the alanine racemase (Wild and Obrepalska, 1982; Wild *et al.*, 1985). The expression of the *dad* operon can be induced by the presence of either L- or D-alanine (Wild and Obrepalska, 1982). Recent studies show that this operon is also regulated by cyclic AMP-CRP and Lrp protein (leucine-responsive regulatory protein) (Lobočka *et al.*, 1994; Mathew *et al.*, 1996; Zhi *et al.*, 1999).

From this information, I do not think overexpression of *dadA* and/or *fadR* should affect cell's morphology.

The function of the remaining gene *ycgB* is unknown. Analysing its protein sequence by eye, I found that there are two putative leucine zipper structures in its C-terminal region (see Table 5.2). Using its protein sequence to run Blast Search at NCBI, I found SpoVR from *B. subtilis* shared significant similarity (about 30%) with YcgB. Interestingly, SpoVR also has a leucine zipper structure, which corresponds to YcgB's second one (see Table 5.2). It was also reported that the two proteins share a potential helix-turn-helix DNA binding motif typical of transcriptional regulators and that both might be cytoplasmic proteins (Beall and Moran, 1994).

spoVR was found by screening for sigma-E dependent promoters in *B. subtilis*. Mutations in this gene cause an increased proportion of cortexless spores and the SpoVR protein is proposed to be involved in cortex formation indirectly, possibly by functioning as a regulator. It was reported that the expression of *spoVR* was initiated at the second hour of sporulation from a sigma E-dependent promoter (Beall and Moran, 1994).

I propose that *friL* is the unidentified *ycgB* and that its name be changed to *friL* in this thesis. The FriL protein might function as a transcriptional repressor of cell division genes.

It has been shown that the combination of *rodA*ts or *pbpA*ts with cell division mutations (such as *ftsZ*ts and *ftsA*ts, etc.) can be used to detect the septation step at which the selected cell division protein functions (Begg and Donachie, 1985). Since the

morphology of C600 (pSB) at 37°C or 42°C is very similar to that of *rodA*ts or *pbpA*ts mutants at non-permissive temperature, the *dsbB* gene in pSWO3 may work similarly to *rodA*ts and *pbpA*ts in estimating the stage of a cell division defect. Therefore, when grown at 37°C or above a C600 strain carrying plasmid pSWO3 should tell us the stage at which septation is blocked by the overexpression of *friL*. When grown at 37°C (Fig. 5.3) or 42°C (data not shown), C600 (pSWO3) formed big spherical or lemon-shaped cells. According to the earlier studies, this means that over-expression of *friL* blocks cell division at a very early stage. So far only *ftsZ* and *ftsW* mutants are known to be blocked at such an early stage (Begg and Donachie, 1985; Khattar *et al.*, 1994). Therefore, FriL may control cell division by regulating the *mra* region. This could be tested easily by over-expressing *friL* in reporter strains such as VIP407 and VIP490 (the strains are shown in chapter 6).

To further study its function, other work should be done such as to delete this gene from the chromosome or to make its expression controllable.

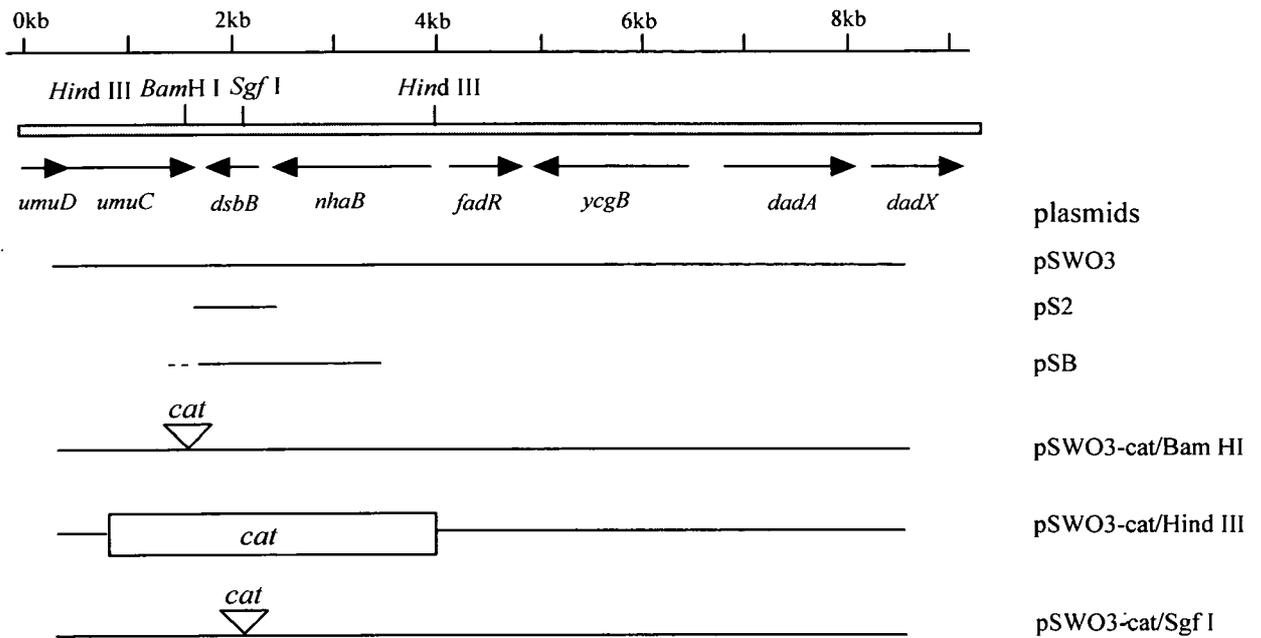


Figure 5.1. Genetic organization around *dsbB* gene in *E. coli* and some plasmids used in this work. The gene organization map was drawn according to data from "the Colibri World-Wide Web Server" on the Netscape. The DNA fragments inserted into *Bam*H I and *Cla* I double digested pACYC177 were shown on the left and the names of the corresponding plasmids were listed on the right.



Figure 5.2. Morphology of C600 (pSWO3) cells grown at 30°C. In this experiment, overnight culture was diluted 1:100 into L broth with Ap (50 $\mu\text{g}/\text{ml}$) and grown at 30°C for 4 hours. Then samples were taken for photographing. Scale is shown by indicating the width of each picture at their left corners.

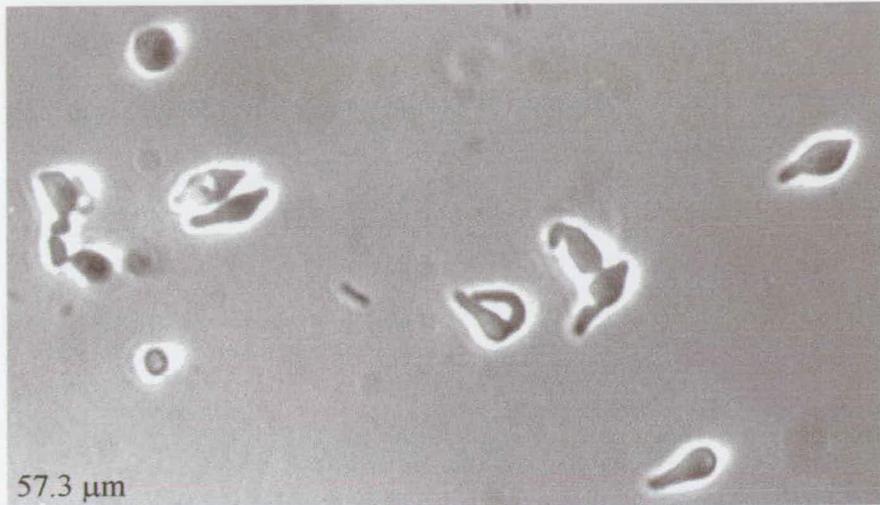


Figure 5.3. Morphology of C600 (pSWO3) cells grown at 37°C. In this experiment, overnight culture was diluted 1:100 into L broth with Ap (50 $\mu\text{g}/\text{ml}$) and grown at 37°C for 3 hours. Then samples were taken for photographing. Scale is shown by indicating the width of each picture at their left corners.

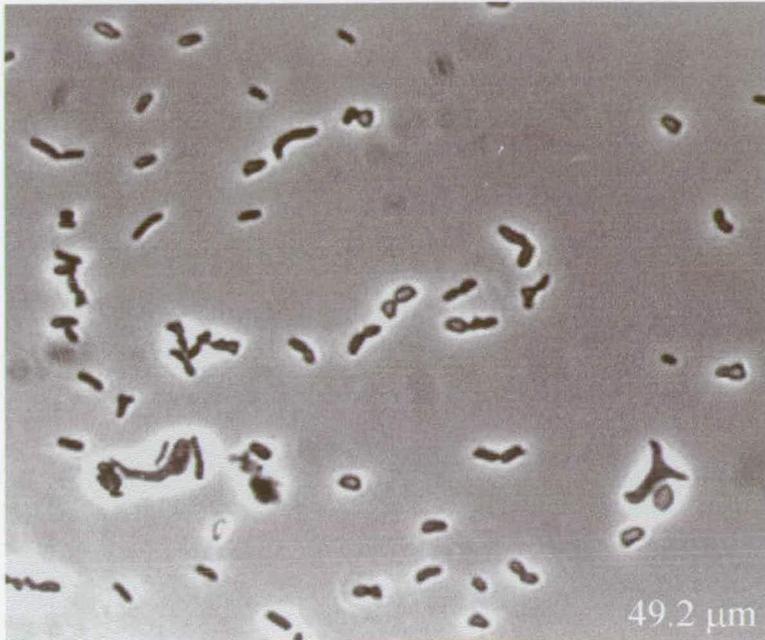


Figure 5.4. Morphology of C600 (pS2) cells grown at 37°C. In this experiment, overnight culture was diluted 1:100 into L broth with Ap (50μg/ml) and grown at 37°C for 3 hours. Then samples were taken for photographing. Scale is shown by indicating the picture's width at its right corner.



Figure 5.5. Morphology of C600 (pF) (5a) and C600 *recA::cm* (pF) (5b). In this experiment, overnight cultures at 30°C were diluted 1:100 into L broth with Ap (50 $\mu\text{g}/\text{ml}$) and grown at 37°C for 2 hours. Then samples were taken for photographing. The scale is shown by indicating the width of each picture at its left corner.



Figure 5.6. Morphology of DH5a (pF). In this experiment, overnight culture at 30°C was 1:100 diluted into L broth with Ap (50μg/ml) and grown at 37°C for 2.5 hours. Sample was then taken for photographing. Scale is shown by indicating the width of the picture at its left corner.

Table 5.2. Alignment of *E. coli* FriL (YcgB) with *B. subtilis* SpoVR. The underlined leucines (L) indicate the putative leucine zipper structure(s): two in FriL and one in SpoVR (this work), and a possible DNA-binding motif is underlined by double lines (Beall B and Moran CP Jr 1994).

Seq1(1>510) FriL-51 (25>497)	Seq2(1>468) SpoVR-BacSu-46 (9>454)	Similarit Inde	Gap Number	Gap Length	Consensus Length
		27.6	15	29	474
v30 LDVYLAEIDRVAKLYRLDTPHQIEVITSEQMMDAYSSVCGMPINYPHWSFGKKFIETERL L: :.EI. :AK : LD YP : E: :.E :: :::: GMP...:HWSFGK:F . . LQRATEEETTELAKGFGLDYFPMRYEICPAE-IITYTFGAYGMPTRFSSHWSFGKQFHMKMLH ^10 ^20 ^30 ^40 ^50 ^60	v40 v50 v60 v70 v80				
v90 YKHGQOGLAYEIVINSNPCIAYLMEENTITMQALVMAHACYGHNSFFKNNYLFRSWIDAS Y. G : YE:VINS:PC A:L::N:: : L::AH. :H .FFKNN. F:: . . YDLGLSKI-YELVINS DPCYAFLLDSNLSIQNKLI VAHV-LAHCDFFKNNCRFQN--TKR ^70 ^80 ^90 ^100 ^110 ^120	v100 v110 v120 v130 v140				
v150 STVDYLI FARKYITTECEERYGVDEVERLLDSCHALMNYGVDRYKRPQKISLQEEKARQKS .:V: : : . I.:.E: .G: .EVE.:LD: :: : . RP .LQ DMVESMAATAERIKHYEQVHGIIKEVESFLDAILSIQEHIDPSLVRP---KLOWSDDEEE ^130 ^140 ^150 ^160 ^170 ^180	v160 v170 v180 v190 v200				
v210 REEYLQSQVNM LWRITL PKREEEKTVAEARRYPSEPOENLLYFMEKNAPLLESWOREILRI E . . : LW. K: : :P: P: : :L F:E: . . . LE:WQR:IL : EIEFAATPYDDLWLSLDEKKPKKQVKKSKKPFPPRPEKDILLFIEEHSRELEFPWORDILITM ^190 ^200 ^210 ^220 ^230 ^240	v220 v230 v240 v250 v260				
v270 VRKVSQYFYFQKQIQVMNEGWAIFWHYITILNHL YDECKVTERFMLEFLHSHIN VVFPQPY :R. YF.PQ :T::MNEGWA::WH I::L .:T. :EF . :.VV MREEMLYFWPQLETKIMNEGWASYWHQRIITREL----DLTSDEATEFAKLNAGVV----- ^250 ^260 ^270 ^280 ^290	v280 v290 v300 v310 v320				
v330 NSFWYSGINPYALGFAMFQDIKRICQSPTEEDKYWFPDIAGSDWLETILHFAMRDFKDE-S P :GINPY LG: :F:DI: : :PTEE K . :S:: E: F:R: . . . : S -QPSKIGINPYLGLKIFEDIEKRYNNPTEEMK-KMGVQPDSCR-EKM-FEVREIESDIS ^300 ^310 ^320 ^330 ^340	v340 v350 v360 v370 v380				
v390 FISQFLSPKVMRDFRFFIVLDDDRHNYLEISATHNEEGYREIRNRLSSQYNLSNLEPNIQ FI.:L: : : : :Y I : . . . :R::L S : . : P : FIRNYLTKELVMREDLY-LFQKQGRDY----KIIDKQ-WKSVRDQLVSMRVNGGF-PYLT ^350 ^360 ^370 ^380 ^390 ^400	v400 v410 v420 v430 v440				
v450 IWNVDLRCGRSLITLRYIPHNRAPLDRGRKEVLKHVHRLWGFDMLEQQNEDGSI : : DL : . . . : . . : . . VL : :LWG .V :E ED . .	v460 v470 v480 v490				

Chapter 6. Cell division is co-ordinated with chromosome replication at the transcriptional level in *Escherichia coli*

6.1. Introduction.

The main processes in the cell cycle of all bacteria are genome replication, segregation of the replicated genome and cell division. These events are normally precisely co-ordinated.

The best known example of the co-ordination of chromosome replication and cell division is probably the inhibition of cell division by SfiA protein through SOS response after DNA damage (Lutkenhaus J.F. 1983, George J. *et al* 1975, Huisman O *et al* 1981, 1984, Mizusawa S. *et al* 1983). Damaged DNA leads to the activation of RecA protein which facilitates the auto-proteolytic cleavage of LexA protein. This in turn leads to derepression of the SOS genes (including *sfiA*), which are normally repressed by the LexA protein. The SfiA protein, in elevated concentration, blocks cell division by targeting FtsZ protein and destabilising its polymerisation at cell division sites (Bi E. and Lutkenhaus J. 1993, Trusca D. *et al* 1998). The formation of the FtsZ ring is one of the earliest detected steps in cell division (Bi EF. and Lutkenhaus J. 1991).

Another putative mechanism has been suggested for many years. In this model, termination of chromosome replication might release a positive signal that would trigger the initiation of cell division (for reviews see Pardee A.B. *et al* 1973, Donachie W.D. *et al* 1973, Jones NC and Donachie WD 1973). However, this idea has not yet been confirmed. Studies on *E. coli* cells have shown that blocking chromosome replication by thymine starvation in

thymine auxotrophs leads to a halt to cell division and the production of filamentous cells as growth continues (Donachie W.D. 1969, Donachie W.D. *et al* 1968). Slowing down the rate of chromosome replication by reducing the concentration of thymine also causes an increase in cell length (Pritchard R.H. and Zaritsky A. 1970, Meacock P. and Pritchard R.H. 1975, Begg K.J. and Donachie W.D. 1978). Experiments involving the interruption of chromosome replication by removal and subsequent readdition of thymine or by changing the initiation rate of chromosome replication, have suggested that a minimum DNA/mass ratio is required for the recommencement of cell division, *i.e.* cells will elongate without division if their DNA content is not sufficient (Donachie W.D. 1969, Botello.E. and Nordstrom K. 1998). However, it is not clear whether all the above phenomena were caused by SOS induction. Studies on *Bacillus subtilis* have shown that completion of chromosome replication is not required for septation but that the central localization of FtsZ ring is affected (McGinness T. and Wake R.G. 1979, 1981, Wu L.J. *et al* 1995, Harry E.J. *et al* 1999). It is also reported that, instead of the termination of chromosome replication, it is the amount of newly replicated chromosomal DNA that determines the frequency of premature septation in germinating spores of *B. subtilis* (McGinness, T. and Wake, R.G. 1981).

In this study we found that even under conditions in which SOS induction was prevented — in a *recA* strain, blocking chromosome replication still caused filamentation in *E. coli*. This suggested that filamentation might be due to lowered expression of the cell division genes. Among the nine known cell division genes in *E. coli*, six of them are clustered at the 2min region (also named *dcw* or *mra* cluster). These genes are contiguous and co-transcribed in the same direction. There are no transcriptional terminator sequences before the last gene (*envA*) (Fig. 6.1). FtsZ and possibly also FtsW are the earliest detectable proteins to localize at cell division sites to initiate septation. They are then rapidly followed by other cell division proteins, including FtsA, ZipA, FtsQ, FtsI, FtsL, FtsK and FtsN, *etc* (Begg K. *et al* 1985, Addinall S.G. 1996, Khattar M.M. *et al* 1994, Boyle D. *et al* 1997, Wang L. *et al* 1998). Because of the importance of these genes in cell division, we decided to test whether their transcription was affected by a block to chromosome replication. In strain VIP407 and its derivatives, the expression of β -galactosidase reflects the native

transcriptional level of the *ftsZ* gene. Since there is no transcriptional terminator between *mraZ* and *ftsZ*, these strains are expected to indicate the total transcriptional activity of this region (Flärdh K. *et al* 1998). Because of the insertion of strong transcription terminators into the *ddlB* gene, the β -galactosidase activity of strain VIP490 and its derivatives will only reflect the activity of promoters lying between *ddlB* and *ftsZ*, *i.e.* *ftsQ2p1p*, *ftsAp* and *ftsZ4p3p2p* promoters (Flärdh K. *et al* 1998). In strain ,Tp8503 λ RWS100, *lacZ* is under the control of *ftsAp* and *ftsZ4p3p2p*, sited on λ RWS100 (Smith RW. *et al* 1993) (Fig. 6.1).

6.2. Materials and Methods.

Strains:

The following *E. coli* K12 strains were used.

C600: *leuB6 hsdR thr-1 lacY1 thi-1 supE44 fhuA21*.

MC1061: *hsdR mcrB araD139 Δ (araABC-leu) Δ lacX74 galU galK rpsL thi*.

VIP407: MC1061 *ftsZ::KfV116[Φ (*ftsZ-lacZ*) bla lacI^Q *tacp-ftsZ*]*.

VIP490: MC1061 *Δ (gal attl bio uvrB) nad::Tn10 ftsZ::pKfV116[Φ (*ftsZ-lacZ*) bla lacI^Q *tacp-ftsZ*] ddlB:: Ω (pKfV122)*.

ME8448: F⁻ *ilv-145 metE46 his-4 trpC3 pro thi thyA::Tn5 thyR mt1-1 malA1 ara-9 galK2 lac114 rpsL ton*.

TP8503: *Δ (lac-proB) leu- thi- supE42 fhuA*.

TP8503 λ RWS100: TP8503 lysogenised with λ RWS100.

ED419: TP8503 *dnaAts46*.

ED100: ED419 λ RWS100.

The following strains were constructed during this work.

- (1). C600 *recA::cm*, VIP407 *sfiA::Tn5*, VIP407 *recA::cm*, TP8503 *recA::cm* λ RWS100 and ED100 *recA::cm* were constructed by transducing *sfiA::Tn5* or *recA::cm* to the respective strains with P1 transduction.
- (2). Thymine auxotrophs of VIP407 were selected as described by Miller (1972). One of them, named VIP407 *thya2* was used in the experiments shown in this paper. *recA::cm* was introduced into it to produce strain VIP407 *thya2 recA::cm*.
- (3). VIP407 *thyA::Tn5* and VIP490 *thyA::Tn5* were constructed by P1 transduction. The P1 lysate was grown on ME8448. The transductants selected on Kan (25 μ g/ml) plate were further tested for Thy⁻ phenotype by streaking onto M9 + CAA + Maltose + MgSO₄ + Thiamine HCl + Ap + Kan + IPTG plates with or without thymine (40 μ g/ml).
- (4). ED419 *rodA*_{ts} was constructed by co-transducing *rodA*_{ts} with a proximal Tn10 (Begg K. *et al* 1985).

Growth conditions:

Overnight cultures were always grown in L broth, supplemented as required with antibiotics (Kan 25 μ g/ml, Cm 20 μ g/ml and/or Spec 25 μ g/ml), thymine(40 μ g/ml) and/or IPTG. For the production of log phase cultures, the overnight cultures were diluted 1:100 (unless otherwise indicated) into fresh medium. Minimal medium (named M9+CAA+Maltose) was M9 + casamino acids (CAA)(5mg/ml) + Maltose(0.2%) + MgSO₄(1mM) + Thiamine-HCl(4 μ g/ml) + Ampicillin(40 μ g/ml) + IPTG. Thymine was used at a final concentration of 40 μ g/ml. The working concentration of IPTG was 8 μ g/ml for VIP407 and its derivatives and 20 μ g/ml for VIP490 and its derivatives. For the log phase growth, OD₆₀₀s were always kept below 0.2 by periodic dilutions. For blocking chromosome replication, nalidixic acid was used at a final concentration of 20 μ g/ml.

Assay of β -galactosidase:

The activity of β -galactosidase was assayed and specific activities (Miller Units) were calculated according to Miller (1972).

6.3. Results.

6.3.1. *recA*-independent filamentation caused by nalidixic acid treatment.

Nalidixic acid (Nal) blocks chromosome replication by targeting the GyrA subunit of DNA gyrase (for review see Cozzarelli NR 1980).

It is well known that when DNA is damaged or chromosome replication is blocked, the SOS response is induced and cell division is inhibited through the action of SfiA and/or SfiC proteins on FtsZ (D'Ari R *et al* 1983, Maguin E *et al* 1986). Here we show that in a *recA* mutant, in which the SOS response cannot be induced, blocking chromosome replication by nalidixic acid still causes cells to filament (Fig. 6.2). A small difference between Nal-treated C600 and C600 *recA::cm* cultures is that the latter cultures had some small or normal sized cells. Most of these cells were anucleate. This is probably due to DNA degradation in *recA*⁻ strains (Skarstad K and Boye E 1993).

6.3.2. Co-ordination of transcription of cell division genes in the *mra* region with chromosome replication.

In order to test if the above filamentation was due to the repression of cell division genes in the *mra* cluster, chromosome replication in the reporter strains was blocked by different methods.

6.3.2.1. Blocking chromosome replication with Nalidixic acid.

After Nal was added to the VIP407 culture, its growth rate slowed (Fig. 6.3a) and the Miller Units of the culture (β -galactosidase as a proportion of cell mass)

dropped quickly to about one quarter of the level of the control grown without Nal (Fig. 6.3b). Total β -galactosidase activity (per ml of culture) increased in direct proportion to the OD600 (cell mass per ml of culture) in the absence of Nal, but, as soon as Nal was added, the synthesis of total enzyme (relative to increase in total cell mass) first slowed and then stopped (Fig. 6.3c). Therefore, transcription of the distal cell division genes in the *mra* region was repressed sharply after chromosome replication was blocked by Nal.

Because SOS induction (dependent on RecA activity) blocks cell division through the binding of SfiA to FtsZ, we investigated whether SfiA and/or RecA were also involved in coupling *mra* transcription to chromosome replication. For this purpose, *sfiA::Tn5* and *recA::cm* were separately introduced into VIP407 by P1 transduction. These strains reacted to Nal treatment in a very similar way to VIP407 (Fig. 6.3). Therefore, co-ordination of *mra* transcription with chromosome replication is *sfiA* and *recA*-independent.

In these strains, FtsZ was expressed constitutively under IPTG control. However, cells of VIP407, VIP407 *sfiA::Tn5* and VIP407 *recA::cm* all became filamentous with nalidixic acid treatment, while without Nal treatment they were quite normal. This suggests that some other cell division proteins were also being under-expressed, although only *ftsZ*'s transcription was directly measured in our experiments.

In order to test whether promoters in a subsection of this region respond to changes in chromosome replication in the same way as the totality of *mra* promoters, VIP490 and TP8503 *recA::cm* (λ RWS100) were also treated with Nal. (The *recA::cm* allele was introduced into TP8503 [λ RWS100] by P1 transduction in order to prevent induction of the λ -prophage during the Nal-induced SOS response.) The behaviour of VIP490 was very similar to VIP407 when treated with nalidixic acid (Fig. 6.4). This suggested that the subset of *mra* promoters in VIP490 (those in the *ddlB-ftsQ-ftsA* region) were repressed after chromosome replication was blocked by Nal.

Tp8503 *recA::cm* λ RWS100, also showed some repression of *lacZ* transcription but the response was much less marked than in VIP407 and VIP490. This might be because the reporter construct is located in the prophage in Tp8503 *recA::cm* (λ RWS100).

6.3.2.2. Blocking chromosome replication by thymine starvation.

To substantiate the above conclusions, thymine auxotrophs were isolated and thymine starvation was used as an alternative way of blocking chromosome replication.

Results showed that the transcriptional levels of *thy*⁻ strains of VIP407 (VIP407 *thya2* and VIP407 *recA::cm thya2*) grown in thymineless medium were only about half of those grown in the presence of thymine. The control *thy*⁺ strain VIP407 did not show any significant differences in transcription of the *mra::lacZ* reporter when grown in media with or without thymine (Fig. 6.5).

The *thyA::Tn5* allele of ME8448 was also introduced into VIP407. It was found that VIP407 *thyA::Tn5* behaved in similar fashion to VIP407 *thya2* during thymine starvation (data not shown).

When grown in thymineless medium, both VIP407 *thya2* and VIP407 *recA::cm thya2* formed filaments (data not shown). Therefore thymine starvation can also cause SOS-independent filamentation.

VIP490 *thyA::Tn5* was constructed in order to test whether the promoters between *ddlB* and *ftsZ* would also respond to thymine starvation. Results again showed that thymine starvation repressed the transcriptional activity of the *mra* promoters in VIP490 *thyA::Tn5* (Fig. 6.6). In contrast, the transcriptional activity of the same promoters in the control strain VIP490 was the same, whether grown in medium with or without thymine (data not shown).

It is known that transcription of *ftsZ* in *E. coli* increases significantly when cells start to enter stationary phase (Aldea M *et al* 1990, Sitnikov DM *et al* 1996). We also found that when VIP407 and VIP490 started to enter stationary phase, the transcription of the *mra* region increased to a very high level (data not shown). This is why the specific level of β -galactosidase dropped first after dilution from overnight cultures into fresh medium (Fig. 6.6). We found that both VIP490 *thyA::Tn5* and VIP490 grew very poorly in M9+CAA+Maltose medium (data not shown). Transcription of *ftsZ*'s proximal promoters is an inverse function of growth rate (Dewar SJ *et al* 1989, Aldea M *et al* 1990, Smith RW *et al* 1993). Therefore, the observed increase in relative transcriptional activity following the initial drop is probably due to the decreasing growth rate of VIP490 *thyA::Tn5* in M9+CAA+Maltose (Fig. 6.6). The difference in relative rates of transcription in the presence and absence of thymine is however quite clear.

Before I got the above thymine starvation results, I tried to grow the VIP407 *thya2* or VIP407 *thyA::Tn5* (from overnight cultures) first in M9+CAA+Glycerol+thymine medium to log phase and then the culture was centrifuged, washed once, resuspended in prewarmed M9+CAA+Glycerol without thymine and then 1:3-1:5 diluted into prewarmed M9+CAA+Glycerol media with or without thymine (final concentration 40 μ g/ml). The result I got was similar to the above: the transcriptional activity of the *mra* promoters of VIP407 *thy* in thymineless medium could be less than a half of that in thymine medium (Fig. 6.7). Total enzyme calculation shows that after the cells were shifted into thymineless medium, the transcription of these genes almost halted (Fig. 6.7c).

Since the cells grew quite slowly in this glycerol medium, I decided to use maltose as the carbon source. However, when this experiment was exactly repeated, except using maltose to replace glycerol, sometimes I could get positive results similar to the above one, but more times I got results only showing slight repression, or the repression only happened in a very short time after the cells were shifted into

thymineless medium. If the cells were diluted into M9+CAA+Maltose medium directly from overnight cultures (instead of growing them in this medium to log phase first), I would get the above clear and repeatable result. The reason for this phenomenon is not clear. It seems that chromosome replication initiation might also play a role in cell division. Possibly, interrupting of replication initiation would cause the activation of some promoters in the *dcw* region. The following study on the *dnaAts* and *dnaEts* mutants seems to support this idea.

6.3.2.3. Blocking chromosome replication by *dnaAts* or *dnaEts* mutation.

In contrast to the above description of the coupling between *ftsZ* transcription and completion of chromosome replication, a former report showed that blocking the initiation of chromosome replication can cause *ftsZ* transcription to increase (Smith *et al.* 1996). I repeated one of the experiments in that publication with strain ED419, a *dnaAts46* mutant and got a similar result. In this *dnaAts* mutant, chromosome replication can be initiated normally at 30°C, but not at 42°C. I found ED419, ED419 *sfiA::Tn5* and also ED419 *recA::cat* filamented at 42°C (data not shown), although the transcription level from a DNA fragment containing promoters *ftsApftsZ4p3p2p* (Smith *et al.*, 1996) was increased. In order to ascertain at which stage cell division was blocked in this case, a *rodAts* allele was introduced into the ED419 mutant. When grown at 42°C, this double mutant formed spherical or lemon-shaped big cells (Fig. 6.8), indicating that cell division was blocked at a very early stage. Only mutations in *ftsZ* or *ftsW* are known to give rise to this phenotype (Begg *et al.*, 1985; Khatter *et al.*, 1994). It therefore, seems likely that the transcription of other genes (especially *ftsW*) is still being repressed by the stop of chromosome replication and this would give rise to filamentation despite the sufficient expression of *ftsZ*. To prove this possibility new reporter strains should be constructed to test whether the native transcriptional activity of *ftsW* or *ftsQ* is repressed after the initiation of chromosome replication is stopped in the *dnaAts* strain at non-permissive temperature. I also tried to block chromosome replication by a *dnaEts* mutant. When *rodAts* was introduced into E486, a *dnaEts*

mutant, the double mutant *dnaEts rodAts* and strain *dnaEts sfiA::Tn5 rodAts* both formed spherical or lemon-shaped big cells at 42°C (Fig. 6.9). An interesting phenomenon when this *dnaEts rodAts* double mutant grown at 42°C is that some of the cells was producing small cells at their ends. I propose that this small cells are equivalent to mini cells produced by filaments with normal diameter. It is known that if *FtsZ* is overexpressed while *FtsA* not then cells will produce mini-cells at ends.

6.4. Summary and Discussion.

It is known that the SOS response, induced by DNA damage or inhibition of DNA replication, can inhibit cell division. Here we have shown that blocking chromosome replication with either nalidixic acid treatment or thymine starvation can also cause SOS-independent inhibition of cell division. In addition, we have shown that transcription of *ftsZ*, and probably the other genes in the *mra* cluster, is repressed by inhibition of chromosome replication, and that this repression is also SOS independent. This suggests the existence of an SOS-independent mechanism coupling transcription of the *mra* locus to chromosome replication. Such coupling would allow the frequency of cell division to be determined by the frequency of chromosome replication and thus ensure orderly duplication of the cell under any growth condition. The function of the SOS dependent system is presumably limited to situations in which DNA is subject to damage.

The finding that the main promoters in the *mra* cluster respond to a block in chromosome replication in a similar fashion (in VIP407 and VIP490) is perhaps not a surprise, because all the gene products in this region are involved in the highly coordinated processes of cell division and peptidoglycan synthesis. It is to be expected that the regulation of their expression should also be well coupled.

It is known that transcription of *ftsZ* is an inverse function of growth rate (Donachie *et al.* 1984, Dewar *et al.* 1989, Aldea *et al.* 1990, Smith *et al.* 1993). Nevertheless we show here that, although blocking chromosome replication causes a progressive reduction in growth rate, which by itself might have been expected to stimulate transcription of *ftsZ*, the relative rate of transcription of the *mra* region is in fact progressively reduced. The coupling between chromosome replication and cell division might therefore appear at first sight to have overridden the mechanism that couples cell division to growth rate. Despite these appearances, it is possible to explain both the growth rate dependence and the dependence on chromosome replication by a single mechanism (Smith *et al.* 1993). Smith *et al.* (1993) described the kinetics of *ftsZ* transcription following a shift from a poor to a rich growth medium. Following such a shift, the total rate of protein synthesis accelerates rapidly but the rate of cell division is maintained at near the preshift rate for about one hour before changing abruptly to equal the rate of mass doubling (Kjeldgaard NO, *et al.* 1958). This is explained by the classic model in which the rate of initiation of new rounds of chromosome replication is directly coupled to cell mass (Donachie WD. 1968) but the rate of cell division is linked to the rate of completion of rounds of chromosome replication which is delayed by about 40 minutes (the "C" period) required for replication forks to travel from the replication origin to the replication terminus of the chromosome, plus about 20 minutes (the "D" period) to complete septation and cell division (Cooper S, Helmstetter CE. 1968). Smith *et al.* (1993) showed that the rate of transcription of *ftsZ* was directly proportional to the rate of completion of rounds of chromosome replication: *i.e.* that it was maintained at the preshift rate for about 40 minutes after the shift from the poor to the rich growth medium. Such a coupling also explains the observed inverse relationship between rate of *ftsZ* transcription and growth rate, because the average number of complete chromosomes/cell mass increases as growth rate decreases, for exactly the same reasons as were used above to explain the kinetics of cell division and *ftsZ* transcription during a shift in growth rate. Our new results are therefore in complete harmony with all these previous observations and models.

Studies on germinating spores of *B. subtilis* have given an indication of similar phenomena (McGinness and Wake 1979, 1981, Harry *et al* 1999). These workers found that, at 180 min after the start of germination in thymine containing medium, 81% of cells had a Z ring, while in low thymine medium, only 35% of cells had a Z ring. The authors only concluded however, that the effect of chromosome replication on cell division was on the central location of the FtsZ ring (Harry *et al.* 1999). However, it is not clear what role,if any, the SOS response plays in the reduction of FtsZ ring formation in this species.

All the *E. coli* experiments therefore suggest that some event associated with the termination of chromosome replication can induce transcription of the *mra* region and that this may be the main mechanism coupling cell division with chromosome replication under normal growth conditions. The signaling pathway of this co-ordination mechanism is not clear and determination of the factors that monitor chromosome replication and regulate the transcription of cell division genes may be a major project for future work.

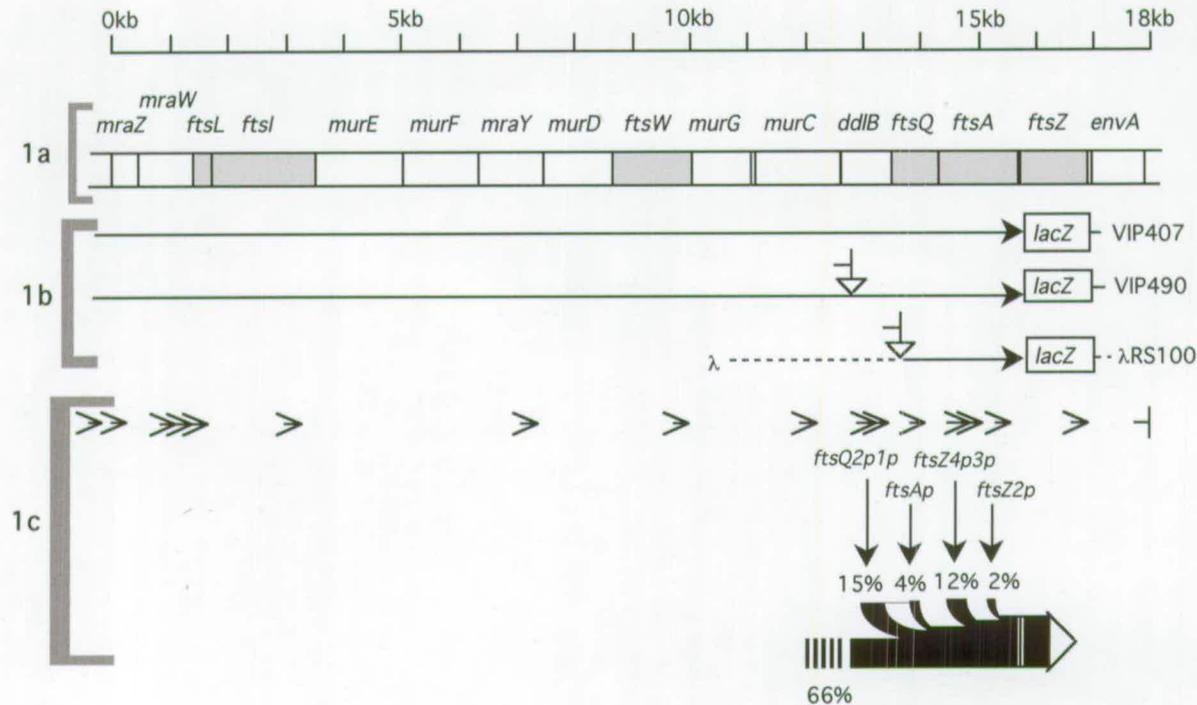
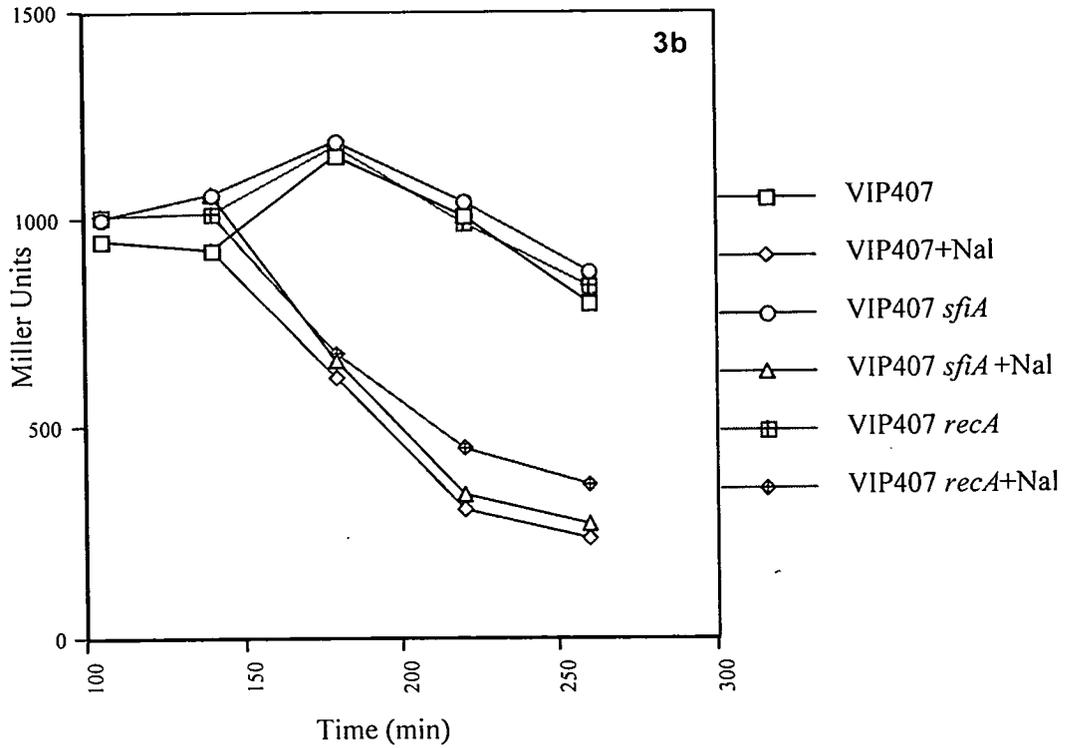
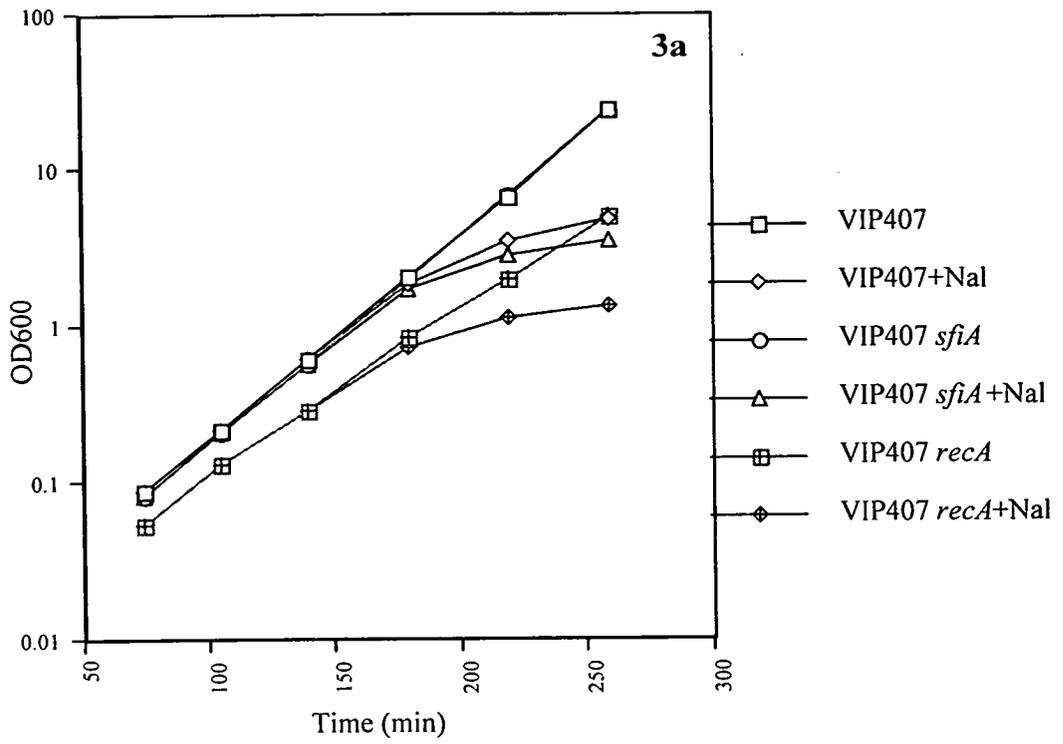


Figure 6.1. Genetic organization of the *dcw* cluster at 2min region in *Escherichia coli* chromosome (1a), transcriptional contributions of promoters measured at *ftsZ* location (1c) and the promoters measured by LacZ expression in different reporter strains (1b) (Flardh *et al.*, 1998; Lutkenhaus and Mukherjee, 1996; Simth *et al.*, 1993). Arrows represent detected or putative promoters. —| : represents transcriptional terminator(s). In VIP407 and VIP490 the constructs are located at the native location of *ftsZ*, while the construct in λRS100 is located in phage λ in *E.coli* chromosome.



Figure 6.2. RecA-independent filamentation caused by nalidixic acid treatment. In this experiment, overnight cultures of C600 and C600 *recA::cm* were 1:100 inoculated into L broth supplied with Nal (20μg/ml) and grown at 37°C with shaking. The pictures show the morphology of C600 (2a) and C600 *recA::cm* (2b) after three hours incubation. The scale is shown by indicating the width of each picture at their left corners.



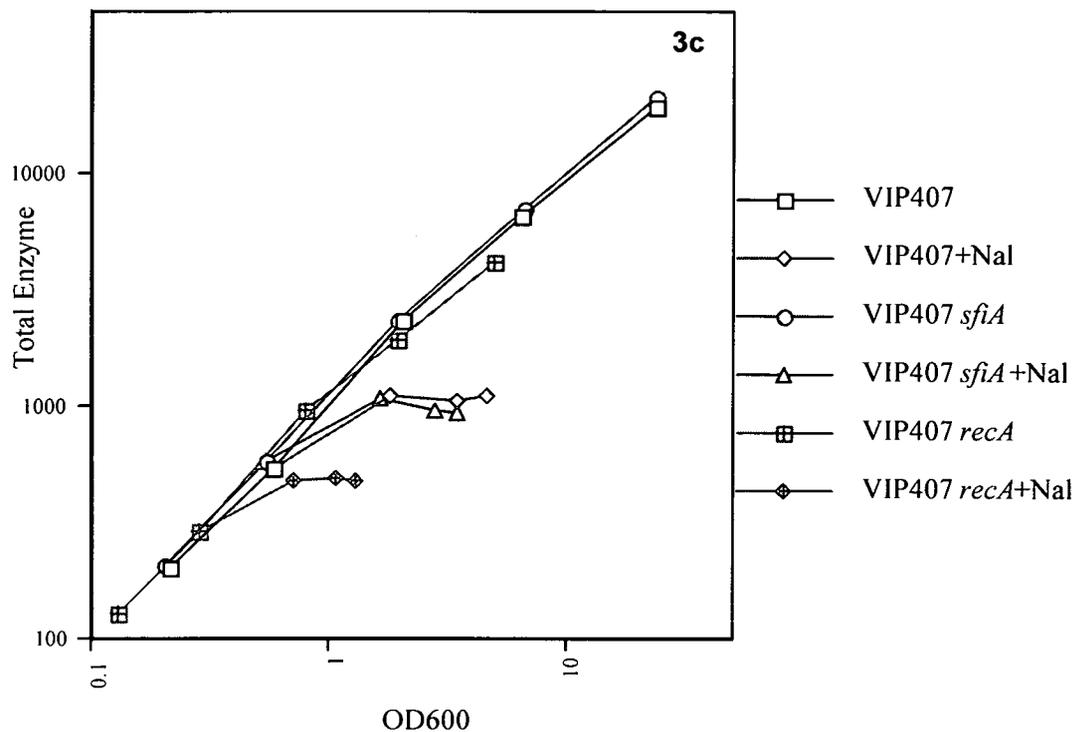


Figure 6.3. The effect of blocking chromosome replication by nalidixic acid on the transcription of cell division genes in VIP407, VIP407 *sfiA::Tn5* and VIP407 *recA::cat*. In this experiment, overnight cultures of VIP 407, VIP407 *sfiA::Tn5* and VIP407 *recA::cat* were diluted 1:100 into LB medium with Ap and IPTG and grown at 37°C with shaking. Nal was added at 145 min. OD600s (3a) and Miller Units (3b) of the cultures were measured at indicated times. Total enzymes were also calculated and then drawn against OD600s (3c).

Note: Total enzyme = MU*OD600.

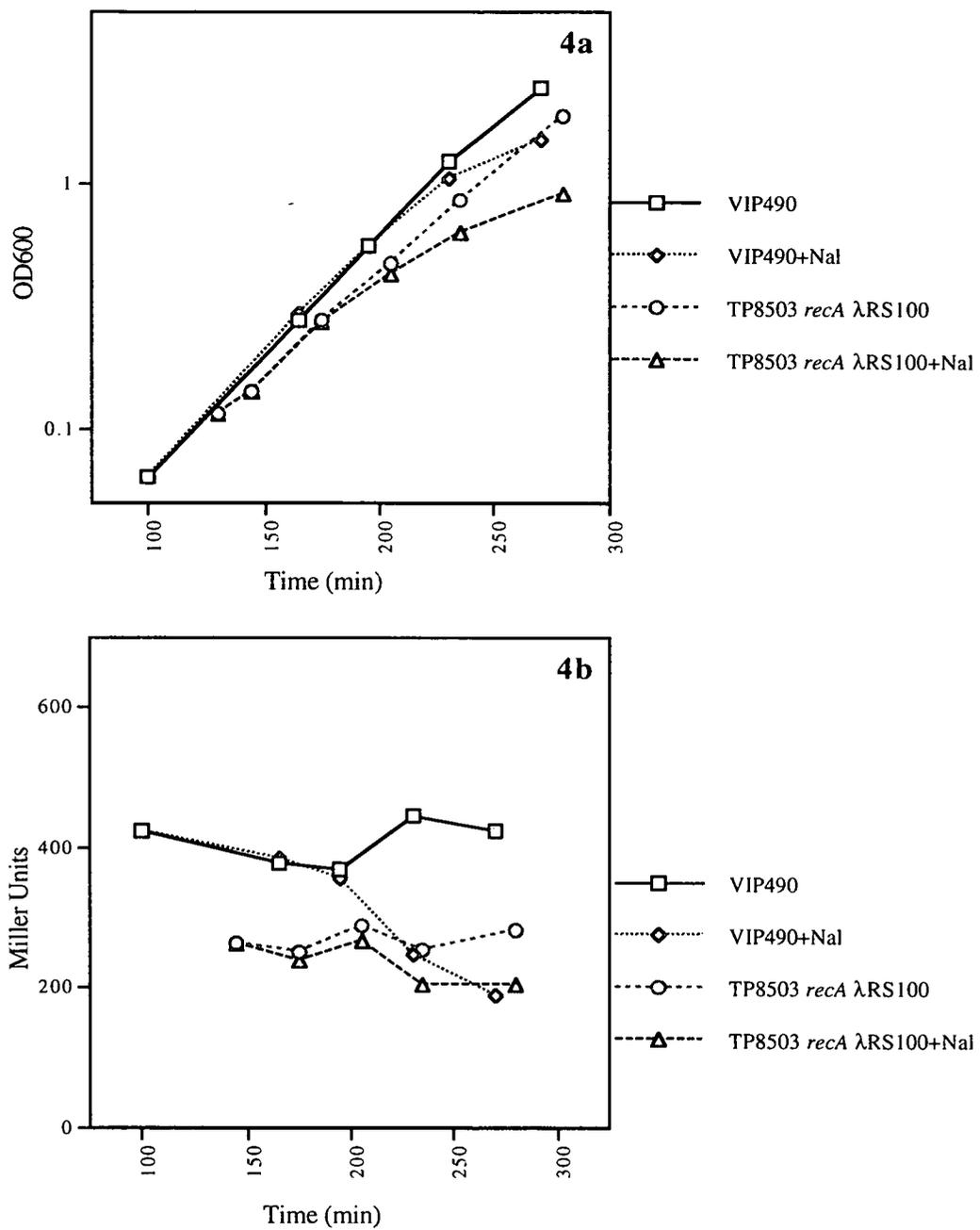
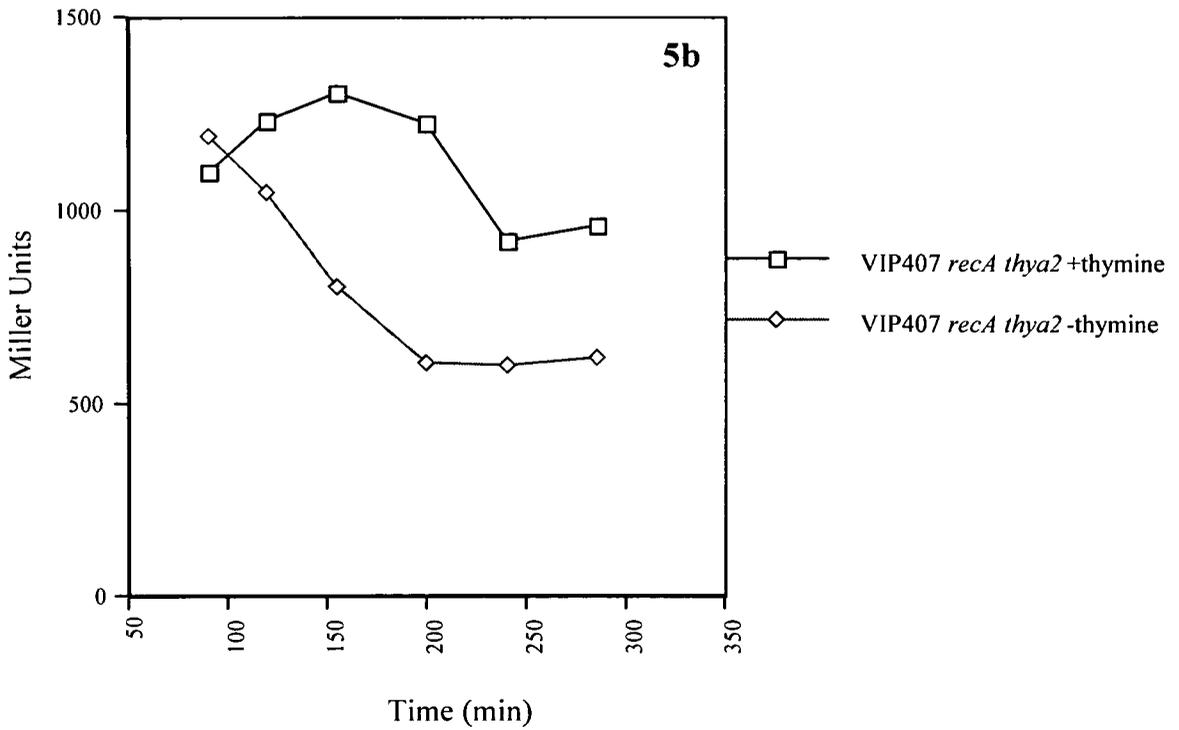
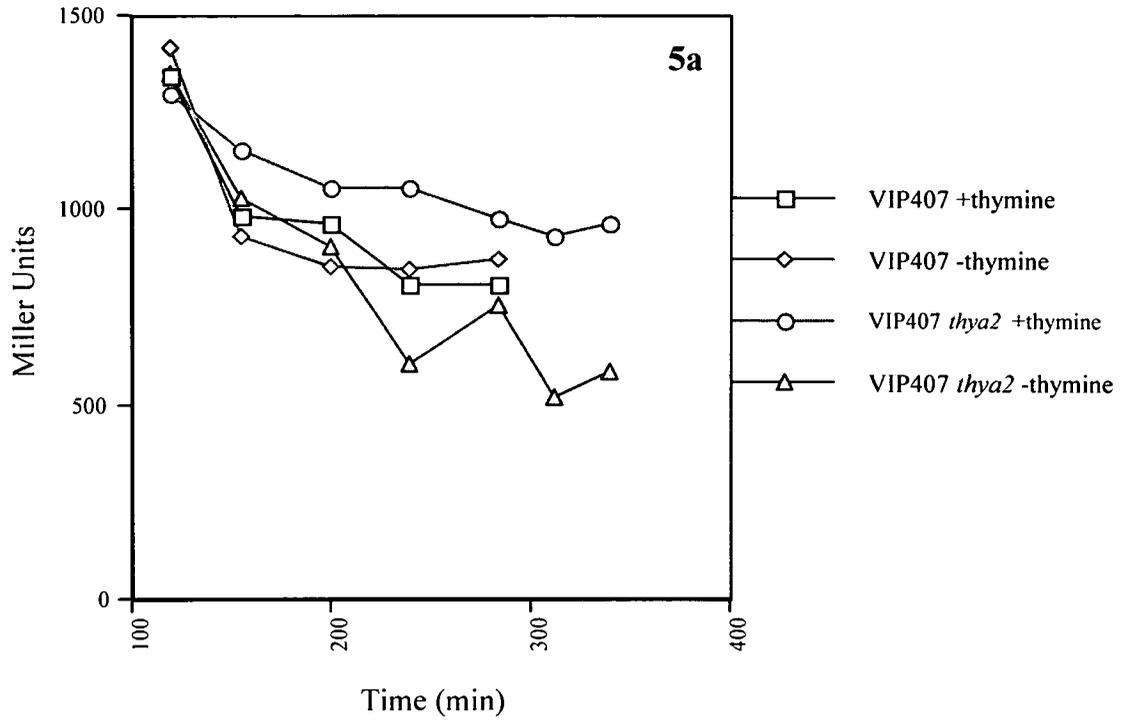


Figure 6.4. Responses of VIP490 and TP8503 *recA::cat* λRS100 to Nal treatment. (4a) shows growth rates (OD600) and (4b) shows Miller Units of the cultures with or without Nal treatment. Nal was added at 180min for VIP490 and at 150min for TP8503 *recA::cat* λRS100.



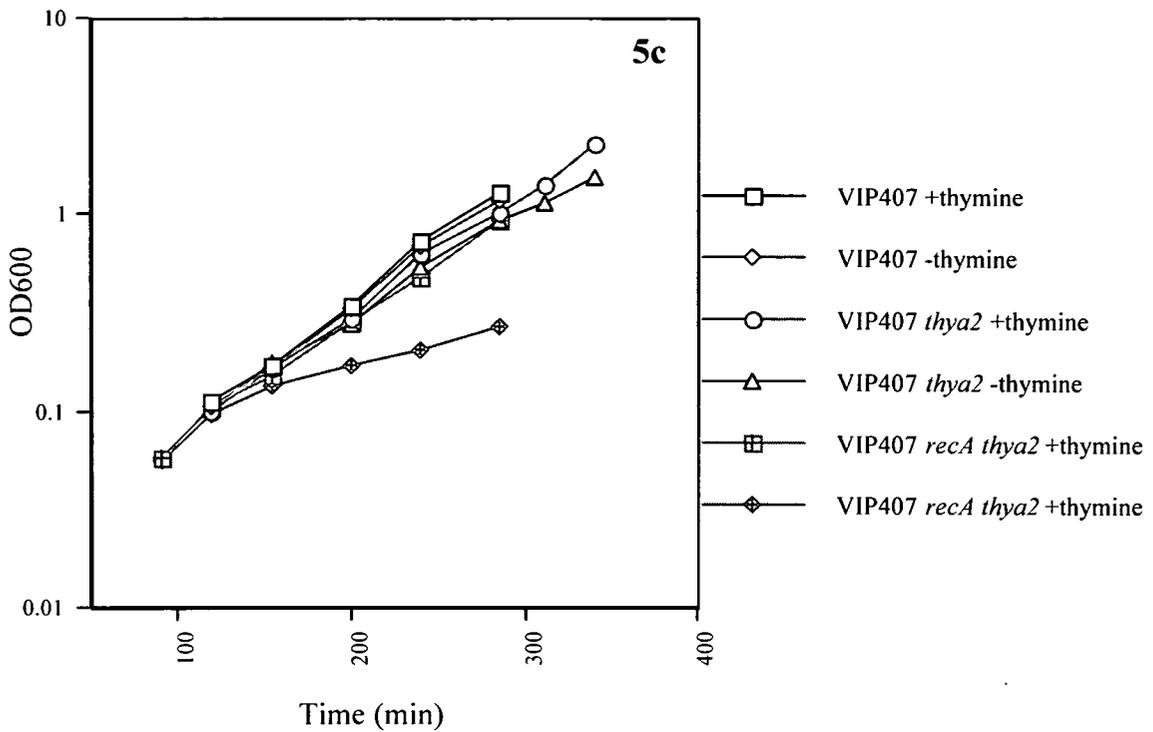


Figure 6.5. The effect of thymine starvation on the transcription activity of cell division genes in VIP407 *thy⁻* strains. In this experiment, 1.2ml overnight cultures of VIP407, VIP407 *thya2* and VIP407 *recA::cat thya2* were spun down and resuspended in 0.6ml L broth. The resuspensions were 1:50 (for VIP407 *recA::cat thya2*, due to its low viability) or 1:100 (for the others) inoculated into M9+CAA+Maltose medium with or without thymine (40ug/ml). (5a) and (5b) show the MUs and (5c) shows the growth rates (OD600) of the cultures in medium with or without thymine.

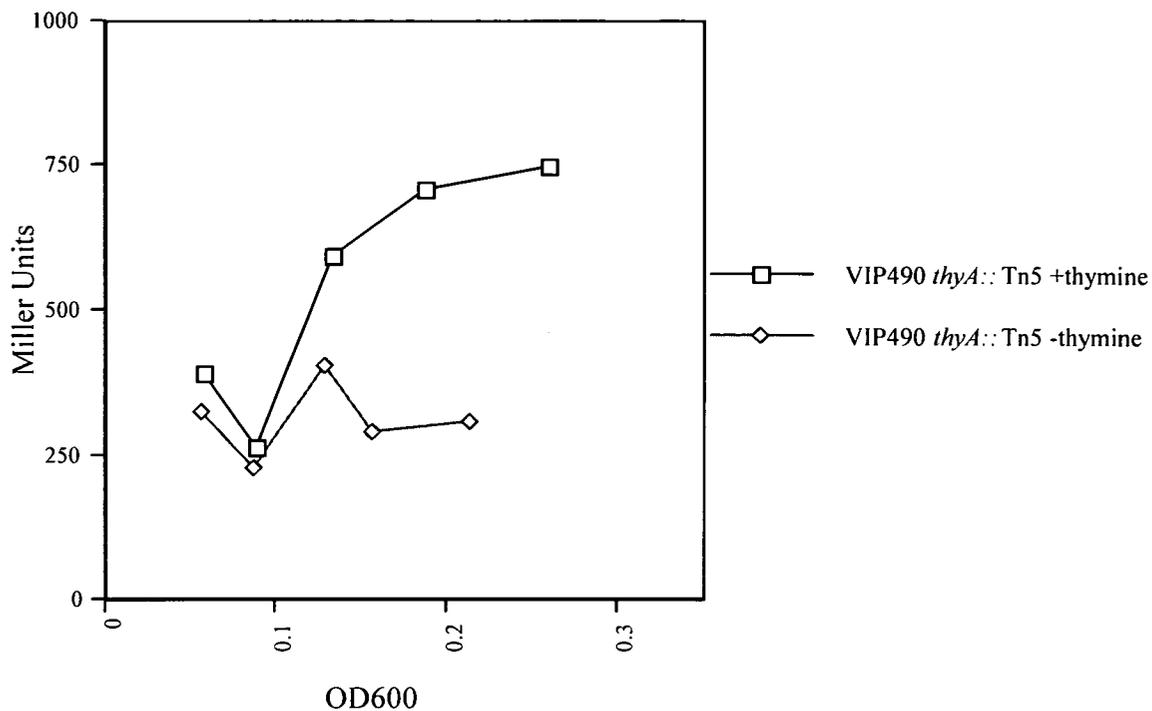


Figure 6.6. The effect of thymine starvation on VIP490 *thy-* strain. In this experiment, 0.6ml overnight of VIP490 *thyA::Tn5* was spun down and resuspended in the same volume of L broth. Then the resuspension was 1:100 inoculated into M9+CAA+Maltose medium supplied with or without thymine(40 μ g/ml). Samples were taken and the specific enzyme activity of β -galactosidase (Miller Units) was assayed.

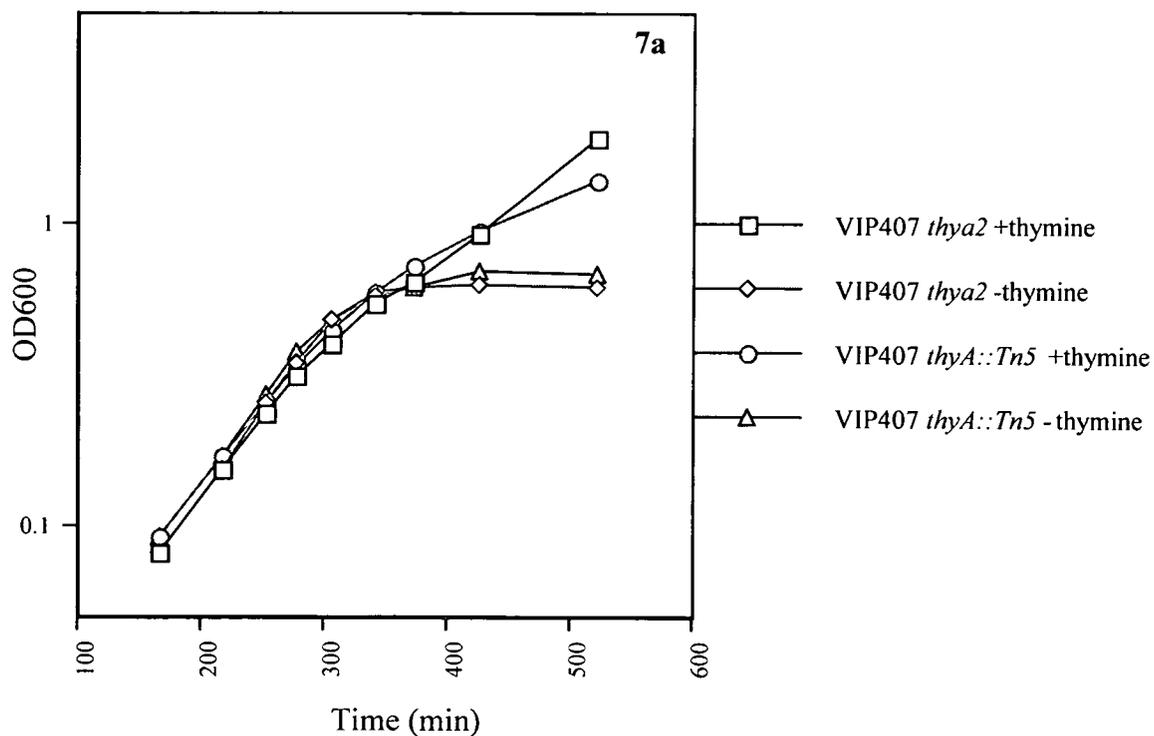
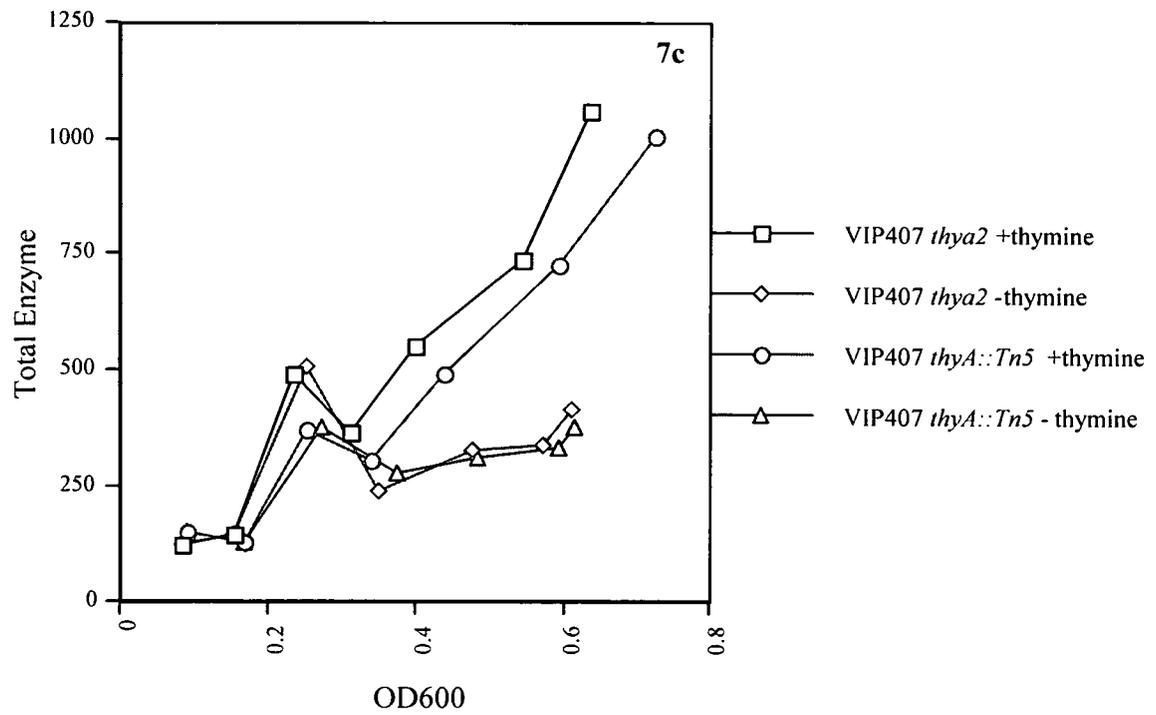
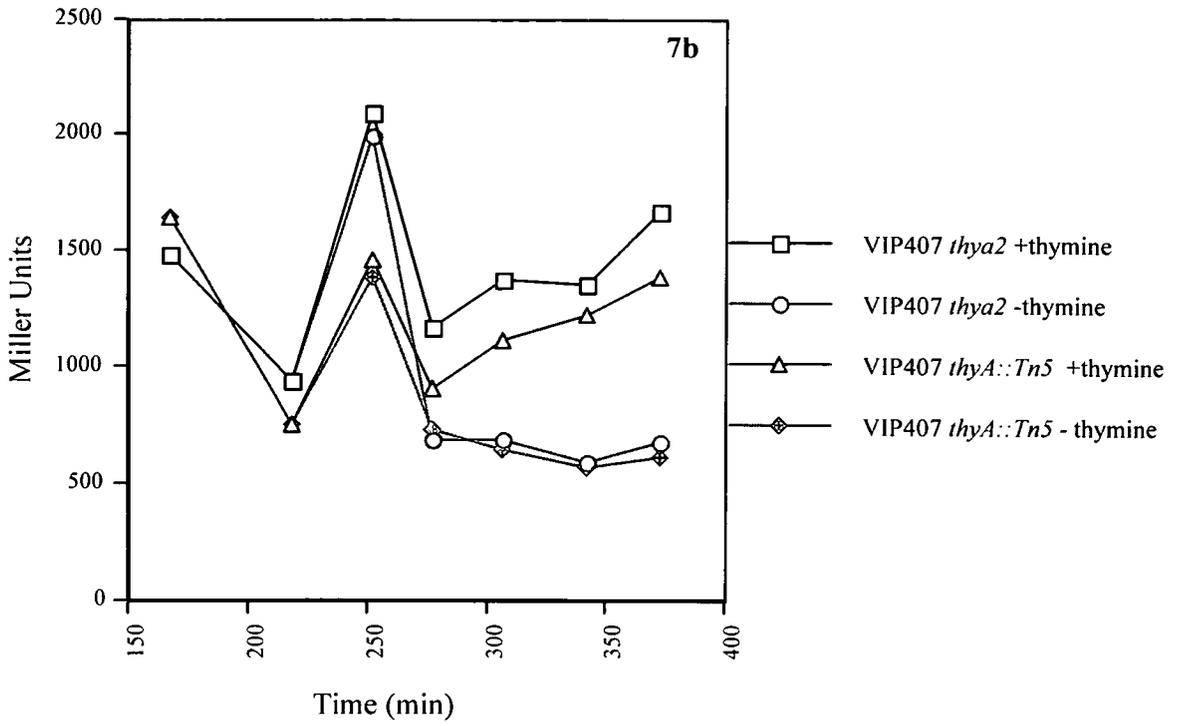


Figure 6.7. Thymine starvation effect on VIP407 *thy*⁻ strains when grown on glycerol medium. In this experiment, overnight cultures of VIP407 *thya2* and VIP407 *thyaA::Tn5* were 1:200 diluted into M9+CAA+Glycerol with thymine (40ug/ml) at 230min when their OD600s were between 0.15-0.2 the cells were spun down and then at 240min 1:2 diluted into prewarmed above medium whith or without thymine. Since then OD600s were always kept below 0.2 by 1:3 dilutions. Their OD600s (7a) and MUs (7b) were measured at indicated times and total enzyme activities were calculated (7c).

$$\text{Total enzyme} = \text{OD600} * \text{MU}.$$





width: 49.4 μ m.

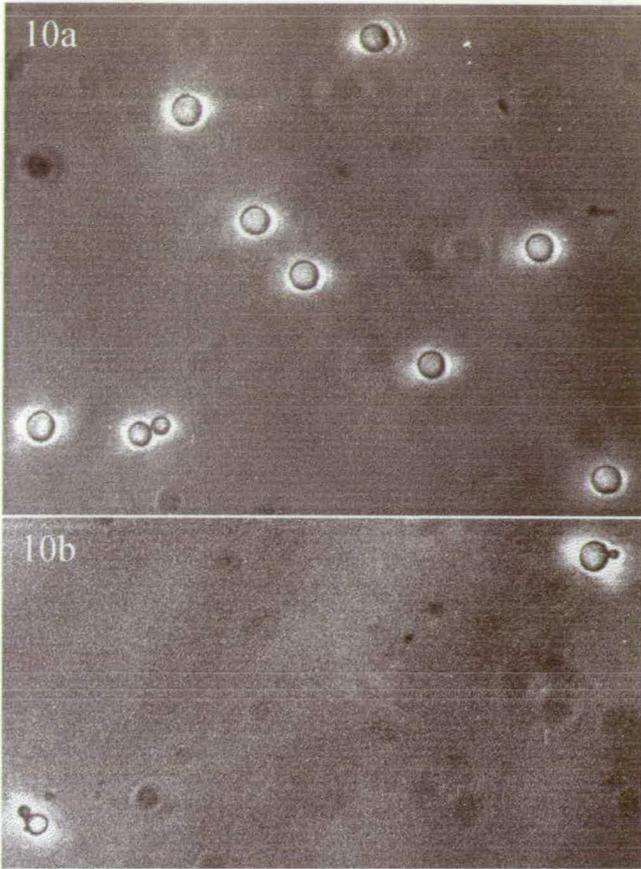


width: 36.6 μ m.

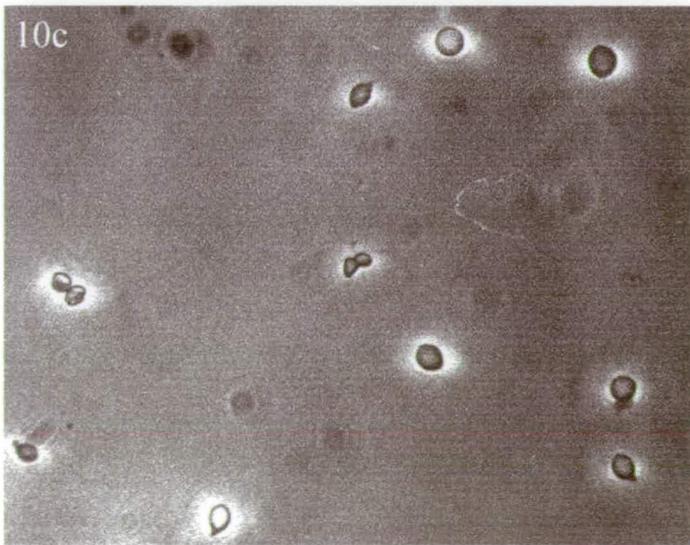


width: 50.2 μ m.

Figure 6.8. Morphology of *dnaAts rodAts* double mutants at 42°C. In this experiment, ED419 *rodAts* (9a) and ED419 *sfiA::Tn5 rodAts* (9b and 9c) were grown at 30°C for two hours first and then shifted to 42°C. After three hours incubation at 42°C samples were taken for photographing. Scale is shown by indicating the width of each picture at their left sides.



width: 41.8 μ m.



width: 45 μ m.

Figure 6.9.

References:

- Adams DE, West SC. Unwinding of closed circular DNA by the *Escherichia coli* RuvA and RuvB recombination/repair proteins. *J Mol Biol* 1995, 247(3):404-17.
- Addinall SG, Bi E, Lutkenhaus J. FtsZ ring formation in *fts* mutants. *J Bacteriol*. 1996, 178(13):3877-84.
- Addinall SG, Cao C, Lutkenhaus J. FtsN, a late recruit to the septum in *Escherichia coli*. *Mol Microbiol*. 1997, 25(2):303-9.
- Addinall SG, Lutkenhaus J. FtsA is localized to the septum in an FtsZ-dependent manner. *J Bacteriol* 1996 Dec;178(24):7167-72
- Aldea M, Garrido T, Pla J, Vicente M. Division genes in *Escherichia coli* are expressed coordinately to cell septum requirements by gearbox promoters *EMBO J* 1990 Nov; 9(11):3787-94.
- Allen GC Jr., Kornberg A. Assembly of the primosome of DNA replication in *Escherichia coli*. *J Biol Chem* 1993 Sep 15;268(26):19204-9
- Andersen CL, Matthey-Dupraz A, Missiakas D, Raina S. A new *Escherichia coli* gene, *dsbG*, encodes a periplasmic protein involved in disulphide bond formation, required for recycling DsbA/DsbB and DsbC redox proteins. *Mol Microbiol* 1997 Oct;26(1):121-32.
- Asai T, Kogoma T. D-loops and R-loops: alternative mechanisms for the initiation of chromosome replication in *Escherichia coli*. *J Bacteriol* 1994 Apr;176(7):1807-12
- Asai T, Kogoma T. Roles of *ruvA*, *ruvC* and *recG* gene functions in normal and DNA damage-inducible replication of the *Escherichia coli* chromosome. *Genetics* 1994 Aug;137(4):895-902
- Asai T, Sommer S, Bailone A, Kogoma T. Homologous recombination-dependent initiation of DNA replication from DNA damage-inducible origins in *Escherichia coli*. *EMBO J* 1993 Aug;12(8):3287-95.

- Bader M, Muse W, Ballou DP, Gassner C, Bardwell JCA. Oxidative protein folding is driven by the electron transport system. *CELL*, 1999, Vol.98, No.2, pp.217-227
- Bainton NJ, Bycroft BW, Chhabra SR, Stead P, Gledhill L, Hill PJ, Rees CED, Winson MK, Salmond GPC, Stewart GSAB and Williams P. A general role for the lux autoinducer in bacterial-cell signaling - control of antibiotic biosynthesis in erwinia. *Gene*. 1992, vol.116, no.1, pp.87-91.
- Ballesteros M, Kusano S, Ishihama A, Vicente M. The *ftsQ1p* gearbox promoter of *Escherichia coli* is a major sigma S-dependent promoter in the *ddlB-ftsA* region. *Mol Microbiol*. 1998 Oct; 30(2):419-30.
- Bardwell JC, Lee JO, Jander G, Martin N, Belin D, Beckwith J. A pathway for disulfide bond formation in vivo. *Proc Natl Acad Sci U S A* 1993 Feb 1;90(3):1038-42
- Barondess JJ, Carson M, Guzman Verduzco LM and Beckwith J. Alkaline phosphatase fusions in the study of cell division genes. *Res Microbiol*. 1991 Feb-Apr; 142(2-3):295-9
- Beall B, Moran CP Jr. Cloning and characterization of spoVR, a gene from *Bacillus subtilis* involved in spore cortex formation *J Bacteriol* 1994 Apr;176(7):2003-12.
- Begg KJ, Dewar SJ, Donachie WD. A new *Escherichia-coli* cell-division gene, *ftsk*. *J Bacteriol*. 1995, vol.177, no.21, pp.6211-6222.
- Begg KJ, Donachie WD. Cell shape and division in *Escherichia coli*: experiments with shape and division mutants. *J Bacteriol*. 1985 Aug; 163(2):615-22 .
- Begg KJ, Donachie WD. Changes in cell size and shape in thymine-requiring *Escherichia coli* associated with growth in low concentrations of thymine. *J Bacteriol*. 1978 Feb; 133(2):452-8.
- Begg KJ, Hatfull GF and Donachie WD. Identification of new genes in a cell envelope-cell division gene cluster of *Escherichia coli*: cell division gene *ftsQ*. *J Bacteriol*. 1980 Oct; 144(1):435-7.

- Begg KJ, Spratt BG, Donachie WD. Interaction between membrane proteins PBP3 and rodA is required for normal cell shape and division in *Escherichia coli* J Bacteriol 1986 Sep;167(3):1004-8.
- Benneria T, Ron EZ. A cluster of cell-division genes maps to the terC region of the chromosome of *Escherichia coli* K-12. *Mol Gen Genetics*, 1995, vol.246, no.5, pp.605-609.
- Bennett RJ, Dunderdale HJ, West SC. Resolution of Holliday junctions by RuvC resolvase: cleavage specificity and DNA distortion. *Cell* 1993 Sep 24;74(6):1021-31
- Bennett RJ, West SC. Resolution of Holliday junctions in genetic recombination: RuvC protein nicks DNA at the point of strand exchange. *Proc Natl Acad Sci U S A* 1996 Oct 29;93(22):12217-22
- Benson F, Collier S, Lloyd RG. Evidence of abortive recombination in *ruv* mutants of *Escherichia coli* K12. *Mol Gen Genet* 1991 Feb;225(2):266-72.
- Benson FE, Illing GT, Sharples GJ, Lloyd RG. Nucleotide sequencing of the *ruv* region of *Escherichia coli* K-12 reveals a LexA regulated operon encoding two genes. *Nucleic Acids Res* 1988 Feb 25;16(4):1541-9
- Bernander R, Nordstrom K. Chromosome-replication does not trigger cell-division in *Escherichia coli*. *Cell*, 1990, vol.60, no.3, pp.365-374.
- Bi E, Lutkenhaus J. Cell division inhibitors SulA and MinCD prevent formation of the FtsZ ring. *J Bacteriol.* 1993 Feb; 175(4):1118-25.
- Bi E, Lutkenhaus J. FtsZ regulates frequency of cell-division in *Escherichia coli*. *J. Bacteriol*, 1990, vol.172, no.5, pp.2765-2768
- Bi E, Lutkenhaus J. FtsZ ring structure associated with division in *Escherichia coli*. *Nature*, 1991, Vol.354, No.6349, pp.161-164.
- Blakely G, Colloms S, May G, Burke M, Sherratt D. *Escherichia coli* Xerc Recombinase Is Required For Chromosomal Segregation At Cell-Division. *New Biologist*, 1991, Vol.3, No.8, Pp.789-798

- Blakely G, May G, Mcculloch R, Arciszewska LK, Burke M, Lovett ST, Sherratt DJ. 2 Related Recombinases Are Required For Site-Specific Recombination At Dif And Cer In Escherichia-Coli K12. *Cell*, 1993, Vol.75, No.2, Pp.351-361
- Blakely G, Sherratt D. Determinants of selectivity in Xer site-specific recombination. *Genes and Dev.* 1996, Vol.10, No.6, pp.762-773
- Bognar A, Pyne C, Yu M, Basi G. Transcription of the folC gene encoding folylpolyglutamate synthetase-dihydrofolate synthetase in Escherichia coli. *J Bacteriol* 1989 Apr;171(4):1854-61.
- Bognar AL, Osborne C, Shane B. Primary structure of the Escherichia coli folC gene and its folylpolyglutamate synthetase-dihydrofolate synthetase product and regulation of expression by an upstream gene. *J Biol Chem* 1987 Sep 5;262(25):12337-43.
- Bognar AL, Osborne C, Shane B, Singer SC, Ferone R. Folylpolyl-gamma-glutamate synthetase-dihydrofolate synthetase. Cloning and high expression of the Escherichia coli folC gene and purification and properties of the gene product. *J Biol Chem* 1985 May 10;260(9):5625-30.
- Bork P, Sander C, Valencia A. An ATPase domain common to prokaryotic cell cycle proteins, sugar kinases, actin, and hsp70 heat shock proteins. *Proc Natl Acad Sci U S A* 1992 Aug 15;89(16):7290-4.
- Botello E and Nordstrom K. Effects of chromosome underreplication on cell division in *Escherichia coli*. *J Bacteriol.* 1998 Dec; 180(23):6364-74.
- Bowler LD, Spratt BG. Membrane topology of penicillin-binding protein 3 of Escherichia coli. *Mol Microbiol* 1989 Sep;3(9):1277-86.
- Boyle DS, Khattar MM, Addinall SG, Lutkenhaus J, Donachie WD. *ftsW* is an essential cell-division gene in Escherichia coli. *Mol Microbiol* 1997 Jun;24(6):1263-73.
- Boyle DS, Khattar MM, Addinall SG, Lutkenhaus J, Donachie WD. *ftsW* is an essential cell-division gene in *Escherichia coli*. *Mol Microbiol.* 1997 Jun; 24(6):1263-73.

- Bramhill D, Thompson CM. GTP-dependent polymerization of *Escherichia coli* FtsZ protein to form tubules. *Proc Natl Acad Sci U S A* 1994 Jun 21;91(13):5813-7
- Broome-Smith JK, Spratt BG. A vector for the construction of translational fusions to TEM beta-lactamase and the analysis of protein export signals and membrane protein topology. *Gene* 1986;49(3):341-9
- Broyles SS, Pettijohn DE. Interaction of the *Escherichia coli* HU protein with DNA. Evidence for formation of nucleosome-like structures with altered DNA helical pitch *J Mol Biol* 1986 Jan 5;187(1):47-60.
- Buddelmeijer N, Aarsman ME, Kolk AH, Vicente M, Nanninga N. Localization of cell division protein FtsQ by immunofluorescence microscopy in dividing and nondividing cells of *Escherichia coli*. *J. Bacteriol.* 1998 Dec; 180(23): 6107-16
- Buddelmeijer N, Aarsman MEG, Kolk AHJ, Vicente M, Nanninga N. Localization of cell division protein FtsQ by immunofluorescence microscopy in dividing and nondividing cells of *Escherichia coli*. *Journal of Bacteriology*, 1998, Vol.180, No.23, pp.6107-6116
- Cam K, Cuzange A, Bouche JP. Sigma S-dependent overexpression of *ftsZ* in an *Escherichia coli* K-12 *rpoB* mutant that is resistant to the division inhibitors DicB and DicF RNA. *Mol Gen Genet* 1995 Jul 28;248(2):190-4.
- Cam K, Rome G, Krisch HM, Bouche JP. RNase E processing of essential cell division genes mRNA in *Escherichia coli*. *NUCLEIC ACIDS RESEARCH*, 1996, Vol.24, No.15, pp.3065-3070
- Carl PL, Bloom L, Crouch RJ. Isolation and mapping of a mutation in *Escherichia coli* with altered levels of ribonuclease H. *J Bacteriol* 1980 Oct;144(1):28-35.
- Carson MJ, Barondess J, Beckwith J. The FtsQ protein of *Escherichia coli*: membrane topology, abundance, and cell division phenotypes due to overproduction and insertion mutations. *J Bacteriol.* 1991 Apr; 173(7):2187-95
- Cashel M, Gentry DR, Hernandez VJ and Vinella D. 1996. The Stringent Response, P1458-1496. In FC Neidhardt *et al.* (ed.), *Escherichia coli* and *Salmonella*:

Cellular and Molecular Biology. American Society for Microbiology, Washington, DC.

Chan SN, Harris L, Bolt EL, Whitby MC, Lloyd RG. Sequence specificity and biochemical characterization of the RusA Holliday junction resolvase of *Escherichia coli*. *J Biol Chem* 1997 Jun 6;272(23):14873-82

Chan SN, Vincent SD, Lloyd RG. Recognition and manipulation of branched DNA by the RusA Holliday junction resolvase of *Escherichia coli*. *Nucleic Acids Res* 1998 Apr 1;26(7):1560-6

Chatterji D, Fujita N, Ishihama A. The mediator for stringent control, ppGpp, binds to the beta-subunit of *Escherichia coli* RNA polymerase. *Genes Cells* 1998 May;3(5):279-87.

Chen JC, Weiss DS, Ghigo JM, Beckwith J. Septal localization of FtsQ, an essential cell division protein in *Escherichia coli*. *J Bacteriol.* 1999 Jan; 181(2):521-30.

Clerget M. site-specific recombination promoted by a short DNA segment of plasmid-R1 and by a homologous segment in the terminus region of the *Escherichia coli* chromosome. *New Biologist*, 1991, vol.3, no.8, pp.780-788.

Contreras A, Maxwell A. *gyrB* mutations which confer coumarin resistance also affect DNA supercoiling and ATP hydrolysis by *Escherichia coli* DNA gyrase. *Mol Microbiol* 1992 Jun;6(12):1617-24.

Cook WR, Rothfield LI. Development of the cell-division site in FtsA- filaments. *Mol Microbiol.* 1994 14(3):497-503.

Cook WR, Rothfield LI. Early stages in development of the *Escherichia coli* cell-division site. *Mol Microbiol* 1994 Nov; 14(3):485-95.

Cook WR, Rothfield LI. Nucleoid-independent identification of cell division sites in *Escherichia coli*. *Journal of Bacteriology*, 1999, Vol.181, No.6, pp.1900-1905.

Cooper S, Helmstetter CE. Chromosome replication and the division cycle of *Escherichia coli* B/r. *J. Mol. Biol.* 1968, 31: 519-40.

- Corrette-Bennett SE, Lovett ST. Enhancement of RecA strand-transfer activity by the RecJ exonuclease of *Escherichia coli*. *J Biol Chem* 1995 Mar 24;270(12):6881-5.
- Cozzarelli NR. DNA gyrase and the supercoiling of DNA *Science* 1980 Feb 29;207(4434):953-60.
- Cronan JE Jr, Subrahmanyam S. FadR, transcriptional co-ordination of metabolic expediency *Mol Microbiol* 1998 Aug;29(4):937-43.
- Dai K, Lutkenhaus J. ftsz is an essential cell-division gene in *Escherichia coli*. *Journal of Bacteriology*, 1991, vol.173, no.11, pp.3500-3506.
- Dai K, Lutkenhaus J. The proper ratio of ftsz to ftsa is required for cell-division to occur in *Escherichia coli*. *Journal of bacteriology*, 1992, vol.174, no.19, pp.6145-6151
- Dai K, Xu Y, Lutkenhaus J. Cloning and characterization of ftsN, an essential cell division gene in *Escherichia coli* isolated as a multicopy suppressor of ftsA12(Ts). *J Bacteriol.* 1993 Jun; 175(12):3790-7.
- Dai K, Xu Y, Lutkenhaus J. Topological characterization of the essential *Escherichia coli* cell division protein FtsN. *J Bacteriol.* 1996 Mar; 178(5):1328-34.
- D'Ari R, Huisman O. Novel mechanism of cell division inhibition associated with the SOS response in *Escherichia coli* *J Bacteriol* 1983 Oct;156(1):243-50.
- Davies AA, West SC. Formation of RuvABC-Holliday junction complexes in vitro. *Curr Biol* 1998 Jun 4;8(12):725-7.
- de Boer P, Crossley R, Rothfield L. The essential bacterial cell-division protein FtsZ is a GTPase. *Nature* 1992 Sep 17;359(6392):254-6
- de Boer PA, Crossley RE, Hand AR, Rothfield LI. The MinD protein is a membrane ATPase required for the correct placement of the *Escherichia coli* division site. *EMBO J* 1991 Dec;10(13):4371-80.
- de Boer PA, Crossley RE, Rothfield LI. A division inhibitor and a topological specificity factor coded for by the minicell locus determine proper placement of the division septum in *E. coli*. *Cell* 1989 Feb 24;56(4):641-9.

- de Boer PA, Crossley RE, Rothfield LI. Isolation and properties of minB, a complex genetic locus involved in correct placement of the division site in *Escherichia coli*. *J Bacteriol* 1988 May;170(5):2106-12.
- de Boer PA, Crossley RE, Rothfield LI. Roles of MinC and MinD in the site-specific septation block mediated by the MinCDE system of *Escherichia coli*. *J Bacteriol* 1992 Jan;174(1):63-70.
- Dewar SJ, Donachie WD. Antisense transcription of the *ftsZ-ftsA* gene junction inhibits cell-division in *Escherichia coli*. *Journal of Bacteriology*, 1993, vol.175, no.21, pp.7097-7101
- Dewar SJ, Kagan-Zur V, Begg KJ, Donachie WD. Transcriptional regulation of cell division genes in *Escherichia coli* *Mol Microbiol* 1989 Oct;3(10):1371-7.
- Dianov G and Lindahl T. Reconstitution of the DNA base excision-repair pathway. *Curr Biol* 1994 Dec 1;4(12):1069-76.
- Diez AA, Farewell A, Nannmark U, Nyström T. A mutation in the *ftsK* gene of *Escherichia coli* affects cell separation, stationary-phase survival, stress adaptation, and expression of the gene encoding the stress protein UspA. *J. Bacteriol.* 1997; 179: 5878-83.
- DiRusso CC, Nystrom T. The fates of *Escherichia coli* during infancy and old age: regulation by global regulators, alarmones and lipid intermediates *Mol Microbiol* 1998 Jan;27(1):1-8 .
- Dixon NE, Kornberg A. Protein HU in the enzymatic replication of the chromosomal origin of *Escherichia coli* *Proc Natl Acad Sci U S A* 1984 Jan;81(2):424-8
- Donachie WD, Hobbs DG, Masters M. Chromosome replication and cell division in *Escherichia coli* 15T after growth in the absence of DNA synthesis. *Nature* 1968 Sep 7;219(158):1079-80.
- Donachie WD, Jones NC and Teather R. *Symp Soc Gen Microbiol*, 1973; 23: 9-44.
- Donachie WD, Martin DT, Begg KJ. Independence of cell division and DNA replication in *Bacillus subtilis*. *Nature New Biol.* 1971 Jun 30;231(26):274-6.

- Donachie WD. Relationship between cell size and time of initiation of DNA replication. *Nature* 1968, 219: 1077-79.
- Donachie WD. Control of cell division in *Escherichia coli*: experiments with thymine starvation. *J Bacteriol.* 1969 Oct; 100(1):260-8.
- Donachie WD. The cell cycle of *Escherichia coli*. *Annu Rev Microbiol.* 1993;47:199-230.
- Draper GC, McLennan N, Begg K, Masters M, Donachie WD. Only the N-terminal domain of FtsK functions in cell division. *J Bacteriol* 1998 Sep;180(17):4621-7.
- Dunderdale HJ, Benson FE, Parsons CA, Sharples GJ, Lloyd RG, West SC. Formation and resolution of recombination intermediates by *E. coli* RecA and RuvC proteins. *Nature* 1991 Dec 19-26;354(6354):506-10
- Dwek R, Orgad S, Rozenhak S, Ron EZ. 2 new cell-division mutants in *escherichia-coli* map near the terminus of chromosome-replication molecular & general genetics, 1984, vol.193, no.2, pp.379-381
- Eggleston AK, Mitchell AH, West SC. In vitro reconstitution of the late steps of genetic recombination in *E. coli*. *Cell* 1997 May 16;89(4):607-17.
- Eichel J, Chang YY, Riesenber D, Cronan JE Jr. Effect of ppGpp on *Escherichia coli* cyclopropane fatty acid synthesis is mediated through the RpoS sigma factor (sigmaS). *J Bacteriol* 1999 Jan;181(2):572-6
- Erickson HP, Taylor DW, Taylor KA, Bramhill D. Bacterial cell division protein FtsZ assembles into protofilament sheets and minirings, structural homologs of tubulin polymers. *Proc Natl Acad Sci U S A* 1996 Jan 9;93(1):519-23
- Ezaki B, Ogura T, Niki H, Hiraga S. Partitioning of a mini-F plasmid into anucleate cells of the mukB null mutant. *J Bacteriol* 1991 Oct;173(20):6643-6.
- Flardh K, Garrido T, Vicente M. Contribution of individual promoters in the *ddlB-ftsZ* region to the transcription of the essential cell-division gene *ftsZ* in *Escherichia coli* *Mol Microbiol* 1997 Jun;24(5):927-36

- Flardh K, Palacios P, Vicente M. Cell division genes *ftsQAZ* in *Escherichia coli* require distant cis-acting signals upstream of *ddlB* for full expression *Mol Microbiol* 1998 Oct; 30(2):305-15.
- Flardh K, Garrido T, Vicente M. Contribution of individual promoters in the *ddlB-ftsZ* region to the transcription of the essential cell-division gene *ftsZ* in *Escherichia coli* *Molecular Microbiology*, 1997, Vol.24, No.5, pp.927-936
- Flardh K, Palacios P, Vicente M. Cell division genes *ftsQAZ* in *Escherichia coli* require distant cis-acting signals upstream of *ddlB* for full expression *molecular microbiology*, 1998, Vol.30, No.2, pp.305-315
- Foster PL, Trimarchi JM, Maurer RA. Two enzymes, both of which process recombination intermediates, have opposite effects on adaptive mutation in *Escherichia coli*. *Genetics* 1996 Jan;142(1):25-37
- Freudl R, Braun G, Honore N, Cole ST. Evolution of the enterobacterial *sulA* gene: a component of the SOS system encoding an inhibitor of cell division *Gene* 1987;52(1):31-40.
- GarciaLara J, Shang LH, Rothfield LI. An extracellular factor regulates expression of *sdiA*, a transcriptional activator of cell division genes in *Escherichia coli* *JOURNAL OF BACTERIOLOGY*, 1996, Vol.178, No.10, pp.2742-2748
- Garriga X, Calero S, Barbe J. Nucleotide sequence analysis and comparison of the *lexA* genes from *Salmonella typhimurium*, *Erwinia carotovora*, *Pseudomonas aeruginosa* and *Pseudomonas putida* *Mol Gen Genet* 1992 Dec;236(1):125-34.
- Gellert M, O'Dea MH, Itoh T, Tomizawa J. Novobiocin and coumermycin inhibit DNA supercoiling catalyzed by DNA gyrase. *Proc Natl Acad Sci U S A* 1976 Dec;73(12):4474-8.
- Gentry DR, Hernandez VJ, Nguyen LH, Jensen DB, Cashel M. Synthesis of the stationary-phase sigma factor sigma s is positively regulated by ppGpp. *J Bacteriol* 1993 Dec;175(24):7982-9.
- George J, Castellazzi M, Buttin G. Prophage induction and cell division in *E. coli*. III. Mutations *sfiA* and *sfiB* restore division in *tif* and *lon* strains and permit the

- expression of mutator properties of *tif*. *Mol Gen Genet*. 1975 Oct 22;140(4):309-332.
- Ghigo JM, Beckwith J. Cell division in *Escherichia coli*: role of FtsL domains in septal localization, function, and oligomerization. *J Bacteriol* 2000 Jan;182(1):116-29.
- Ghigo JM, Weiss DS, Chen JC, Yarrow JC, Beckwith J. Localization of FtsL to the *Escherichia coli* septal ring. *Mol Microbiol* 1999 Jan;31(2):725-37.
- Glaser P, Sharpe ME, Raether B, Perego M, Ohlsen K, Errington J. Dynamic, mitotic-like behavior of a bacterial protein required for accurate chromosome partitioning. *Genes Dev* 1997 May 1;11(9):1160-8
- Gordon GS, Sitnikov D, Webb CD, Teleman A, Straight A, Losick R, Murray AW, Wright A. Chromosome and low copy plasmid segregation in *E. coli*: visual evidence for distinct mechanisms. *Cell* 1997 Sep 19;90(6):1113-21
- Grossman N, Rosner E, Ron EZ. Termination of dna-replication is required for cell-division in *Escherichia coli*. *J. bacteriol*, 1989, vol.171, no.1, pp.74-79
- Guzman LM, Barondess JJ, Beckwith J. FtsL, an essential cytoplasmic membrane protein involved in cell division in *Escherichia coli*. *J Bacteriol* 1992 Dec;174(23):7716-28.
- Guzman LM, Belin D, Carson MJ, Beckwith J. Tight regulation, modulation, and high-level expression by vectors containing the arabinose PBAD promoter. *J Bacteriol* 1995 Jul;177(14):4121-30.
- Guzman LM, Weiss DS, Beckwith J. Domain-swapping analysis of FtsI, FtsL, and FtsQ, bitopic membrane proteins essential for cell division in *Escherichia coli*. *J Bacteriol* 1997 Aug;179(16):5094-103.
- Hale CA, de Boer PA. Direct binding of FtsZ to ZipA, an essential component of the septal ring structure that mediates cell division in *E. coli*. *Cell* 1997 Jan 24;88(2):175-85
- Hara H, Yasuda S, Horiuchi K, Park JT. A promoter for the first nine genes of the *Escherichia coli mra* cluster of cell division and cell envelope biosynthesis genes, including *ftsI* and *ftsW*. *J Bacteriol* 1997 Sep; 179(18):5802-11.

- Harry EJ, Rodwell J, Wake RG. Co-ordinating DNA replication with cell division in bacteria: a link between the early stages of a round of replication and mid-cell Z ring assembly. *Mol Microbiol.* 1999 Jul;33(1):33-40.
- Hengge-Aronis R. Back to log phase: sigma S as a global regulator in the osmotic control of gene expression in *Escherichia coli*. *Mol Microbiol* 1996 Sep;21(5):887-93.
- Higashitani A, Higashitani N, Horiuchi K. A cell-division inhibitor *slsA* of *Escherichia coli* directly interacts with *ftsZ* through gtp hydrolysis *Biochemical And Biophysical Research Communications*, 1995, vol.209, no.1, pp.198-204
- Hiraga S. Chromosome and plasmid partition in *Escherichia coli*. *Annu Rev Biochem* 1992; 61:283-306.
- Hiraga S, Niki H, Ogura T, Ichinose C, Mori H, Ezaki B, Jaffé A. Chromosome partitioning in *Escherichia coli*: novel mutants producing anucleate cells. *J. Bacteriol.*1989; 171: 1496-505.
- Hoffman CS, Wright A. Fusions of secreted proteins to alkaline phosphatase: an approach for studying protein secretion. *Proc Natl Acad Sci U S A* 1985 Aug;82(15):5107-11
- Hu Z, Lutkenhaus J. Topological regulation of cell division in *Escherichia coli* involves rapid pole to pole oscillation of the division inhibitor MinC under the control of MinD and MinE. *Mol Microbiol* 1999 Oct;34(1):82-90.
- Hu Z, Mukherjee A, Pichoff S, Lutkenhaus J. The MinC component of the division site selection system in *Escherichia coli* interacts with FtsZ to prevent polymerization. *Proc Natl Acad Sci U S A* 1999 Dec 21;96(26):14819-24
- Huisman O, D'Ari R. An inducible DNA replication-cell division coupling mechanism in *E. coli*. *Nature*. 1981 Apr 30; 290(5809):797-9.
- Huisman O, D'Ari R. Effect of suppressors of SOS-mediated filamentation on *sfiA* operon expression in *Escherichia coli*. *J Bacteriol* 1983 Jan; 153(1):169-75.
- Huisman O, D'Ari R, Gottesman S. Cell-division control in *Escherichia coli*: specific induction of the SOS function SfiA protein is sufficient to block septation. *Proc Natl Acad Sci U S A.* 1984 Jul; 81(14):4490-4.

- Hussain K, Begg KJ, Salmond GPC, Donachie WD. *ParD*: a new gene coding for a protein required for chromosome partitioning and septum localization in *Escherichia coli*. *Molec. Microbiol.* 1987; 1: 73-81.
- Ikeda M, Sato T, Wachi M, Jung HK, Ishino F, Kobayashi Y, Matsuhashi M. Structural similarity among *Escherichia coli* FtsW and RodA proteins and *Bacillus subtilis* SpoVE protein, which function in cell division, cell elongation, and spore formation, respectively. *J Bacteriol* 1989 Nov;171(11):6375-8.
- Ireton K, Gunther NW 4th, Grossman AD. *spo0J* is required for normal chromosome segregation as well as the initiation of sporulation in *Bacillus subtilis*. *J Bacteriol* 1994 Sep;176(17):5320-9.
- Ishino F, Jung HK, Ikeda M, Doi M, Wachi M, Matsuhashi M. New mutations *fts-36*, *fts-33*, and *ftsW* clustered in the *mra* region of the *Escherichia coli* chromosome induce thermosensitive cell growth and division. *J Bacteriol* 1989 Oct;171(10):5523-30.
- Ishino F, Matsuhashi M. Peptidoglycan synthetic enzyme activities of highly purified penicillin-binding protein 3 in *Escherichia coli*: a septum-forming reaction sequence. *Biochem Biophys Res Commun* 1981 Aug 14;101(3):905-11.
- Ishioka K, Fukuoh A, Iwasaki H, Nakata A, Shinagawa H. Abortive recombination in *Escherichia coli* *ruv* mutants blocks chromosome partitioning. *Genes Cells* 1998 Apr;3(4):209-20.
- Itoh T, Tomizawa J. Formation of an RNA primer for initiation of replication of ColE1 DNA by ribonuclease H. *Proc Natl Acad Sci U S A* 1980 May;77(5):2450-4
- Iwasaki H, Shiba T, Makino K, Nakata A, Shinagawa H. Overproduction, purification, and ATPase activity of the *Escherichia coli* RuvB protein involved in DNA repair. *J Bacteriol* 1989 Oct;171(10):5276-80.
- Iwasaki H, Takahagi M, Nakata A, Shinagawa H. *Escherichia coli* RuvA and RuvB proteins specifically interact with Holliday junctions and promote branch migration. *Genes Dev* 1992 Nov;6(11):2214-20

- Iwasaki H, Takahagi M, Shiba T, Nakata A, Shinagawa H. Escherichia coli RuvC protein is an endonuclease that resolves the Holliday structure. *EMBO J* 1991 Dec;10(13):4381-9
- Jones NC, Donachie WD. Chromosome replication, transcription and control of cell division in *Escherichia coli* *Nat New Biol* 1973 May 23; 243(125):100-3.
- Joseleau-Petit D, Thevenet D, D'Ari R. ppGpp concentration, growth without PBP2 activity, and growth-rate control in *Escherichia coli*. *Mol Microbiol* 1994 Sep;13(5):911-7
- Kaiser D, Losick R. How and why bacteria talk to each other *Cell* 1993;73(5):873-85
- Kanemori M, Yanagi H, Yura T. The ATP-dependent HslVU ClpQY protease participates in turnover of cell division inhibitor SulA in *Escherichia coli* *Journal of Bacteriology*, 1999, Vol.181, No.12, pp.3674-3680
- Kaplan HB, Plamann L A. *Myxococcus xanthus* cell density-sensing system required for multicellular development *FEMS Microbiol Lett* 1996 Jun 1;139(2-3):89-95
- Katayama T, Takata M, Sekimizu K. CedaA is a novel *Escherichia coli* protein that activates the cell division inhibited by chromosomal DNA over-replication *Molecular Microbiology*, 1997, Vol.26, No.4, pp.687-697
- Kato J, Nishimura Y, Imamura R, Niki H, Hiraga S, Suzuki H. New topoisomerase essential for chromosome segregation in *E. coli* *Cell* 1990 Oct 19;63(2):393-404.
- Kato J, Nishimura Y, Yamada M, Suzuki H, Hirota Y. Gene organization in the region containing a new gene involved in chromosome partition in *Escherichia coli* *J Bacteriol* 1988 Sep;170(9):3967-77.
- Khattar MM, Begg KJ, Donachie WD. Identification of FtsW and characterization of a new ftsW division mutant of *Escherichia coli*. *J Bacteriol.* 1994 Dec; 176(23):7140-7.
- Khattar MM. Overexpression of the hslVU operon suppresses SOS-mediated inhibition of cell division in *Escherichia coli*. *FEBS LETTERS*, 1997, Vol.414, No.2, pp.402-404

- Khattar MM, Addinall SG, Stedul KH, Boyle DS, Lutkenhaus J, Donachie WD. Two polypeptide products of the *Escherichia coli* cell division gene *ftsW* and a possible role for FtsW in FtsZ function. *J. Bacteriol*, 1997, Vol.179, No.3, pp.784-793
- King GF, Rowland SL, Pan B, Mackay JP, Mullen GP, Rothfield LI. The dimerization and topological specificity functions of MinE reside in a structurally autonomous C-terminal domain. *Mol Microbiol* 1999 Feb;31(4):1161-9.
- Kjeldgaard NO, Maaløe O, Schaechter M. The transition between different physiological states during balanced growth of *Salmonella typhimurium*. *J. Gen. Microbiol.* 1958, 19: 607-16.
- Kogoma T, Barnard KG, Hong X. RecA, Tus protein and constitutive stable DNA replication in *Escherichia coli* *rnhA* mutants. *Mol Gen Genet* 1994 Sep 1;244(5):557-62.
- Kogoma T, Cadwell GW, Barnard KG, Asai T. The DNA replication priming protein, PriA, is required for homologous recombination and double-strand break repair. *J Bacteriol* 1996 Mar;178(5):1258-64
- Kogoma T, Hong X, Cadwell GW, Barnard KG, Asai T. Requirement of homologous recombination functions for viability of the *Escherichia coli* cell that lacks RNase HI and exonuclease V activities. *Biochimie* 1993;75(1-2):89-99.
- Kogoma T, Subia NL, von Meyenburg K. Function of ribonuclease H in initiation of DNA replication in *Escherichia coli* K-12. *Mol Gen Genet* 1985;200(1):103-9
- Kuempel P, Hogaard A, Nielsen M, Nagappan O, Tecklenburg M. Use of a transposon (Tndif) to obtain suppressing and nonsuppressing insertions of the dif resolvase site of *Escherichia coli* (vol 10, pg 1162, 1996) *Genes & Development*, 1996, Vol.10, No.13, p.1709
- Kuempel P, Hogaard A, Nielsen M, Nagappan O, Tecklenburg M. Use of a transposon (Tndif) to obtain suppressing and nonsuppressing insertions of the dif resolvase site of *Escherichia coli* *GENES & DEVELOPMENT*, 1996, Vol.10, No.9, pp.1162-1171

- Kuempel PL, Henson JM, Dircks L, Tecklenburg M, Lim DF. Dif, A RecA-Independent Recombination Site In The Terminus Region Of The Chromosome Of Escherichia-Coli. *New Biologist*, 1991, Vol.3, No.8, Pp.799-811
- Lee EH, Kornberg A. Replication deficiencies in priA mutants of Escherichia coli lacking the primosomal replication n' protein. *Proc Natl Acad Sci U S A* 1991 Apr 15;88(8):3029-32
- Lee EH, Masai H, Allen GC Jr, Kornberg A. The priA gene encoding the primosomal replicative n' protein of Escherichia coli. *Proc Natl Acad Sci U S A* 1990 Jun;87(12):4620-4
- Lewis PJ, Errington J. Direct evidence for active segregation of oriC regions of the Bacillus subtilis chromosome and co-localization with the SpoOJ partitioning protein. *Mol Microbiol* 1997 Sep;25(5):945-54
- Lin DC, Grossman AD. Identification and characterization of a bacterial chromosome partitioning site. *Cell* 1998 Mar 6;92(5):675-85
- Lin DCH, Levin PA, Grossman AD. Bipolar localization of a chromosome partition protein in Bacillus subtilis. *Proc Natl Acad Sci U S A* 1997 Apr 29;94(9):4721-6.
- Liu J, Marians KJ. PriA-directed assembly of a primosome on D loop DNA. *J Biol Chem* 1999 Aug 27;274(35):25033-41
- Liu J, Xu L, Sandler SJ, Marians KJ. Replication fork assembly at recombination intermediates is required for bacterial growth. *Proc Natl Acad Sci U S A* 1999 Mar 30;96(7):3552-5.
- Liu Z, Mukherjee A, Lutkenhaus J. Recruitment of ZipA to the division site by interaction with FtsZ. *Mol Microbiol* 1999 Mar;31(6):1853-61
- Lloyd RG. Conjugal recombination in resolvase-deficient ruvC mutants of Escherichia coli K-12 depends on recG. *J Bacteriol* 1991 Sep;173(17):5414-8
- Lloyd RG, Benson FE, Shurvinton CE. Effect of ruv mutations on recombination and DNA repair in Escherichia coli K12. *Mol Gen Genet* 1984;194(1-2):303-9.

- Lloyd RG, Buckman C, Benson FE. Genetic analysis of conjugational recombination in *Escherichia coli* K12 strains deficient in RecBCD enzyme. *J Gen Microbiol* 1987 Sep;133 (Pt 9):2531-8
- Lloyd RG, Evans NP, Buckman C. Formation of recombinant lacZ+ DNA in conjugational crosses with a recB mutant of *Escherichia coli* K12 depends on recF, recJ, and recO. *Mol Gen Genet* 1987 Aug;209(1):135-41.
- Lloyd RG, Porton MC, Buckman C. Effect of recF, recJ, recN, recO and ruv mutations on ultraviolet survival and genetic recombination in a recD strain of *Escherichia coli* K12. *Mol Gen Genet* 1988 May;212(2):317-24.
- Lloyd RG, Sharples GJ. Dissociation of synthetic Holliday junctions by *E. coli* RecG protein. *EMBO J* 1993 Jan;12(1):17-22
- Lobocka M, Hennig J, Wild J, Klopotoski T. Organization and expression of the *Escherichia coli* K-12 dad operon encoding the smaller subunit of D-amino acid dehydrogenase and the catabolic alanine racemase *J Bacteriol* 1994 Mar;176(5):1500-10 .
- Loewen PC, Hu B, Strutinsky J, Sparling R. Regulation in the rpoS regulon of *Escherichia coli*. *Can J Microbiol* 1998 Aug;44(8):707-17.
- Lovett ST, Clark AJ. Genetic analysis of the recJ gene of *Escherichia coli* K-12. *J Bacteriol* 1984 Jan;157(1):190-6
- Lovett ST, Kolodner RD. Identification and purification of a single-stranded-DNA-specific exonuclease encoded by the recJ gene of *Escherichia coli*. *Proc Natl Acad Sci U S A* 1989 Apr;86(8):2627-31.
- Lutkenhaus JF, Wolf-Watz H, Donachie WD. Organization of genes in the ftsA-envA region of the *Escherichia coli* genetic map and identification of a new fts locus (ftsZ) *J Bacteriol* 1980 May;142(2):615-20 .
- Lutkenhaus JF. Coupling of DNA replication and cell division: *sulB* is an allele of *ftsZ*. *J Bacteriol.* 1983 Jun; 154(3):1339-46.
- Ma X, Ehrhardt DW, Margolin W. Colocalization of cell division proteins FtsZ and FtsA to cytoskeletal structures in living *Escherichia coli* cells by using green

- fluorescent protein. *Proc Natl Acad Sci U S A* 1996 Nov 12;93(23):12998-3003
- Magee TR, Asai T, Malka D, Kogoma T. DNA damage-inducible origins of DNA replication in *Escherichia coli*. *EMBO J* 1992 Nov;11(11):4219-25
- Maguin E, Lutkenhaus J, D'Ari R. Reversibility of SOS-associated division inhibition in *Escherichia coli* *J Bacteriol* 1986 Jun;166(3):733-8.
- Mahdi AA, Sharples GJ, Mandal TN, Lloyd RG. Holliday junction resolvases encoded by homologous *rusA* genes in *Escherichia coli* K-12 and phage 82. *J Mol Biol* 1996 Apr 5;257(3):561-73
- Makise M, Sakamoto K, Tsuchiya T, Mizushima T. Identification of a high-copy-number plasmid suppressor of a lethal phenotype caused by mutant DnaA protein which has decreased intrinsic ATPase activity. *Biol Pharm Bull* 1999 Sep;22(9):904-9.
- Mandal TN, Mahdi AA, Sharples GJ, Lloyd RG. Resolution of Holliday intermediates in recombination and DNA repair: indirect suppression of *ruvA*, *ruvB*, and *ruvC* mutations. *J Bacteriol* 1993 Jul;175(14):4325-34.
- Marians KJ. PriA: at the crossroads of DNA replication and recombination. *Prog Nucleic Acid Res Mol Biol* 1999;63:39-67.
- Marston AL, Errington J. Dynamic movement of the ParA-like Soj protein of *B. subtilis* and its dual role in nucleoid organization and developmental regulation. *Mol Cell* 1999 Nov;4(5):673-82
- Masai H, Arai K. Mechanisms of primer RNA synthesis and D-loop/R-loop-dependent DNA replication in *Escherichia coli*. *Biochimie* 1996;78(11-12):1109-17
- Masai H, Asai T, Kubota Y, Arai K, Kogoma T. *Escherichia coli* PriA protein is essential for inducible and constitutive stable DNA replication. *EMBO J* 1994 Nov 15;13(22):5338-45
- Masai H, Deneke J, Furui Y, Tanaka T, Arai KI. *Escherichia coli* and *Bacillus subtilis* PriA proteins essential for recombination-dependent DNA replication: involvement of ATPase/helicase activity of PriA for inducible stable DNA replication. *Biochimie* 1999 Aug-Sep;81(8-9):847-57

- Mathew E, Zhi J, Freundlich M. Lrp is a direct repressor of the dad operon in *Escherichia coli* J Bacteriol 1996 Dec;178(24):7234-40 .
- Maxwell A. The interaction between coumarin drugs and DNA gyrase. Mol Microbiol 1993 Aug;9(4):681-6.
- McGinness T, Wake RG. A fixed amount of chromosome replication needed for premature division septation in *Bacillus subtilis*. J Mol Biol. 1981 Feb 15;146(1):173-7.
- McGinness T, Wake RG. Division septation in the absence of chromosome termination in *Bacillus subtilis*. J Mol Biol. 1979 Oct 25; 134(2):251-64.
- Meacock PA, Pritchard RH. Relationship between chromosome replication and cell division in a thymineless mutant of *Escherichia coli* B/r. J Bacteriol. 1975 Jun; 122(3):931-42.
- Melby TE, Ciampaglio CN, Briscoe G, Erickson HP. The symmetrical structure of structural maintenance of chromosomes (SMC) and MukB proteins: long, antiparallel coiled coils, folded at a flexible hinge. J Cell Biol 1998 Sep 21;142(6):1595-604.
- MenginLecreulx D, Ayala J, Bouhss A, VanHeijenoort J, Parquet C, Hara H. Contribution of the P-mra promoter to expression of genes in the *Escherichia coli* mra cluster of cell envelope biosynthesis and cell division genes Journal of Bacteriology, 1998, Vol.180, No.17, pp.4406-4412
- Milija J, Lilic M, Janjusevic R, Jovanovic G, Savic DJ. tRNA synthetase mutants of *Escherichia coli* K-12 are resistant to the gyrase inhibitor novobiocin. J Bacteriol 1999 May;181(9):2979-83
- Miller JF. 1972. *Experiments in Molecular Genetics*. Cold Spring Harbour, NY: Cold Spring Harbour Laboratory Press.
- Mitchell AH, West SC. Hexameric rings of *Escherichia coli* RuvB protein. Cooperative assembly, processivity and ATPase activity. J Mol Biol 1994 Oct 21;243(2):208-15
- Mizusawa S, Court D, Gottesman S. Transcription of the *sulA* gene and repression by LexA. J Mol Biol. 1983 Dec 15; 171(3):337-43.

- Mukherjee A, Donachie WD. Differential translation of cell division proteins J Bacteriol 1990 Oct;172(10):6106-11.
- Mukherjee A, Lutkenhaus J. Analysis of FtsZ assembly by light scattering and determination of the role of divalent metal cations. J Bacteriol 1999 Feb;181(3):823-32
- Mukherjee A, Lutkenhaus J. Dynamic assembly of FtsZ regulated by GTP hydrolysis. EMBO J 1998 Jan 15;17(2):462-9
- Mukherjee A, Lutkenhaus J. Guanine nucleotide-dependent assembly of FtsZ into filaments. J Bacteriol 1994 May;176(9):2754-8
- Mukherjee A, Dai K, Lutkenhaus J. Escherichia-coli cell-division protein ftsZ is a guanine-nucleotide binding-protein *proc natl acad sci u s a* 1993, vol.90, no.3, pp.1053-1057
- Muller B, Tsaneva IR, West SC. Branch migration of Holliday junctions promoted by the Escherichia coli RuvA and RuvB proteins. I. Comparison of RuvAB- and RuvB-mediated reactions. J Biol Chem 1993a Aug 15;268(23):17179-84.
- Muller B, Tsaneva IR, West SC. Branch migration of Holliday junctions promoted by the Escherichia coli RuvA and RuvB proteins. II. Interaction of RuvB with DNA. J Biol Chem 1993b Aug 15;268(23):17185-9
- Nanninga N. cell-division and peptidoglycan assembly in escherichia-coli *Molecular Microbiology*, 1991, vol.5, no.4, pp.791-795
- Neidhardt FC. *et al.* (ed.), 1996, Escherichia coli and Salmonella: Cellular and Molecular Biology. American Society for Microbiology, Washington, DC.
- Neu HC. Penicillin-binding proteins and role of amdinocillin in causing bacterial cell death. *Am J Med* 1983 Aug 29;75(2A):9-20.
- Niki H, Imamura R, Kitaoka M, Yamanaka K, Ogura T, Hiraga S. E.coli MukB protein involved in chromosome partition forms a homodimer with a rod-and-hinge structure having DNA binding and ATP/GTP binding activities. EMBO J 1992 Dec;11(13):5101-9
- Niki H, Jaffe A, Imamura R, Ogura T, Hiraga S. The new gene mukB codes for a 177 kd protein with coiled-coil domains involved in chromosome

- Niki H, Jaffe A, Imamura R, Ogura T, Hiraga S. The new gene mukB codes for a 177 kd protein with coiled-coil domains involved in chromosome partitioning of *E. coli* EMBO J 1991 Jan;10(1):183-93.
- Nishimura A, Moriya S, Ukai H, Nagai K, Wachi M, Yamada Y. Diadenosine 5',5'''-P1,P4-tetraphosphate (Ap4A) controls the timing of cell division in *Escherichia coli* Genes to Cells, 1997, Vol.2, No.6, pp.401-413
- Nurse P, Zavitz KH and Marians KJ. Inactivation of the *Escherichia coli* priA DNA replication protein induces the SOS response. J Bacteriol 1991 Nov;173(21):6686-93
- Ogawa T, Okazaki T. Function of RNase H in DNA replication revealed by RNase H defective mutants of *Escherichia coli*. Mol Gen Genet 1984;193(2):231-7.
- Ogura T, Niki H, Kano Y, Imamoto F, Hiraga S. Maintenance of plasmids in HU and IHF mutants of *Escherichia coli*. Mol Gen Genet 1990 Jan;220(2):197-203
- Otsuji N, Iyehara H, Hideshima Y. Isolation and characterization of an *Escherichia coli* ruv mutant which forms nonseptate filaments after low doses of ultraviolet light irradiation. J Bacteriol 1974 Feb;117(2):337-44
- Pardee AB, Wu PC and Zusman DR. 1973. In *Bacterial membranes and walls* (Lieve L, ed.) pp357-412 Marcel Dekker Inc. New York.
- Parsons CA, Stasiak A, Bennett RJ, West SC. Structure of a multisubunit complex that promotes DNA branch migration. Nature 1995; Mar 23;374(6520):375-8.
- Parsons CA, Tsaneva I, Lloyd RG, West SC. Interaction of *Escherichia coli* RuvA and RuvB proteins with synthetic Holliday junctions. Proc Natl Acad Sci U S A 1992 Jun 15;89(12):5452-6.
- Perego M, Hoch JA. Cell-cell communication regulates the effects of protein aspartate phosphatases on the phosphorelay controlling development in *Bacillus subtilis* Proc Natl Acad Sci U S A 1996; 93(4):1549-53
- Pichoff S, Alibaud L, Guedant A, Castanie MP, Bouche JP. An *Escherichia coli* gene (yaeO) suppresses temperature-sensitive

- Pichoff S, Vollrath B, Touriol C, Bouche JP. deletion analysis of gene mine which encodes the topological specificity factor of cell-division in escherichia-coli molecular microbiology, 1995, vol.18, no.2, pp.321-329
- Pla J, Sanchez M, Palacios P, Vicente M, Aldea M. Preferential cytoplasmic location of FtsZ, a protein essential for Escherichia coli septation. Mol Microbiol 1991 Jul;5(7):1681-6
- Powell BS, Court DL. Control of ftsZ expression, cell division, and glutamine metabolism in Luria-Bertani medium by the alarmone ppGpp in Escherichia coli Journal of Bacteriology, 1998, Vol.180, No.5, pp.1053-1062
- Pritchard RH, Zaritsky A. Effect of thymine concentration on the replication velocity of DNA in a thymineless mutant of *Escherichia coli*. *Nature* 1970 Apr 11; 226(241):126-31.
- Pruss BM, Markovic D, Matsumura P. The Escherichia coli flagellar transcriptional activator flhD regulates cell division through induction of the acid response gene *cadA* JOURNAL OF BACTERIOLOGY, 1997, Vol.179, No.11, pp.3818-3821
- Pyne C, Bognar AL. Replacement of the *folC* gene, encoding folylpolyglutamate synthetase-dihydrofolate synthetase in Escherichia coli, with genes mutagenized in vitro. J Bacteriol 1992 Mar;174(6):1750-9.
- Rafferty JB, Sedelnikova SE, Hargreaves D, Artymiuk PJ, Baker PJ, Sharples GJ, Mahdi AA, Lloyd RG, Rice DW. Crystal structure of DNA recombination protein RuvA and a model for its binding to the Holliday junction. *Science* 1996 Oct 18;274(5286):415-21.
- Raina S, Missiakas D. Making and breaking disulfide bonds *Annu Rev Microbiol* 1997;51:179-202.
- Raskin DM, de Boer PA. Rapid pole-to-pole oscillation of a protein required for directing division to the middle of Escherichia coli. *Proc Natl Acad Sci U S A* 1999 Apr 27;96(9):4971-6.

- Raskin DM, de Boer PA. The MinE ring: an FtsZ-independent cell structure required for selection of the correct division site in *E. coli*. *Cell* 1997 Nov 28;91(5):685-94.
- Raskin DM, de Boer PA. MinDE-dependent pole-to-pole oscillation of division inhibitor MinC in *Escherichia coli*. *J Bacteriol* 1999 Oct;181(20):6419-24.
- RayChaudhuri D. ZipA is a MAP-Tau homolog and is essential for structural integrity of the cytokinetic FtsZ ring during bacterial cell division. *EMBO J* 1999 May 4;18(9):2372-83
- RayChaudhuri D, Park JT. *Escherichia coli* cell-division gene *ftsZ* encodes a novel GTP-binding protein. *Nature* 1992 Sep 17;359(6392):251-4.
- Robinson AC, Kenan DJ, Hatfull GF, Sullivan NF, Spiegelberg R, Donachie WD. DNA sequence and transcriptional organization of essential cell division genes *ftsQ* and *ftsA* of *Escherichia coli*: evidence for overlapping transcriptional units *J Bacteriol* 1984 Nov;160(2):546-55.
- Robinson AC, Kenan DJ, Sweeney J, Donachie WD. Further evidence for overlapping transcriptional units in an *Escherichia coli* cell envelope-cell division gene cluster: DNA sequence and transcriptional organization of the *ddl ftsQ* region. *J Bacteriol* 1986 Sep;167(3):809-17.
- Rouviere-Yaniv J, Kjeldgaard NO. Native *Escherichia coli* HU protein is a heterotypic dimer *FEBS Lett* 1979 Oct 15;106(2):297-300
- Rowland SL, Fu X, Sayed MA, Zhang Y, Cook WR, Rothfield LI. Membrane redistribution of the *Escherichia coli* MinD protein induced by MinE. *J Bacteriol* 2000 Feb;182(3):613-9.
- Saleh AZ, Yamanaka K, Niki H, Ogura T, Yamazoe M, Hiraga S. Carboxyl terminal region of the MukB protein in *Escherichia coli* is essential for DNA binding activity. *FEMS Microbiol Lett* 1996 Oct 1;143(2-3):211-6
- Sambrook, J., E.F. Fritsch, and T, Manjatis. 1989. *Molecular cloning: a laboratory manual*, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

- Sanchez M, Valencia A, Ferrandiz MJ, Sander C, Vicente M. Correlation between the structure and biochemical activities of FtsA, an essential cell division protein of the actin family. *EMBO J* 1994 Oct 17;13(20):4919-25.
- Sargentini NJ and Smith KC. Quantitation of the involvement of the *recA*, *recB*, *recC*, *recF*, *recJ*, *recN*, *lexA*, *radA*, *radB*, *uvrD*, and *umuC* genes in the repair of X-ray-induced DNA double-strand breaks in *Escherichia coli*. *Radiat Res* 1986 Jul;107(1):58-72.
- Schreiber G, Metzger S, Aizenman E, Roza S, Cashel M, Glaser G. Overexpression of the *relA* gene in *Escherichia coli*. *J Biol Chem* 1991 Feb 25;266(6):3760-7.
- Schreiber G, Ron EZ, Glaser G. ppGpp-mediated regulation of dna-replication and cell-division in *Escherichia coli* *Current Microbiology*, 1995, vol.30, no.1, pp.27-32.
- Seigneur M, Bidnenko V, Ehrlich SD, Michel B. RuvAB acts at arrested replication forks. *Cell* 1998 Oct 30;95(3):419-30
- Seong IS, Oh JY, Yoo SJ, Seol JH, Chung CH. ATP-dependent degradation of *SulA*, a cell division inhibitor, by the
- Shah R, Cosstick R, West SC. The RuvC protein dimer resolves Holliday junctions by a dual incision mechanism that involves base-specific contacts. *EMBO J* 1997 Mar 17;16(6):1464-72
- Sharpe ME, Errington J A. fixed distance for separation of newly replicated copies of *oriC* in *Bacillus subtilis*: implications for co-ordination of chromosome segregation and cell division. *Mol Microbiol* 1998 Jun;28(5):981-90.
- Sharples GJ, Benson FE, Illing GT, Lloyd RG. Molecular and functional analysis of the *ruv* region of *Escherichia coli* K-12 reveals three genes involved in DNA repair and recombination. *Mol Gen Genet* 1990 Apr;221(2):219-26
- Sharples GJ, Chan SN, Mahdi AA, Whitby MC, Lloyd RG. Processing of intermediates in recombination and DNA repair: identification of a new endonuclease that specifically cleaves Holliday junctions. *EMBO J* 1994 Dec 15;13(24):6133-42

- Sharples GJ, Lloyd RG. Resolution of Holliday junctions in *Escherichia coli*: identification of the *ruvC* gene product as a 19-kilodalton protein. *J Bacteriol* 1991 Dec;173(23):7711-5
- Shiba T, Iwasaki H, Nakata A, Shinagawa H. SOS-inducible DNA repair proteins, RuvA and RuvB, of *Escherichia coli*: functional interactions between RuvA and RuvB for ATP hydrolysis and renaturation of the cruciform structure in supercoiled DNA. *Proc Natl Acad Sci U S A* 1991 Oct 1;88(19):8445-9.
- Shinagawa H, Iwasaki H. Processing the holliday junction in homologous recombination. *Trends Biochem Sci* 1996 Mar;21(3):107-11
- Shinagawa H, Makino K, Amemura M, Kimura S, Iwasaki H, Nakata A. Structure and regulation of the *Escherichia coli* *ruv* operon involved in DNA repair and recombination. *J Bacteriol* 1988 Sep;170(9):4322-9
- Shlomai J and Kornberg A. An *Escherichia coli* replication protein that recognizes a unique sequence within a hairpin region in phi X174 DNA. *Proc Natl Acad Sci U S A* 1980 Feb;77(2):799-803.
- Shurvinton CE, Lloyd RG. Damage to DNA induces expression of the *ruv* gene of *Escherichia coli*. *Mol Gen Genet* 1982;185(2):352-5
- Silhavy TJ, Beckwith JR. Uses of lac fusions for the study of biological problems. *Microbiol Rev* 1985 Dec;49(4):398-418.
- Silhavy, TJ., Berman, M.L. and Enquist, L.W. 1984. Experiments with Gene Fusions. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
- Simons RW, Houman F and Kleckner N. Improved single and multicopy *lac*-based cloning vectors for protein and operon fusions. *Gene* 1987; 53:85-96.
- Sitnikov DM, Schineller JB, Baldwin TO. Control of cell division in *Escherichia coli*: regulation of transcription of *ftsQA* involves both *rpoS* and *SdiA*-mediated autoinduction *Proc Natl Acad Sci U S A* 1996 Jan 9; 93(1):336-41.
- Skarstad K, Boye E. Degradation of individual chromosomes in *recA* mutants of *Escherichia coli* *J Bacteriol* 1993 Sep; 175(17):5505-9.
- Smith RW, Masters M, Donachie WD. Cell division and transcription of *ftsZ* *J Bacteriol* 1993 May; 175(9):2788-91.

- Smith RW, McAteer S, Masters M. The coupling between *ftsZ* transcription and initiation of DNA replication is not mediated by the DnaA-boxes upstream of *ftsZ* or by DnaA *Mol Microbiol* 1996 Jul; 21(2):361-72.
- Spratt BG, Boyd A, Stoker N. Defective and plaque-forming lambda transducing bacteriophage carrying penicillin-binding protein-cell shape genes: genetic and physical mapping and identification of gene products from the lip-dacA-rodA-pbpA-leuS region of the Escherichia coli chromosome *J Bacteriol* 1980 Aug;143(2):569-81.
- Stacey KA, Lloyd RG. Isolation of rec- mutants from an F-prime merodiploid strain of Escherichia coli K-12. *Mol Gen Genet* 1976 Jan 16;143(2):223-32
- Steiner W, Liu G, Donachie WD, Kuempel P. The cytoplasmic domain of FtsK protein is required for resolution of chromosome dimers. *Mol Microbiol* 1999 Jan;31(2):579-83.
- Steiner WW, Kuempel PL. Cell division is required for resolution of dimer chromosomes at the dif locus of Escherichia coli *Molecular Microbiology*, 1998, Vol.27, No.2, pp.257-268.
- Steiner WW, Kuempel PL. Sister chromatid exchange frequencies in Escherichia coli analyzed by recombination at the dif resolvase site. *J Bacteriol*, 1998, Vol.180, No.23, pp.6269-6275
- Stirling CJ, Colloms SD, Collins JF, Szatmari G, Sherratt DJ. XerB, an Escherichia coli gene required for plasmid ColE1 site-specific recombination, is identical to pepA, encoding aminopeptidase-a, a protein with substantial similarity to bovine lens leucine aminopeptidase. *EMBO J*, 1989, vol.8, no.5, pp.1623-1627.
- Stirling CJ, Szatmari G, Stewart G, Smith MCM, Sherratt DJ. The arginine repressor is essential for plasmid-stabilizing site-specific recombination at the cole1 cer locus. *EMBO J*, 1988; vol.7, no.13, pp.4389-4395
- Storts DR, Aparicio OM, Schoemaker JM, Markovitz A. Overproduction and identification of the ftsQ gene product, an essential cell division protein in Escherichia coli K-12 *J Bacteriol* 1989 Aug;171(8):4290-7.

- Summers DK. Derivatives of *cole1* *cer* show altered topological specificity in site-specific recombination *EMBO J*, 1989; vol.8, no.1, pp.309-315.
- Swift S, Winson MK, Chan PF, Bainton NJ, Birdsall M, Reeves PJ, Rees CED, Chhabra SR, Hill PJ, Throup JP, Bycroft BW, Salmond GPC, Williams P, Stewart GSAB. A novel strategy for the isolation of *luxI* homologs - evidence for the widespread distribution of a *luxR luxI* superfamily in enteric bacteria. *Molecular Microbiology*, 1993, vol.10, no.3, pp.511-520
- Takahagi M, Iwasaki H, Nakata A, Shinagawa H. Molecular analysis of the *Escherichia coli* *ruvC* gene, which encodes a Holliday junction-specific endonuclease. *J Bacteriol* 1991 Sep;173(18):5747-53
- Tecklenburg M, Naumer M, Nagappan O, Kuempel P. The *Dif* Resolvase Locus Of The *Escherichia-Coli* Chromosome Can Be Replaced By A 33-Bp Sequence, But Function Depends On Location *Proc Natl Acad Sci U S A* 1995, Vol.92, No.5, Pp.1352-135
- Trun NJ, Gottesman S. Characterization of *Escherichia coli* mutants with altered ploidy *Res Microbiol* 1991 Feb-Apr;142(2-3):195-200.
- Trun NJ, Gottesman S. On the bacterial cell cycle: *Escherichia coli* mutants with altered ploidy *Genes Dev* 1990 Dec;4(12A):2036-47.
- Trusca D, Scott S, Thompson C, Bramhill D. Bacterial SOS checkpoint protein *SulA* inhibits polymerization of purified *FtsZ* cell division protein. *J Bacteriol.* 1998 Aug; 180(15):3946-53.
- Tsaneva IR, Illing G, Lloyd RG, West SC. Purification and properties of the *RuvA* and *RuvB* proteins of *Escherichia coli*. *Mol Gen Genet* 1992 Oct;235(1):1-10
- Tsaneva IR, Muller B, West SC. ATP-dependent branch migration of Holliday junctions promoted by the *RuvA* and *RuvB* proteins of *E. coli*. *Cell* 1992b Jun 26;69(7):1171-80
- Tsaneva IR, Muller B, West SC. *RuvA* and *RuvB* proteins of *Escherichia coli* exhibit DNA helicase activity in vitro. *Proc Natl Acad Sci U S A* 1993 Feb 15;90(4):1315-9

- Ukita T and Ikeda H. Role of the recJ gene product in UV-induced illegitimate recombination at the hotspot. *J Bacteriol* 1996 Apr;178(8):2362-7.
- Ulitzur S, Dunlap PV. Regulatory circuitry controlling luminescence autoinduction in *vibrio fischeri* photochemistry and photobiology, 1995, vol.62, no.4, pp.625-632
- van Gool AJ, Hajibagheri NM, Stasiak A, West SC. Assembly of the Escherichia coli RuvABC resolvase directs the orientation of holliday junction resolution. *Genes Dev* 1999 Jul 15;13(14):1861-70.
- van Gool AJ, Shah R, Mezard C, West SC. Functional interactions between the holliday junction resolvase and the branch migration motor of Escherichia coli. *EMBO J* 1998 Mar 16;17(6):1838-45.
- Vicente M, Gomez MJ, Ayala JA. Regulation of transcription of cell division genes in the *Escherichia coli* *dcw* cluster *Cell Mol Life Sci* 1998 Apr; 54(4):317-24.
- Vicente M, Kushner SR, Garrido T, Aldea M. The role of the 'gearbox' in the transcription of essential genes. *Mol Microbiol* 1991 Sep;5(9):2085-91
- Vinella D, D'Ari R, Jaff A. Penicillin binding protein 2 is dispensable in Escherichia coli when ppGpp synthesis is induced. *EMBO J* 1992 Apr; 11(4):1493-501. Published erratum appears in *EMBO J* 1992 Dec; 11(13):5137
- Vinella D, Joseleaupeitit D, Thevenet D, Bouloc P, Dari R. Penicillin-binding protein-2 inactivation in escherichia-coli results in cell-division inhibition, which is relieved by *ftsZ* overexpression *journal of bacteriology*, 1993, vol.175, no.20, pp.6704-6710
- Viswanathan M and Lovett ST. Single-strand DNA-specific exonucleases in Escherichia coli. Roles in repair and mutation avoidance. *Genetics* 1998 May;149(1):7-16.
- von Meyenburg K, Boye E, Skarstad K, Koppes L, Kogoma T. Mode of initiation of constitutive stable DNA replication in RNase H-defective mutants of Escherichia coli K-12. *J Bacteriol* 1987 Jun;169(6):2650-8

- Wada M, Kano Y, Ogawa T, Okazaki T and Imamoto F. Construction and characterization of the deletion mutant of *hupA* and *hupB* genes in *Escherichia coli* *J Mol Biol* 1988 Dec 5;204(3):581-91
- Wang L, Khattar MK, Donachie WD, Lutkenhaus J. FtsI and FtsW are localized to the septum in *Escherichia coli*. *J Bacteriol.* 1998 Jun; 180(11):2810-6.
- Wang L, Lutkenhaus J. FtsK is an essential cell division protein that is localized to the septum and induced as part of the SOS response. *Mol Microbiol* 1998 Aug;29(3):731-40.
- Wang XD, De Boer PAJ, Rothfield LI. A factor that positively regulates cell-division by activating transcription of the major cluster of essential cell-division genes of *Escherichia coli* *EMBO Journal*, 1991, Vol.10, No.11, pp.3363-3372.
- Webb CD, Graumann PL, Kahana JA, Teleman AA, Silver PA, Losick R. Use of time-lapse microscopy to visualize rapid movement of the replication origin region of the chromosome during the cell cycle in *Bacillus subtilis*. *Mol Microbiol* 1998 Jun;28(5):883-92.
- Webb CD, Teleman A, Gordon S, Straight A, Belmont A, Lin DC, Grossman AD, Wright A, Losick R. Bipolar localization of the replication origin regions of chromosomes in vegetative and sporulating cells of *B. subtilis*. *Cell* 1997 Mar 7;88(5):667-74.
- Weiss DS, Chen JC, Ghigo JM, Boyd D, Beckwith J. Localization of FtsI (PBP3) to the septal ring requires its membrane anchor, the Z ring, FtsA, FtsQ, and FtsL. *J Bacteriol* 1999 Jan;181(2):508-20.
- Weiss DS, Pogliano K, Carson M, Guzman LM, Fraipont C, Nguyen-Disteche M, Losick R, Beckwith J. Localization of the *Escherichia coli* cell division protein FtsI (PBP3) to the division site and cell pole. *Mol Microbiol* 1997 Aug;25(4):671-81.
- Weitao T, Nordstrom K, Dasgupta S. Mutual suppression of *mukB* and *seqA* phenotypes might arise from their opposing influences on the *Escherichia coli* nucleoid structure. *Mol Microbiol* 1999 Oct;34(1):157-68.

- Whitby MC, Lloyd RG. Branch migration of three-strand recombination intermediates by RecG, a possible pathway for securing exchanges initiated by 3'-tailed duplex DNA. *EMBO J* 1995 Jul 17;14(14):3302-10
- Whitby MC, Lloyd RG. Targeting Holliday junctions by the RecG branch migration protein of *Escherichia coli*. *J Biol Chem* 1998 Jul 31; 273(31):19729-39
- Whitby MC, Ryder L, Lloyd RG. Reverse branch migration of Holliday junctions by RecG protein: a new mechanism for resolution of intermediates in recombination and DNA repair. *Cell* 1993 Oct 22;75(2):341-50
- Whitby MC, Vincent SD, Lloyd RG. Branch migration of Holliday junctions: identification of RecG protein as a junction specific DNA helicase. *EMBO J* 1994 Nov 1;13(21):5220-8
- Wild J, Hennig J, Lobočka M, Walczak W, Kłopotowski T. Identification of the *dadX* gene coding for the predominant isozyme of alanine racemase in *Escherichia coli* K12 *Mol Gen Genet* 1985;198(2):315-22 .
- Wild J, Kłopotowski T. D-Amino acid dehydrogenase of *Escherichia coli* K12: positive selection of mutants defective in enzyme activity and localization of the structural gene *Mol Gen Genet* 1981;181(3):373-8.
- Wild J, Obrepalska B. Regulation of expression of the *dadA* gene encoding D-amino acid dehydrogenase in *Escherichia coli*: analysis of *dadA-lac* fusions and direction of *dadA* transcription *Mol Gen Genet* 1982;186(3):405-10 .
- Withers HL, Nordstrom K. Quorum-sensing acts at initiation of chromosomal replication in *Escherichia coli* *Proc Natl Acad Sci U S A* 1998, Vol.95, No.26, pp.15694-15699
- Wu LJ, Errington J. *Bacillus subtilis* SpoIIIE protein required for DNA segregation during asymmetric cell division. *Science* 1994; 264: 572-75.
- Wu LJ, Franks AH, Wake RG. Replication through the terminus region of the *Bacillus subtilis* chromosome is not essential for the formation of a division septum that partitions the DNA. *J Bacteriol.* 1995 Oct; 177(19):5711-5.

- Wu LJ., P.J. Lewis, R. Allmansberger, P.M. Hauser, and J. Errington. A conjugation-like mechanism for prespore chromosome partitioning during sporulation in *Bacillus subtilis*. *Gene Dev.* 1995; 9: 1316-1326.
- Yamanaka K, Ogura T, Niki H, Hiraga S. Identification of two new genes, mukE and mukF, involved in chromosome partitioning in *Escherichia coli* *Mol Gen Genet* 1996 Feb 25;250(3):241-51.
- Yamazaki K, Nagata A, Kano Y, Imamoto F. Isolation and characterization of nucleoid proteins from *Escherichia coli*. *Mol Gen Genet* 1984;196(2):217-24
- Yamazoe M, Onogi T, Sunako Y, Niki H, Yamanaka K, Ichimura T, Hiraga S. Complex formation of MukB, MukE and MukF proteins involved in chromosome partitioning in *Escherichia coli*. *EMBO J* 1999 Nov 1;18(21):5873-84.
- Yi QM, Rockenbach S, Ward JE Jr, Lutkenhaus J. Structure and expression of the cell division genes *ftsQ*, *ftsA* and *ftsZ* *J Mol Biol* 1985 Aug 5;184(3):399-412 .
- Yoshioka, Y., Y. Fujita, and E. Ohtsubo. Nucleotide sequence of the promoter-distal region of the *tra* operon of plasmid R100, including *traI* (DNA helicase I) and *traD* genes. *J. Mol. Biol.* 1990, 214:39-53.
- Yu XC, Tran AH, Sun Q, Margolin W. Localization of cell division protein FtsK to the *Escherichia coli* septum and identification of a potential N-terminal targeting domain. *J Bacteriol* 1998 Mar;180(5):1296-304.
- Yu XC, Weihe EK, Margolin W (b). Role of the C terminus of FtsK in *Escherichia coli* chromosome segregation. *J Bacteriol* 1998 Dec;180(23):6424-8
- Zerbib D, Mezard C, George H, West SC. Coordinated actions of RuvABC in Holliday junction processing. *J Mol Biol* 1998 Aug 28;281(4):621-30.
- Zhao CR, de Boer PA, Rothfield LI. Proper placement of the *Escherichia coli* division site requires two functions that are associated with different domains of the MinE protein. *Proc Natl Acad Sci U S A* 1995 May 9;92(10):4313-7.
- Zhi J, Mathew E, Freundlich M. Lrp binds to two regions in the *dadAX* promoter region of *Escherichia coli* to repress and activate transcription directly *Mol Microbiol* 1999 Apr;32(1):29-40 .

Appendix. Protocols.

1. DNA Techniques.

1.1. Plasmid Miniprep.

—Wizard Plus SV Minipreps DNA Purification System protocol (From promega company).

1. Pellet 1-10ml of bacterial culture by centrifugation for 5 minutes at 10,000 x g in a tabletop centrifuge. Pour off the supernatant.
2. Add 250µl of Wizard Plus SV Minipreps Cell Resuspension Solution and completely resuspend the cell pellet by vortexing well.
3. Add 250µl of Wizard Plus SV Minipreps Cell Lysis Solution and mix by inverting the tube 4 times (DO NOT VORTEX). Observe suspension for clearing to insure lysis is complete.
4. Add 10µl of Alkaline Protease Solution and mix by inverting the tube 4 times. Incubate for 5 minutes at room temperature.
5. Add 350µl of Wizard Plus SV Minipreps Neutralization Solution and mix by inverting the tube 4 times (DO NOT VORTEX).
6. Centrifuge the bacterial lysate at 14000 x g in a microcentrifuge for 10 minutes at room temperature.
7. Transfer the cleared lysate, approximately 850ul, by decanting into the Wizard Plus SV Minipreps Spin Column inserted into a 2ml Collection Tube.
8. Centrifuge the cleared lysate at 14,000 x g in a microcentrifuge for 1 minute at room temperature. Remove the Wizard Plus SV Minipreps Spin Column from the tube and discard the flow through from the Collection Tube.
9. Add 750µl of Wizard Plus SV Minipreps Column Wash Solution, previously diluted with 95% ethanol, to the Wizard Plus SV Minipreps Spin Column.

10. Centrifuge at 14000 x g in a microcentrifuge for 1 minute at room temperature. Remove the Wizard Plus SV Minipreps Spin Column from the tube and discard the flow through from the Collection Tube.
11. Add 250µl of Wizard Plus SV Minipreps Column Wash Solution to the Wizard Plus SV Minipreps Spin Column.
12. Centrifuge at 14000 x g in a microcentrifuge for 2 minutes at room temperature.
13. Transfer the Wizard Plus SV Minipreps Spin Column to a clean, sterile 1.5ml microcentrifuge tube.
14. Elute the plasmid DNA by adding 100µl of Nuclease-Free Water to the Wizard Plus SV Minipreps Spin Column. Centrifuge at 14000 x g for 1 minute at room temperature in a microcentrifuge.

1.2. Small-scale preparation of chromosomal DNA.

Inoculate 5 ml of media containing the appropriate selective antibiotics with a single bacterial colony. Incubate overnight at the permissive temperature. Spin 1.5 ml of the culture in a microcentrifuge for 2 minutes at 15000 rpm, or until a compact pellet is formed and discard the supernatant. Resuspend the pellet in 567 µl of TE buffer and add 3µl of proteinase K (20 mg/ml) and 30 µl 10% SDS. Mix thoroughly and incubate at 37°C for 1 hour. Add 100 µl of 5M NaCl and mix thoroughly. Add 80 µl of hexadecyltrimethyl ammonium bromide (CTAB)/NaCl solution, mix thoroughly and incubate for 10 minutes at 65°C. Add an equal volume of chloroform/isoamyl alcohol (1:1 v/v), mix thoroughly and spin in a microcentrifuge for 5 minutes. Transfer the aqueous upper layer to a new tube and add an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1 v/v), mix thoroughly and spin in a microcentrifuge. Transfer the supernatant to a fresh tube and add 0.6 volume of isopropanol. Mix the contents and spin in a microcentrifuge for 5 minutes at room

temperature. Remove the supernatant and wash the pellet in 1 ml 70% ethanol, then spin in a microcentrifuge for 5 minutes at room temperature. Discard the supernatant and briefly dry the pellet in a vacuum desiccator. Resuspend the pellet in 100 μ l of TE buffer and use 15 μ l per restriction digest.

CTAB/NaCl solution (10% CTAB in 0.7 M NaCl)

Adjust the final volume to 100 ml. Dissolve 4.1 g NaCl in 80 ml H₂O and slowly add 10 g CTAB (hexadecyltrimethyl ammonium bromide) while heating and stirring. If necessary, heat to 65°C to dissolve.

1.3. DNA precipitation.

A 1/10 volume of 3 M sodium acetate (pH 5.0) was added to the DNA solution and then mixed by vortexing. To this add 3 volumes of absolute ethanol, mix thoroughly and leave on ice for 10 minutes. Spin in a microcentrifuge at 15000 rpm for at least 15 minutes and discard the supernatant. Add 1 ml of 70% ethanol and vortex vigorously to wash the pellet. Spin in a microcentrifuge for 5 minutes at 15000 rpm, remove the supernatant and dry the pellet for 3 minutes in a vacuum desiccator.

1.4. Determination of DNA concentrations.

To determine the concentration of DNA solutions, diluted aliquots had their adsorption measured at 260nm. An OD₂₆₀ value of 1.0 represents a concentration of 50 mg/ml for double-stranded DNA.

The purity of the DNA can be calculated by measuring the adsorption of the DNA solution at 280nm. The 260/280 ratio should be 1.75 to 2.0 to represent protein free double-stranded DNA (and 2.0 for single-stranded DNA).

1.5. Restriction of DNA.

The digestion of DNA using restriction endonucleases was performed usually in 50-100 μ l volumes depending on the concentration of the DNA sample, normally 1-10 mg/ml. The digest solution contains 1x concentration of the appropriate buffer and the restriction enzyme was present in two to five fold excess, i.e. 2-5 Units of enzyme per microgram of DNA. The final volume of the digest were made up using d H₂O. For restriction enzymes requiring BSA, this too was added in accordance with the manufacturers instructions, usually 10 mg/ml. For complete restriction of the DNA the digests were incubated for 3-5 hours at the restriction enzymes optimum temperature, 37°C for most enzymes used. The reactions were then analysed by agarose gel electrophoresis or phenol extracted, ethanol precipitated and resuspended in a suitable volume of TE buffer for further manipulations.

1.6. 'Filling in' of recessed 3' termini.

The Klenow enzyme was used to 'fill in' the 3' recess, formed after digestion of DNA with certain restriction endonucleases, to create blunt ended DNA molecules. The Klenow reactions were performed in 20 μ l volumes containing 1 μ g DNA, 1x nick translation buffer (Boehringer Mannheim), each dNTP at a final concentration of 20 mM, 2 Units of Klenow enzyme and TE buffer to make up the final volume to 20 microlitres. The samples were incubated at 37°C for 30 minutes and then heat

inactivated at 70°C for 15 minutes. TE buffer was added to increase the final volume to 100 microlitres. The DNA was ethanol precipitated and then resuspended in a suitable volume of TE buffer.

1.7. Extraction of DNA from agarose gel.

—QIAquick Gel Extraction Kit Protocol

1. Excise DNA fragment from the agarose gel with a clean, sharp scalpel.
2. Weigh the gel slice in a colorless tube. Add 3 volumes of Buffer QC to 1 volume of gel (100mg-100ul).
3. Incubate at 50°C for 10 min (or until the gel slice has completely dissolved). To help dissolve gel, mix by vortexing the tube every 2-3 min during the incubation.
4. After the gel slice has dissolved completely, check that the color of the mixture is yellow (similar to Buffer QC without dissolved agarose).
5. Add 1 gel volume of isopropanol to the sample and mix.
6. Place a QIAquick spin column in a provided 2-ml collection tube.
7. To bind DNA, apply the sample to the QIAquick column, and centrifuge for 1 min.
8. Discard flow-through and place QIAquick column back in the same collection tube.
9. Add 0.5 ml of Buffer QC to QIAquick column and centrifuge for 1 min.
10. To wash, add 0.75 ml of Buffer PE to QIAquick column and centrifuge for 1 min.

Note: If the DNA will be used for salt sensitive applications, such as blunt-end ligation direct sequencing, let the column stand 2-5 min after addition of Buffer PE, before centrifuging.

11. Discard the flow-through and centrifuge the QIAquick column for an additional 1 min at $\geq 10,000 \times g$ (~13,000 rpm).
12. Place QIAquick column into a clean 1.5-ml microfuge tube.
13. To elute DNA, add 50 μ l of Buffer EB(10 mM Tris-Cl, pH8.5) or H₂O to the center of the QIAquick membrane and centrifuge the column for 1 min at maximum speed.

Alternatively, for increased DNA concentration, add 30 μ l elution buffer to the center of the QIAquick membrane, let the column stand for 1 min, and then centrifuge for 1 min.

1.8. PCR reaction.

The PCR reaction mixture was made in the following way.

40 pmol each primer:	8 μ l (5 pmol/ μ l) 8 μ l (5 pmol/ μ l)
10 x Vent buffer:	10 μ l
200 μ M dNTPs:	4 μ l
BSA 100 μ g/ μ l:	10 μ l BSA (10 x)
Template:	10 μ l (dilutions of DNA)
2u vent pol:	1 μ l
dH ₂ O to 100 μ l:	49 μ l
<hr/>	
	100 μ l

1.9. DNA Sequencing.

— Protocol for Cycle Sequencing from PE Applied Biosystems, a division of Perkin-Elmer.

(1). Mixing the Reagents

For each reaction, mix the following reagents in a labeled tube of the appropriate size:

Reagent	Quantity
Terminator Ready Reaction mix	8.0 μ l
Template	
single-stranded DNA, 0.1 μ g/ μ l	0.5-1.0 μ l
double-stranded DNA, 0.2 μ g/ μ l	1.5-2.5 μ l
PCR product, 10-30 ng/ μ l	3-6 μ l
Primer	3.2 pmole
dH ₂ O	
Final Reaction Volume	20 μ l

(2). Cycle Sequencing on the DNA Thermal Cycler (TC1) and the DNA Thermal Cycler Model 480

1. Place the tubes in a thermal cycler, and begin thermal cycling as follows:

 Rapid thermal ramp to 96°C

 96°C for 30 seconds

 Rapid thermal ramp to 50°C

 50°C for 15 seconds

 Rapid thermal ramp to 60°C

 60°C for 4 minutes

2. Repeat Step 1 for 28 cycles.

3. Rapid thermal ramp to 4°C and hold.

4. Proceed with Purifying Extension Products by Ethanol Precipitation.

(3). Purifying Extension Products by Ethanol Precipitation

1. For each reaction, prepare a 0.5 ml microcentrifuge tube by adding the following:

2.0µl 3M Sodium acetate, pH5.2

50µl 95% ethanol

2. Transfer the entire 20 µl contents of the reaction tubes to the microcentrifuge tubes containing the ethanol solution. Vortex and place on ice for 10 minutes.
3. Centrifuge in a microcentrifuge at maximum speed for 15-30 minutes.
4. Carefully aspirate the ethanol solution with a micropipetter. Remove as completely as possible.
5. Rinse the pellet by adding 250 µl 70% ethanol. At this point it is not necessary to centrifuge.
6. Carefully aspirate all the alcohol solution with a micropipetter. Use a KimWipe to remove any alcohol from the sides of the tube. Be careful not to disturb the pellet, which may or may not be visible.
7. Dry the pellet in a vacuum centrifuge.

The product was then submitted for automatic sequencing to get the DNA sequence.

1.10. Southern blotting of DNA.

(1). Transferring of DNA to nylon membrane.

This was a method used to detect the target sequence(s) on the chromosome. Genomic DNAs (10 µg) were cut with appropriate restriction enzymes to produce fragments of calculated size. Plasmid DNA controls (50 ng) were cut with the same enzymes so that comparisons could be made. 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 millilitres of 0.25M HCl for 15 minutes to depurinate the DNA. Next the gel was washed in 200 millilitres of 0.5M

This method is adapted from that described by Feinberg and Vogelstein, 1984. The required probe DNAs were always cloned in plasmids. 1 microgram of the plasmid DNA was cut with appropriate restriction enzymes and the fragment isolated by agarose gel electrophoresis. The gel was stained in EtBr (0.5µg/ml) and after destaining 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 50 nanograms of the fragment DNA to the following mixture:

DNA	~50 ng
OLB	10 µl
BSA (10mg/ml)	2 µl
α[32]P dCTP (10µCi/µl)	5 µl
Klenow enzyme (2 Units)	1 µl
d.H2O to 50µl	

Incubate overnight at room temperature to allow polymerization to occur in a lead containment vessel. The DNA will now be randomly labelled.

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 pd(N)₆ (in TE buffer)
 2 ml BSA (10 mg/ml)

(3). Probe hybridization using phosphate buffer

Preheat the hybridization oven (Techne) to 65°C. Place the nylon filter blot in a glass hybridization tube with the blotted side facing towards the centre of the tube. Add 20 millilitres of SDS-phosphate buffer and prehybridize the filter at 65°C for 1 hour. Add the labelled probe solution and leave to hybridize with the filter overnight incubating at 65°C. Pour the hybridization mixture into a universal bottle and store at -20°C(*). Remove one of the tube caps and add 30 millilitres of SDS-phosphate wash buffer. Reseal the tube and allow the buffer to wash for 30 minutes at 65°C. Pour the wash solution into a waste bottle and monitor the activity from the filter. If the cps is ~20 the the filter may be put down in a autoradiography cassette, if the count is still high (>30 cps) then add a further 30 millilitres of wash buffer and incubate again for 30 minutes at 65°C. This too is then disposed of into the waste bottle. The filter was removed from the tube and placed in a polythene bag. This was then secured to an autorad cassette with a reflector pad. An X-ray film was inserted and the cassette was stored at -70°C. The time of exposure of the film to the labelled filter was dependant on how strong the signal was from the labelled probe .

(*). The frozen buffer/labelled probe mix will retain sufficient activity to be reused for further hybridizations up to one week after labelling the probe. However it must first be placed in boiling water for 5 minutes and then allowed to cool to 50°C before being introduced to the prehybridized filter. This is to prevent the stripping of DNA from the filter.

SDS-phosphate buffer solution 50mls

Phosphate buffer (pH 7.2) 25 mls

20% SDS.	17.5 mls
d.H ₂ O.	7.4 mls
0.5 M EDTA.	100 µl

1 M Phosphate buffer recipe:

Na ₂ HPO ₄	354.92 g
NaH ₂ PO ₄	156.01 g
Adjust pH to 7.2	
Add distilled water to 3 litres	

(4). Stripping probes from nylon filters

Once the Southern blot has been probed, a second different probe may be required and before this can be hybridized to the blot the previous labelled probe must be removed. Incubate the blot at 45°C for 30 minutes in 30 ml 0.4 M NaOH and then wash in 40 ml 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 now be negligible and the blot is ready to be hybridized with the next labelled probe.

2. Bacteriophage techniques.

2.1. Hfr matings.

Fresh overnight cultures of the Hfr donors were diluted 1:100 into prewarmed L broth and incubated at 37°C without shaking until OD₆₀₀ reached 0.3 to 0.4. Mating was started by mixing 1ml of every of the donors with an equal volume of freshly prepared

log phase F⁻ recipient, and allowing the incubation to proceed without agitation at 37°C for an hour. Mating was disrupted by vortexing vigorously. Samples were removed and different dilutions were spread onto selective plates.

2.2. Production of bacteriophage P1 lysates.

Inoculate 5 ml of L broth containing 2.5 mM CaCl₂ with a single colony of the host strain and incubate overnight without shaking. Mix 1 ml of the overnight culture with 5 x 10⁵ P1 in a large sterile test tube and incubate for 30 minutes to allow the phage to be absorbed into the cells. Prepare a cells only control. Add 3 ml of L broth and 3 ml of molten LC top agar, cooled to 45°C, containing 2.5 mM CaCl₂ and mix. Immediately pour the mixture onto LC bottom agar plates with 2.5 mM CaCl₂ and gently swirl the mixture until the surface has an even covering of the top agar. Leave at room temperature for 15 minutes to allow the agar to set and then incubate overnight. The cells only control should have an even lawn of cells whilst the infected culture will be partly or completely lysed, resulting in cleared top agar. Scrape this off with a sterile scalpel blade into a sterile universal containing 4 ml of L broth over 100 microlitres of chloroform. Vortex for 30 seconds and incubate at 30°C with vigorous agitation for 30 minutes. Spin the universals in a bench centrifuge at 5000 rpm for 15 minutes to pellet the top agar and transfer the supernatant, using a pipette, to a 1/2 ounce Bijou bottle. Add a few drops of chloroform to the lysate and store at 4°C.

2.3. Transduction of cells by bacteriophage P1.

Inoculate 5 millilitres of L broth containing 2.5 mM CaCl₂ with 1 millilitre of a fresh overnight culture of the recipient strain. Incubate at a permissive temperature until

the O.D.600 reaches ~1.0. Aliquot four 1 millilitre volumes into Eppendorff tubes, and spin for 15 seconds in a microcentrifuge to pellet the cells. Remove 0.9 millilitres of supernatant from each tube and resuspend the pellet in the remaining 0.1 millilitres of supernatant by vortexing. Take 130 microlitres of P1 lysate and vortex in an Eppendorff tube with a drop of chloroform and then spin in a microcentrifuge for 1 minute to pellet any debris. Lysate aliquots of 50, 10 and 1 microlitre were added to the cell suspension and include a cells only control. A phage only control of 100 microlitres of lysate was also used. Incubate all the tubes for 30 minutes before adding L broth plus sodium citrate (10 mM) and incubate for 1-2 hours. When using auxotrophs, 1 millilitre of phage buffer was added to the cells instead of L broth and then they were plated on minimal selective media without having an incubation period. The cells were concentrated tenfold before plated by pelleting the cells in a microcentrifuge, removing all but 100 microlitres of the supernatant and then resuspending the pellet by vortexing. 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 (1 millimetre).

2.4. Preparation and selection of λ lysogens.

A culture of the desired strain to be lysogenized was grown up in L-broth, supplemented with 0.20 mM MgSO₄ and 0.2% maltose, to mid-log phase. 0.3 millilitres of the culture was used to inoculate millilitres L-top agar. To this was also added λ lysate dilutions to approximately 200 λ phage particles per plate and the mixture poured onto a fresh L-agar plate and left to set. This was incubated overnight at 37°C. The aim here 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 L-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. A lysogenized bacterium will now be 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 L-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 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. β -Galactosidase assays.

The method used to determine the β -galactosidase activity from a promoter-*lacZ* fusion, was as described by Miller (1972). Take 1 millilitre of culture and measure the OD600; from this take 0.5 millilitres and add to 0.5 millilitres of Z buffer. If the promoter activity is known to be high then add 0.1 millilitres of culture to 0.9 millilitres Z buffer. Add 50 microlitres of chloroform and vortex for 30 seconds. Store the samples at 4°C until all sampling has been performed. Add 200 microlitres of ONPG (4 mg/ml) to each sample and vortex. Prepare a control of 0.5 millilitres of the culture medium used and 0.5 millilitres of Z buffer. After addition of ONPG place the samples on ice until all have been treated. Place the samples in a 30°C waterbath noting the exact time of the start of incubation. Check the tubes every 10 minutes and when yellowing occurs add 0.5 millilitres of 1 M Na₂CO₃, vortex and make a note of the time taken for the colour change and store the sample on ice. When all the tubes have reacted measure

the OD420 and OD550 of the samples using the control as a blank. The β -galactosidase activity of the samples can now be calculated and expressed as Miller Units using the equation:

$$\frac{\text{O.D.420} - 1.75 \times \text{O.D.550}}{\text{O.D.600} \times 0.5\text{ml} \times T} \times 1000$$

Where T = time in minutes for colour change.

0.5ml is the sample volume taken from the original culture.

Z buffer recipe:	Na ₂ HPO ₄	4.26 g
	NaH ₂ PO ₄ .H ₂ O	3.11 g
	KCl	0.375 g
	MgSO ₄ .7H ₂ O	0.123 g
	β -mercaptoethanol	1.35 ml
	SDS (10%)	0.25 ml
	Add distilled water to 500 ml	

2.6. Counting and sizing of bacterial cells.

A coulter Counter ZB (Coulter Electronics, Herts.) and Coulter Channelyser (model C-1000) were used to count and determine the size of bacterial cells. Cultures were grown in filtered LB (5 micrometre membrane filter, Millipore) and 500 microlitre samples were fixed in filtered fixing solution. Samples were then stored at 4°C until use. When measuring cells, a volume, usually 100-200 microlitres was made up to a total volume of 6 millilitres with sodium azide solution in a glass vial. The sample volume for counting cells was 50 microlitres. The channelyser places cells in different channels depending on their size. From this the distribution of different cell sizes, the mode and median cell size of a culture could be measured.

Fixing solution: 80% bacterial buffer.
 20% formaldehyde.

Filtered through 5µm filter (Millipore).

Sodium azide solution: 0.85% NaCl.
 0.085% sodium azide.
 Dissolved in distilled water.

Filtered through 5µm filter (Millipore).

2.7. DAPI staining of chromosome DNA.

Take 1ml of early log phase culture (OD600 around 0.2) or 200 µl of higher density culture. Spin and wash once 1XPBS. Resuspend in the same volume of 1XPBS. Spot 10 µl to a glass slide and air-dry it. The slide is then merged into methanol for 5min. Bring the slide up and down in tap water six times and air-dry again. DAPI solution is then added to the spot. Cover it with a cover slipper. Now it is ready to observe the chromosome location of *E. coli* chromosome under a fluorescence and phase-contrast combined microscope.

2.8. 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.

3. Protein Techniques.

3.1. Preparation of SDS-PAGE gels.

The type of gel apparatus used for all SDS-PAGE work was the Hoefer SE600 dual cooled vertical slab unit which is able to run one or two 16 x 18 cm gels. The gel plates are washed in distilled water and then wiped with absolute alcohol. The gel plates are laid together, separated by 0.75 mM spacers, and then clamped together. The plates are then clamped into the baseplate of the gel apparatus and are now ready to receive the acrylamide mix. The resolving gel was poured first. All the ingredients bar the TEMED and the ammonium sulphate were mixed in 50 ml glass beaker and then the latter two added. The ammonium persulphate 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 stacking buffer) and left to polymerize 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. This is left at room temperature for 30 minutes for the stacking gel to polymerize. The comb is then

removed and each well rinsed three times with Tris-glycine running buffer to remove any unpolymerized acrylamide. The gel is now ready to be used to run protein samples.

Recipe for 10% resolving acrylamide gel (40 ml):

Acrylamide (40% v/v)	10 ml.
4x Resolving buffer	10 ml.
d.H ₂ O	19.2 ml.
10% SDS	400 µl.
7.5% Ammonium Persulphate	400 µl.
TEMED	25 µl.

Recipe for 4% stacking gel (10 ml):

Acrylamide (40% v/v)	1.3 ml
4x Stacking buffer	2.5 ml
d.H ₂ O	6.0 ml
10% SDS	100 µl.
7.5% Ammonium persulphate	100 µl.
TEMED	10 µl.

3.2. 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 are spun at 15,000 rpm in a microfuge for 5 minutes. Remove the comb and rinse each well three times with with 1x running buffer to remove any unpolymerized acrylamide. Remove half the Tris-glycine running buffer from each well to be loaded. Aliquot 30 microlitres of each sample and 8 microlitres of the marker proteins into separate wells. Carefully fill the remaining space in the wells with running buffer and clamp on the upper reservoir. Turn on the water

cooling system and fill the lower reservoir with running buffer. Remove the base from the gel plates and place the gel plates and upper reservoir into the tank. Fill the upper reservoir and place the lid with electrodes on the top. Run the gel at 30 mA until the marker dyes have migrated from the stacking gel into the resolving gel, the power can now be increased to 40-45 mA. Run the gel until the marker dye reach the bottom of the gel and then switch off the power and remove the gels.

Unscrew the clamps and using a wedge, prise open the plates. Place each gel in a polythene sandwich box (25 x 25 x 8cm) and add 70 millilitres of Coomassie stain to each and incubate at 37°C with gentle shaking for 20 minutes. Pour off the stain and rinse the gel in 50 millilitres destain to remove traces of the stain from the gel and the box. Add 100 millilitres of destaining solution and foam bungs to absorb the coomassie stain leaching from the gel. Leave this at 37°C with gentle shaking until the marker bands are clearly visible. Remove the destain and add 60 millilitres of fixing solution, this is essential as the gel will will shrink and crack during vacuum drying without the presence of glycerol. Incubate at 37°C with gentle shaking for at least 20 minutes. Pour off the fixative and place a sheet of blotting paper, soaked with distilled water, over the gel. Lay a dry sheet of the paper on this and carefully lift the gel/paper sandwich up. Lay the sandwich on the bed of a vacuum gel dryer gel side up and lay a sheet of Saran wrap over the gel. Dry the gel for 1 hour under vacuum at 60°C. Once dry the gel can be taped in an autoradiogram cassette and an x-ray film added. The gels were usually left overnight to expose the film before developing. The position of the non-labelled marker proteins can be marked on the film by over laying it on the dried gel.

3.3. Solutions used in SDS-PAGE.

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

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

10x loading buffer:	0.25% bromophenol blue
	0.25% xylene cyanol
	25% Ficoll (type 400) in H ₂ O
	Distilled water to 1 litre

Tris glycine electrophoresis buffer:

Working solution:

25 mM Tris·HCl
250 mM glycine (pH 8.0)
0.1% SDS

4x stacking-gel buffer.

15.25 g of Tris base dissolved in two hundred millilitres of distilled water adjusted to pH6.8 with concentrated HCl and made up to two hundred and fifty millilitres with distilled water, filtered and sterilized.

4x resolving gel buffer.

45.5 g Tris base dissolved in two hundred millilitres of distilled water adjusted to pH8.8 with concentrated HCl. Made up to two hundred and fifty millilitres with distilled water, filtered and sterilized.

Coomassie staining solution.

Coomassie brilliant blue (type R250)	0.25 g
Methanol : H ₂ O (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: As destain solution containing 5% glycerol (v/v)

3.4. Western Blotting of SDS-PAGE gels.

1. Remove stacking gel portion and cut nitrocellulose sheet to the size of the resolving gel.
2. Soak nitrocellulose in d.H₂O for 15 min.
3. Soak nitrocellulose in transfer buffer for 10 min.
Transfer buffer: SDS-PAGE running buffer without SDS 20% MetOH (v/v).
4. Soak gel in transfer buffer for 10 min.
5. Lay nitrocellulose sheet on gel (check no bubbles) and lay 3 sheets of blotting paper soaked in transfer buffer. Invert and place another 3 sheets of blotting paper soaked in transfer buffer on the other side of the sandwich. Ensure there's no air bubbles.
6. Place in wet electroblotter in the following order:
 - a) Black electrode.
 - b) Gel/nitrocellulose sandwich.
 - c) Red electrode.
7. Run at 100 V with cooling for 2 hours OR 30V overnight without cooling.
8. Disassemble sandwich and soak nitrocellulose in 1x Ponceau S solution for 5 min with gentle shaking to check for transfer. Destain in d.H₂O for 10-15 min.
9. Block filter in P.T. buffer + 5% dried milk(w/v) with gentle shaking for at least 1 hour at r.t. with gentle shaking or o/n at 40C (P.T. buffer - 0.1% Tween in 1 x PBS)
10. Rinse in 125 ml P.T. buffer twice.
11. Wash once for 15 min, twice at 5 min in P.T. buffer at r.t. with gentle shaking.

12. Dilute 1o antibody to appropriate conc. using P.T. buffer and incubate at r.t. for 1 hour with gentle shaking. Rinse in P.T. buffer.
13. Wash once for 15 min, twice at 5 min in P. T. buffer at r.t. with gentle shaking.
14. Dilute 2o antibody to appropriate conc. using P.T. buffer and incubate at r.t. for 1 hour with gentle shaking. Rinse in P.T. buffer.
15. Wash once for 15 min, four times at 5 min in P.T. buffer at r.t. with gentle shaking.
16. Detection. Add 3mls of solution A and then 3mls of solution B, pour onto the blot and leave at r.t. for 1 minute (don't leave any longer)
17. Remove from solution and remove excess liquid from the filter. Go to dark room and expose filter to x-ray film for varying periods of time (i.e. 5,10, 15, 30, 45, and 60 sec.). Deveop film and compare to coomassie stained duplicate.

ECL detection reagents:

Solution A. 1 ml luminol.
 440µl p-coumaric acid (stock solution dissolved in DMSO)
 10µl 1M Tris. HCl pH8.5 (pH 8.0 is fine)
 d.H₂O to 100ml (N.B. Only make up a maximum of 20
 ml as the soln goes off)

Solution B. 10 ml 1M Tris.HCl pH8.5 (pH8.0 is fine)
 60 µl 30% H₂O₂
 d.H₂O to 100 ml (N.B. Only make up a maximum of 20
 ml as the H₂O₂ degrades within 2 weeks).

NB ·Store both solutions in the ECL Western Kit in the fridge at 4°C.

4. Commonly used buffers and solutions

Bacterial buffer.	MgSO ₄ ·7H ₂ O	2 g
	Na ₂ HPO ₄	7 g
	KH ₂ PO ₄	3 g
	NaCl	4 g

Distilled water to 1 litre

Phage buffer.	Na ₂ HPO ₄	7 g
	KH ₂ PO ₄	3 g
	NaCl	5 g
	MgSO ₄ (1.0 M)	1 ml
	CaCl ₂ (0.1 M)	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.	Working solution :
	40 mM Tris-Acetate
	2 mM EDTA

50 x conc. stock solution:	Tris base	242 g
	Glacial acetic acid	57.1 ml
	0.5 M EDTA (pH 8.0)	100 ml
	Distilled water to 1 litre	

TBE buffer.	
	Working solution:
	89 mM Tris-borate
	89 mM Boric acid

5x conc. stock solution:	Tris base	54 g
	Boric acid	27.5 g
	0.05 M EDTA (pH 8.0)	20 ml

Antibiotic Solutions:

Antibiotic Solutions are listed in the following table.

Antibiotics	Abbreviation	Solvent	Conc. of stock solution (mg/ml)	Final conc. in media ($\mu\text{g/ml}$)
Ampicillin	Amp	H ₂ O	100	50-100
Chloramphenicol	Chl	Ethanol	20	25
Kanamycin sulphate	Kan	H ₂ O	25	25-50
Spectinomycin dihydrochloride	Spc	H ₂ O	50	25-50
Streptomycin sulphate	Str	H ₂ O	100	20
Tetracycline hydrochloride	Tet	50% ethanol	10	10
Trimethoprim	Tmp	Methanol	5	50

THE END