Localization of the cell division proteins in filaments resulting from a lack of S-adenosylmethionine in *Escherichia coli* K-12

Shan Wang

A Thesis

in

The Department

of

Biology

Presented in Partial Fulfillment of the Requirements

for the Degree of Master of Science at

Concordia University

Montreal, Quebec, Canada

August 2004

© Shan Wang, 2004

Library and Archives Canada

Published Heritage Branch

395 Wellington Street
Ottawa ON K1A 0N4
Canada

Bibliothèque et Archives Canada

Direction du Patrimoine de l'édition

395, rue Wellington
Ottawa ON K1A 0N4
Canada

Your file Votre référence
ISBN: 0-612-94663-0
Our file Notre référence
ISBN: 0-612-94663-0

The author has granted a non-exclusive license allowing the Library and Archives Canada to reproduce, loan, distribute or sell copies of this thesis in microform, paper or electronic formats.

L'auteur a accordé une licence non exclusive permettant à la Bibliothèque et Archives Canada de reproduire, prêter, distribuer ou vendre des copies de cette thèse sous la forme de microfiche/film, de reproduction sur papier ou sur format électronique.

The author retains ownership of the copyright in this thesis. Neither the thesis nor substantial extracts from it may be printed or otherwise reproduced without the author's permission.

L'auteur conserve la propriété du droit d'auteur qui protège cette thèse. Ni la thèse ni des extraits substantiels de celle-ci ne doivent être imprimés ou aturement reproduits sans son autorisation.

In compliance with the Canadian Privacy Act some supporting forms may have been removed from this thesis.

Conformément à la loi canadienne sur la protection de la vie privée, quelques formulaires secondaires ont été enlevés de cette thèse.

While these forms may be included in the document page count, their removal does not represent any loss of content from the thesis.

Bien que ces formulaires aient inclus dans la pagination, il n'y aura aucun contenu manquant.

Canada

ABSTRACT

Localization of the cell division proteins in filaments resulting from a lack of S-adenosylmethionine in *Escherichia coli* K-12

Shan Wang

The *metK* gene of *Escherichia coli* encodes S-adenosylmethionine synthetase, which catalyzes the biosynthesis of S-adenosylmethionine (SAM), the primary methyl group donor. A partial defect in *metK* expression caused by a point mutant in its promoter leads to a decrease in the cellular SAM concentration, resulting in a block in cell division. Thus *metK* mutants form filaments with regularly distributed nucleoids.

This thesis aims to find out the division step at which cell division process stops. Herein I examine localization of 8 division proteins in *metK84* filaments. The results show that early division proteins, FtsZ, FtsA and ZipA, are able to establish themselves in the septum, while the late proteins, FtsQ, FtsW, FtsI and FtsN, fail to localize. FtsK localizes to the septum at some extent, but the result is uncertain. Further study of FtsK localization in *metK84* filaments is required. This localization study suggests that *metK84* filamentation is caused by the failure of FtsQ or FtsK to localize to the septum during the cell division process.

ACKNOWLEDGEMENTS

There is a long list of persons I would like to thank for the help and encouragement during my Master's study.

On top of this list I would like to express my special thanks to my supervisor, Dr. E.B. Newman for her scientific guidance, encouragement, financial support and patience.

My sincere thanks go also to Dr. W. Zerges and Dr. P. Gulick for being my committee members and for providing helpful advice and suggestions.

I am deeply grateful to Dr. Whiteway and Anne Marcil from the Biotechnology Research Institute (BRI), and Aleks Spurmanis from the biology department, for their constant support and technical help for the fluorescent microscope.

I am thankful also to all colleagues from past and present of our lab: Dr. Xia Zhao, Dr. Jingjang Fan, Dr. Hongshen Su, Dr. Vlad Yerko, Liliana Perdomo, Rodrigo Reyes Lanothe, Anne Monette, Li Luo, Yeman Tang and Eileen Colella for their helpful suggestion, assistances and wonderful friendship.

Finally, I want to thank my husband and family for their endless love, the understanding and the encouragements, which support me to finish my study.

Table of contents

List of Figures....................xi

List of Tables....................xiii

Introduction....................1

1. SAM, *metK* gene and cell division in *Escherichia coli*....................2

1.1 SAM is required for a multitude of pathways in all organisms....................2

1.2 SAM is a predominant methyl donor in all organisms....................5

1.2.1. DNA methylation....................5

1.2.2. Protein methylation....................7

1.3 *metK*, the only gene encoding SAM synthetase in *E.coli*....................7

1.3.1. Regulation of *metK* in *E. coli*....................8

1.3.2. *metK* is an essential gene *in E.coli*....................8

1.4 SAM, *metK* gene and cell division in *Escherichia coli*....................9

2. The assembly of the division septum in *Escherichia coli*....................9

2.1 Determination of the potential division sites....................10

2.1.1. Nucleoid occlusion....................10

2.1.2. The min system....................11

2.2 The assembly of septum involves a recognizable order of addition of proteins....................12

2.2.1. FtsZ, a key in coordination of the whole process of division....................14

2.2.2. FtsA, an early division protein interacting with FtsZ....................15

2.2.3. ZipA, a membrane anchor for FtsZ..15

2.2.4. FtsK, a bifunctional protein in *E. coli*..16

2.2.5. FtsL, FtsB (YgbQ) and FtsQ, form a complex before
migrating to the potential division site..17

2.2.6. FtsW, a member of the SEDS family of proteins 18

2.2.7. FtsI, an enzyme involved in peptidoglycan synthesis 19

2.2.8. FtsN and AmiC, the last two known proteins for cell division..19

2.3.A defined amount of each division protein is necessary
for correct cell division 20

3. Current conventional methods for division protein
localization experiments..23

3.1. Green Fluorescent protein (GFP) acts as a marker for protein localization......23

3.2. Merodiploid strains constructed by a single-copy gene expression system......24

4. The purpose of this study..25

Materials and Methods..27

1. Bacterial strains and plasmids..27

2. Media, buffers and solutions..27

2.1 Recipes for media..27

2.1.1. Luria-Bertani medium (LB)..27

2.1.2. Minimal medium (NIV)..27

2.1.3. R-top agar for transduction..30

2.1.4. SOC medium for electro-transformation..30

2.1.5. The carbon sources for NIV minimal medium....30

2.2 Buffers....30

2.2.1. SM buffer....30

2.2.2. Phosphate-buffered saline (PBS) (pH7.4)....31

2.2.3. Sodium phosphate buffer (pH7.4)....31

2.3 Solutions....31

2.3.1. Antibiotics....31

2.3.2. Inducer for the expression of various GFP fusion proteins....31

2.3.3. DAPI (4'-6-Diamidino-2-phenylindole), a DNA staining reagent....32

2.3.4. Fix solution for microscopy....32

2.3.5. Cell division inhibitor- aztreonam....33

3. Others....33

4. P1 phage transduction....33

5. Growth and harvesting of cells for protein localization....33

5.1 Preparation of dividing cells....33

5.2 Preparation of filaments....34

5.3 Septum formation in the presence of aztreonam....34

6. Fixing of cells and fluorescence microscope methods....35

6.1 Fixing cells to visualize GFP....35

6.2 Staining of DNA with DAPI....35

6.3 Fluorescence microscopy....36

7. Plasmid isolation and restriction enzyme digestion....36

7.1 Plasmid isolation....36

7.2 Restriction enzyme digestion..37

7.3 DNA gel electrophoresis analysis..37

8. Electro-transformation...37

9. Construction of strains..37

9.1 Construction of merodiploids using P1 mediated transduction....................37

9.2 Construction of *metK84/ gfp-ftsK* using electro-transformation...................38

Results..40

Part I. Localization of the cell division proteins in the *metK84* mutants 41

1. The growth conditions for *metK84* and the control strain, Cu1008..................41

1.1 Demonstration that methodology used is adequate..................................41

1.2 *metK84* is a leucine-requiring strain..42

2. Nucleoid segregation in *metK84* filaments..42

3. Localization of early division proteins in *metK84* filaments.........................47

3.1 Constructions of merodiploid strains..48

3.2 Localization of the first division protein, FtsZ, in the *metK84* filaments.........48

3.3 Localization of FtsA and ZipA, two independent

division proteins, in the *metK84* filaments..49

3.3.1 FtsA localization in the *metK84* filaments...50

3.3.2 Localization of ZipA, the membrane anchor for Z ring,

in the *metK84* filaments..50

4. FtsK localization in *metK84* filaments..56

4.1 Expression of GFP-FtsK fusion under a plasmid-carried pBAD promoter.......56

4.1.1 Plasmid confirmation by enzyme digestion analysis..............................56

4.1.2 Growth condition for *metK84* containing gfp-ftsK..............................57

4.2 Partial localization of FtsK in *metK84* filaments..................................57

4.3 A change in filamentation morphology observed

in glycerol minimal medium..58

4.4 Further investigations on the presence of single cells

in glycerol minimal medium..60

5. Late division proteins, FtsQ, FtsW, FtsI and FtsN,

fail to be recruited to the septum in *metK84* filaments..............................68

5.1 Localization of FtsQ in *metK84* filaments...68

5.2 Localization of FtsW in *metK84* filaments...69

5.3 Localization of FtsI in *metK84* filaments..69

5.4 Localization of the last division protein, FtsN, in *metK84* filaments.............69

Part II. Localization Recovery for late division proteins,

FtsK, FtsQ, FtsW, FtsI and FtsN, in *metK84* background...............................76

1. The growth conditions for Merodiploids/ transformants

with aztreonam in *metK84* background...77

2. Aztreonam treatment does not interrupt the assembly of division septum.........77

2.1 Aztreonam blocks cell division by interacting with division protein FtsI.........77

2.2 Aztreonam does not affect the presence of early division proteins

in *metK84* filaments..78

3. Localization recovery for late division proteins.....................................80

3.1 Localization of FtsK in *metK84* filaments in the presence of aztreonam.........80

3.2 Localization of FtsQ in *metK84* filaments in the presence of aztreonam.........81

3.3 Localization of FtsW in *metK84* filaments in the presence of aztreonam........81

3.4 Localization of FtsI in *metK84* filaments in the presence of aztreonam...........82

3.5 Localization of FtsN in *metK84* filaments in the presence of aztreonam.........82

Discussion...91

1. Localization of division proteins in *metK84* filaments...............................92

1.1 Three early division proteins localize into the potential division sites............92

1.2 All late division proteins, from FtsQ to FtsN,

failed to assemble the septum..92

1.3 Morphology of *metK84* filaments...93

1.4 Synthesis of septa in aztreonam-treated filaments...................................94

1.5 Effects of a specific fts fusion: FtsQ...95

1.6 FtsK localization is uncertain...97

1.6.1 Filamentation due to SAM deficiency is affected

by the carbon source supplied..98

1.6.2 Other ways to decide FtsK localization in

future studies of *metK84* filaments 99

2. Potential methylation targets involved in cell division..............................99

Summary...102

References...104

List of Figures

Figure 1. Metablic roles of S-adenosylmethionine 3

Figure 2. Order of localization of cell division proteins to the septum in *E. coli*..........13

Figure 3. Organization of the cell division apparatus at mid cell............................21

Figure 4. *metK84* is a leucine-requiring strain 44

Figure 5. Nucleoid segregation in *metK84* filaments...46

Figure 6. FtsZ localizes to potential division sites in *metK84* filaments....................52

Figure 7. FtsA localizes to potential division sites in *metK84* filaments....................53

Figure 8. ZipA localizes to potential division sites in *metK84* filaments....................54

Figure 9. FtsK partially localizes to potential division sites in *metK84* filaments.........62

Figure 10. Cell morphology of *metK84/gfp-ftsK* grown with glycerol.......................64

Figure 11. The distribution of cells grown in glucose on different plates...................66

Figure 12. The distribution of cells grown in glycerol on different plates..................67

Figure 13. Localization of division protein FtsQ in *metK84* filaments......................71

Figure 14. Localization of division protein FtsW in *metK84* filaments.....................72

Figure 15. Localization of division protein FtsI in *metK84* filaments......................73

Figure 16. Localization of division protein FtsN in *metK84* filaments......................74

Figure 17. Localization of FtsA in *metK84* filaments

with aztreonam treatment...79

Figure 18. Localization recovery for FtsK in *metK84* filaments.............................84

Figure 19. Localization recovery for FtsQ in *metK84* filaments.............................85

Figure 20. Localization recovery for FtsW in *metK84* filaments.............................86

Figure 21. Localization recovery for FtsI in *metK84* filaments...............................87

Figure 22. Localization recovery for FtsN in *metK84* filaments.............................88

List of Tables

Table 1. Important SAM-dependent transmethylations.....4

Table 2. Bacterial strains and plasmids 28

Table 3. Nucleoid Segregation in *metK84* filaments 45

Table 4. Localization frequencies of GFP fusions with early division proteins in *metK84* filaments.....55

Table 5. Localization frequency of GFP-FtsK in *metK84* filaments.....63

Table 6. Morphology of *metK84* cells grown in minimal medium with different carbon sources.....65

Table 7. Localization frequencies of GFP fusions with late division proteins in *metK84* filaments.....75

Table 8. Localization frequencies of GFP fusions with late division proteins in *metK84* filaments with aztreonam treatment.....89

Introduction

The process of cell division is essential for survival of the organism and is one of the most complex and least understood phenomena in the cell biology of bacteria. In *Escherichia coli,* this process requires a set of at least eleven proteins to coordinate invagination of the cell membrane, inward growth of the peptidoglycan layer and the outer membrane, and finally, promote separation of daughter cells (Buddelmeijer and Beckwith, 2002). Depletion or a specific mutant in any division protein prevents the assembly of the septum at mid cell, which then blocks the cell division.

S-adenosylmethionine (SAM) is a central metabolite, which functions as a donor required for many biochemical reactions and biosynthesis pathways in all organisms. An earlier study reported that *metK84* mutants of *E.coli* cells deficient in SAM synthetase activity undergo extensive filamentation under certain growth conditions, indicating that SAM might play a role in cell division (Newman *et al.*, 1998). However, the point at which cell division was blocked was yet unknown.

In this thesis, I performed a further study of the cell division problem in *metK84* mutants: examination of localization of the division proteins in *metK84* filaments to find out which division proteins, if any, were absent from the division site in *metK84* filaments.

Before presenting my work, I will review the relevant literature in three sections. Firstly, I will survey the general information of SAM, *metK* gene encoding SAM synthetase, and the finding of cell division defect in *metK84* mutant in *E.coli*. In this part, I will focus on the SAM-dependent methylation since SAM is a ubiquitous intracellular methyl donor. Secondly, the details of cell division in *E.coli* will be introduced, including the determination of division sites, known cell division proteins, and their regulation. Finally, I will introduce the methods used in this study for protein localization. At the end of this introduction, I will present the aim of the study in this thesis.

1. SAM, *metK* gene and cell division in *Escherichia coli*

1.1 SAM is required for a multitude of pathways in all organisms

S-adenosylmethionine (SAM) is synthesized from methionine and ATP in a reaction catalyzed by the enzyme, SAM synthetase, and is a central metabolite involved in a myriad of pathways in all cells as showed in Figure 1. SAM plays various biological roles. It is the primary methyl group donor in all organisms, and it is a precursor for the polyamines, biotin and lipoic acid (Markham *et al.*, 2002). However, its significant influence is probably as a methyl donor to various cellular constituents, including DNA, RNA, proteins, and small molecules (Miller *et al.*, 2003).

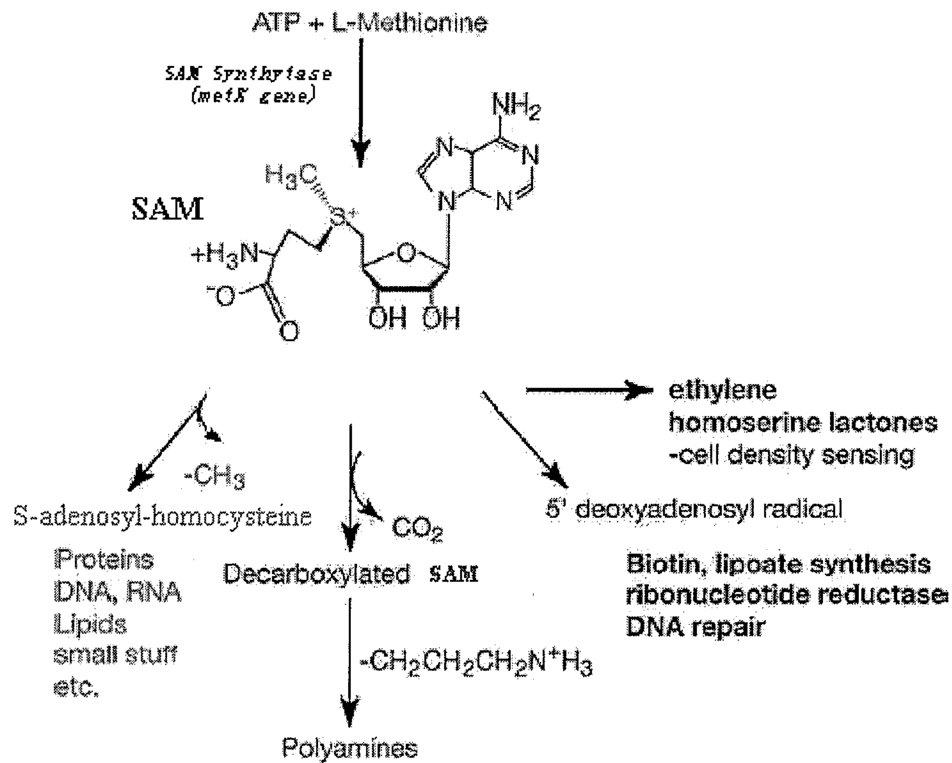


Figure 1. Metabolic roles of S-adenosylmethionine

The image was modified from the picture taken from Markham *et al.*, Scientific Report 2002, Fox Chase Cancer Center.

Table 1. Important SAM-dependent transmethylations

Methyl Group Acceptor	Methylated Product
Norepinephrine	Epinephrine
Guanidinoacetic acid	Creatine
Phosphatidylethanolamine	Phosphatidylcholine
DNA-adenine or -cytosine	DNA-*N*-methyladenine or 5-methylcytosine
tRNA bases	Methylated tRNA bases
Nicotinamide	N^1-Methylnicotinamide
Protein amino acid residues	Methylated amino acid residues

a. Substrates of SAM-dependent methylation range from small metabolites, like norepinephrine, to polymers, such as DNA, RNA, or proteins.

b. The table was taken from Biochemistry *third edition* (Mathews *et al.*).

1.2 SAM is a predominant methyl donor in all organisms

The SAM-dependent methylations provide an essential role in controlling and modulating important cellular activities. Substrates for methylations range from small metabolites, such as norepinephrine, to polymers, such as DNA, RNA and proteins. Some important SAM-dependent transmethylations are listed in Table 1 (Mathews *et al.*, Biochemistry *third edition*). Among these activities, methylations of DNA play a significant role in several important biological processes. Methylation affects the state of the nucleic acid, e.g. restriction and modification of DNA, mismatch error correction (a DNA repair process), and the control of eukaryotic gene expression. It also affects proteins, e.g. methylation of chemotaxis proteins which is involved in signal transduction (Hughes *et al.*, 1987). More details about these activities will be discussed in the following sections.

Once SAM donates its methyl group to the substrates, it breaks down to form S-adenosylhomocysteine (AdoHcy), which is then hydrolyzed to adenosine and homocysteine by AdoHcy hydrolase. Homocysteine can be remethylated to methionine, which can be involved in SAM synthesis again.

1.2.1 DNA methylation

DNA methylation is one of several post-synthetic modifications that normal DNA goes through after each replication. The process of methylation is mediated by enzymes known as DNA methyltransferases.

In eukaryotic DNA, of the four types of base pairs only the Cytosine-Guanine (CG) base pair is methylated. In animals, C in such a sequence is methylated first, followed by methylation of the corresponding G in the complementary strand. In plant DNA, the methylated sequence is CpNpGp, where N can be any of the four bases. In prokaryotic DNA, the major methylated bases are N^6-Methyladenine (mA) and N^4-methylcytosine. Methylation in bacteria occurs at specific sites. In *E. coli*, methylation of A residues in the sequence 5′-GATC-3′ is involved in mismatch error correction, and it plays a role in controlling initiation of DNA replication. Methylation at other sites protects DNA against cleavage by restriction endonucleases (Mathews *et al.*, Biochemistry *third edition*).

DNA methylation has two main significant biological functions:

1) Protection from endonucleases that are designed to destroy foreign DNA, particularly in prokaryotes. Bacterial DNAs are methylated at specific sequences throughout the genome. Foreign DNAs, such as those coming from virus or bacteriophage, are not methylated according to the same pattern. If foreign DNAs enter the cell, they subsequenly are degraded by host endonucleases. This mechanism acts as a defense for bacteria to protect themselves from invading DNAs.

2) Regulation of gene expression during the cell cycle. CpG sites are quite rare in a eukaryotic genome except in regions near the promoter of a eukaryotic gene. These regions are known as CpG islands. The state of methylation of these CpG sites is critical for gene activity and expression. In prokaryotes,

the best-known examples are Dam and Dcm methyltransferases. Dam DNA methylation in *E. coli* has multiple roles, influencing gene expression, chromosome structure (Lobner-Olesen *et al.*, 2003), and even chromosome replication (Boye and Lobner-Olesen, 1990).

1.2.2 Protein methylation

In eukaryotic cells, proteins can be methylated on the side-chain nitrogens of the amino acids arginine, lysine, histidine, and residues containing free carboxyl groups. Methylation of the chromatin proteins, the histones, has been studied in detail in eukaryotic cells. It takes place at specific arginine and lysine residues, and plays a major role in the regulation of gene activity (Kouzarides, 2002).

1.3 *metK*, the only gene encoding SAM synthetase in *E.coli*

The *metK* gene, encoding an enzyme that catalyzes the biosynthesis of S-adenosylmethionine (SAM), has been identified in most microbial genomes. In yeast, there are two genes, *sam1* and *sam2*, which carry out the enzymatic reaction of SAM synthesis. Only one such gene is found in *E.coli*. *metK* is mapped at min 66.492 on the *E.coli* genome and is transcribed clockwise. The open reading frame extends 1152 bases and codes for a 384-residue protein of 41.9KD (Markham *et al.*, 1984). SAM synthetase is a tetramer composing of 4 identical MetK subunits. It catalyzes the following reaction:

L-methionine +ATP+H_2O → S-adenosylmethionine(SAM) +phosphate + pyrophosphate

1.3.1 Regulation of *metK* in *E.coli*

Our lab reported that both leucine and the leucine-responsive regulatory protein (Lrp), a global regulator governing expression of the Leucine/Lrp regulon, have effects on SAM synthetase activity: Lrp represses the *metK* gene expression, while the presence of leucine antagonizes Lrp to release the repression (Newman *et al.*, 1998). Through searching the *metK* promoter, several possible Lrp binding sites are located around the –10 and –35 regions of the *metK*, providing the possibility for Lrp to bind directly to the promoter. However, a CRP (Catabolite Regulatory Protein) binding site was also found at this promoter, 60bp upstream of the *metK* transcription initiation site, implying that CRP might also be involved in the regulation of *metK* expression (Wei, Master thesis, 2001).

1.3.2 *metK* is an essential gene *in E.coli*

The study of Yuhong Wei showed that the *metK* was an essential gene in *E.coli.* She demonstrated that cells could not grow if *metK* was deleted from the chromosome, unless a complementary *metK* expression was available from a rescue plasmid (Wei and Newman, 2002). This supports the view that *metK* is the only gene in *E. coli* which encodes SAM synthetase, and of course, is consistent with the involvement of SAM in so many biological reactions.

1.4 SAM, *metK* gene and cell division in *Escherichia coli*

Previous work from our lab showed that a partial defect in *metK* expression leads to a decrease in the cellular SAM concentration, resulting in a block in cell division. These cells can metabolize for some time, but cannot divide, and therefore, form long filaments (Newman *et al.*, 1998; Wei and Newman, 2002).

mraW, a gene located at the beginning of a *mra* cluster of genes, is involved in cell division and cell wall biosynthesis in most bacteria. It has been reported that MraW exhibited a SAM-dependent methyltransferase activity on membrane-located targets in *E.coli* (Carion *et al.*, 1999). However, several labs have reported that *mraW* and its neighboring gene *mraZ* can be deleted from the chromosome (Arigoni *et al.*, 1998; Merlin *et al.*, 2002).

Filamentation could be explained by a SAM-dependent methylation involved in cell division mechanism. Could MraW be the methyltransferase responsible for that reaction? At present, this has not been determined.

2. The assembly of the division septum in *Escherichia coli*

Two segments compose the bacterial cell cycle: a DNA cycle that includes DNA replication and chromosome segregation; and a division cycle that leads to cytokinesis and cell separation arising after the DNA cycle is complete (Rothfield and Justice, 1997). Both of them are essential for survival of the organism, and remain among the most complex and least understood phenomena in the cell biology of bacteria. Currently, the

division cycle can be divided into several steps and some of them can now be recognized in bacteria, such as *Escherichia coli* (Bi and Lutkenhaus, 1991).

For the cells to divide, the following steps must tale place: (1) selection of the potential division sites; (2) recruitment of the division proteins; (3) invagination of the cytoplasmic membrane accompanied by peptidoglycan; and finally, (4) separation of the daughter cells. Details of steps 1 and 2 follow.

2.1 Determination of the potential division sites

Currently, the combination of two negative regulatory systems is used to explain the selection of the division ring in the model bacterium, *E. coli* (Errington *et al.*, 2003; Norris *et al.*, 2004).

2.1.1 Nucleoid occlusion

The mechanism of nucleoid occlusion was revealed by the observation that cell division was largely inhibited in the cells in which DNA replication and nucleoid segregation failed (Mulder and Woldringh, 1989). When cells are going to divide, there are three sites where DNA is absent or reduced in concentration: one is at mid cell, between the replicated and segregated nucleoids; the other two lie at the cell poles. It is supposed that bacteria may develop a mechanism that senses the sites from which DNA is absent and other sites are occluded. As a result, three spaces are free of DNA and thus,

potential division sites are available: the correct site at mid cell and one at each of the two cell poles.

Optimum nucleoid occlusion activity may require specific organization and structure of the nucleoid. Blocks in transcription, translation or protein secretion influence nucleoid structure, and leading to defective nucleoid occlusion (Sun and Margolin, 2004).

2.1.2. The *min* system

The second mechanism for division site selection is the *min* system, so called because certain mutants of *E. coli* gave rise to aberrant division near the cell poles, resulting in formation of the minicells.

In *E. coli*, the *min* system consists of three proteins, MinC, MinD and MinE. MinC inhibits division ring assembly in areas determined by its interaction with MinD to form MinCD, a negative regulator of septum assembly. The MinCD inhibitor oscillates from pole to pole, associates with the cytoplasmic membrane at the cell poles and finally prevents FtsZ polymerization. MinE also oscillates within a narrow area, moving from site to site about mid cell. It displays topological specificity to the MinCD inhibitor and prevents it from working at mid cell (Corbin *et al.,* 2002).

Through the combined action of these two negative regulatory systems, the correct potential division site is located at mid cell.

2.2 The assembly of septum involves a recognizable order of addition of proteins

2.2 The assembly of septum involves a recognizable order of addition of proteins

Bacterial cell division involves a large number of proteins that localize to the constriction site or septum. These proteins coordinate invagination of the cell membrane, inward growth of the peptidoglycan layer, constriction of the outer membrane and finally, separation of the daughter cells (Buddelmeijer and Beckwith, 2002). In *E. coli*, cell division requires the assembly of at least 11 currently known proteins at mid cell: FtsZ, FtsA, ZpA, FtsK, FtsQ, FtsL, FtsB (previously called YabQ), FtsW, FtsI (also called PBP3), FtsN and AmiC. Many division genes are named *fts*, filamenting temperature sensitive, because they were originally recognized as conditional mutants forming long, aseptate filaments under non-permissive conditions, for instance, temperature-sensitive mutants in *ftsA*, *ftsK* and *ftsI* (Eberhardt *et al.*, 2003).

In *E. coli*, depletion for each of the division proteins revealed a set of dependency relationships that reflect the order in which the division proteins are recruited to the potential division site (Figure 2). FtsZ is the first protein, which localizes to the potential division site, polymerizing to form the Z-ring. ZipA and FtsA localize next, independently of each other but requiring a direct interaction with FtsZ (Poccff and Lutkenhaus, 2002). Next come FtsK and FtsQ; then FtsL and FtsB localizations are dependent on each other and each of them depends on FtsQ too (Buddelmeijer and Beckwith, 2002). Finally, FtsW, FtsI and FtsN are recruited to the septum one after another. FtsN had been thought to be the last of the proteins, but AmiC has now been shown to follow it (Bernhardt and de Boer, 2003). There is no reason to suppose that all

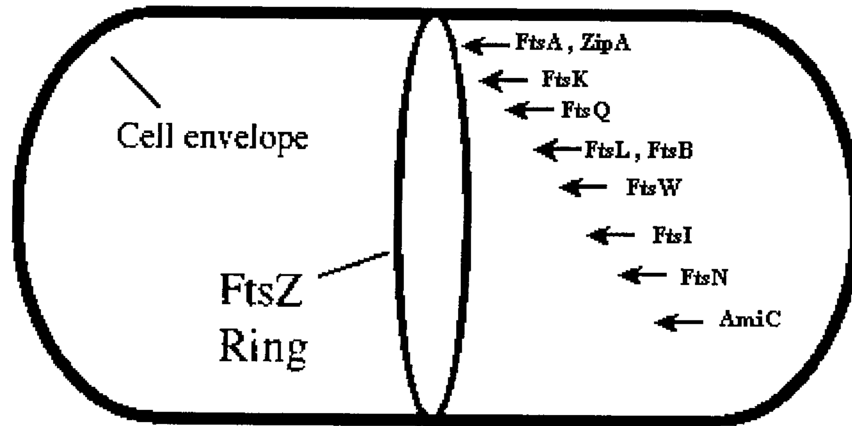


Figure 2. Order of localization of cell division proteins to the septum in *E. coli*

The first event is polymerization of FtsZ into the FtsZ ring. Subsequently, the remaining proteins localize in the order of FtsA, ZipA, FtsK, FtsQ, FtsL and FtsB, FtsW, FtsI, FtsN. The AmiC is the last one to be recruited to the septum.

the proteins involved have been identified.

Despite decades of work about cell division, there is however, little information available on their roles in the division process. In the following parts, I will summarize what we know currently about each of the division proteins.

2.2.1 FtsZ, a key in coordination of the whole process of division

FtsZ is a cytoplasmic protein with a wide distribution and a high degree of sequence conservation in all bacterial and most archaeal species, as well as in the organelles of many eukaryotes. FtsZ is now recognized to play a key role in the assembly of the division septum and in the process of cytokinesis because the Z-ring provides a framework for the subsequent division proteins (Rothfield and Justice, 1997). In *E. coli*, FtsZ is the most abundant of the cell division proteins with about 10,000-20,000 copies per cell.

FtsZ is homologous to tubulin, a cytoskeletal protein in the eukaryotes, both undergoing ATP-dependent polymerization. FtsZ polymerizes to form a ring-like structure at the site of cell constriction. Purified FtsZ exhibits ATP-dependent self-assembly into long polymeric structure *in vitro* (Bramhill and Thompson, 1994). Another review stated that formation of the Z-ring initiates from a specific nucleation site at mid cell, expanding bidirectionally around the circumference (Addinall and Holland, 2002). After formation of Z-Ring, the next two proteins, FtsA and ZipA, interact with FtsZ through its conserved carboxyl terminus (Figure 3).

The crucial role of FtsZ is also indicated by the fact that the well-known endogenous cell division inhibitor, SulA, which is turned on during stress responses, acts by preventing FtsZ polymerization (Mukherjee *et al.*, 1998).

2.2.2 FtsA, an early division protein interacting with FtsZ

FtsA is a member of the actin/DnaK/sugar kinase family of proteins. It is the only known cell division protein, other than FtsZ, that lacks a membrane-spanning domain. There are many fewer molecules of FtsA in the *E.coli* cells, about 100-200 molecules per cell. The cellular ratio of FtsA to FtsZ (1:100) is important for correct division in *E. coli* cells (Dai and Lutkenhaus, 1992).

Protein-protein interaction and localization studies demonstrated that FtsA interacts directly with FtsZ, and its localization completely depends on FtsZ (Wang *et al.*, 1997; Pichoff and Lutkenhaus, 2002).

2.2.3 ZipA, a membrane anchor for FtsZ

Unlike FtsZ and FtsA, ZipA is a membrane protein with an amino-terminal transmembrane segment, a flexible Pro-Gln tether sequence, and a large cytoplasmic carboxy-terminal FtsZ-binding domain (Hale and de Boer, 1999). ZipA is 10-100-fold less abundant than FtsZ.

As shown in Figure 3, the current model for septum constriction requires the ring to be tightly bound to the membrane. ZipA is considered the first candidate to act as a

membrane anchor for FtsZ ring. The reasons are: (1) ZipA is a protein that assembles early in the division site; (2) Topology study shows that ZipA has a large cytoplasmic portion and an amino-terminal transmembrane domain. So ZipA could insert into the membrane and then bind to FtsZ through its large cytoplasmic domain, thus bringing the Z-ring to the membrane by its N-terminal membrane anchor (Errington *et al.*, 2003).

Like FtsA, ZipA interacts directly with FtsZ at mid cell at a very early stage of the division cycle. Binding of ZipA to FtsZ requires a conserved sequence near the C-terminus of FtsZ. ZipA and FtsA localize to the Z ring independently of each other, but they probably do not compete for binding on the same FtsZ molecules. Either one is enough to support formation or stabilization of the Z rings (Pichoff and Lutkenhaus, 2002).

2.2.4 FtsK, a bifunctional protein in *E. coli*

FtsK is one of the largest proteins in *E. coli.* Its open reading frame extends 3990 bases and codes for a 1329-residue protein of 146.4KD. It has a large soluble cytoplamic domain that is highly conserved almost throughout the eubacteria, a N-terminal membrane domain containing four membrane spans and an extremely variable linker that separates two domains (Errington *et al.*, 2003).

In *E. coli,* FtsK is a bifunctional protein required both for proper chromosome segregation and cell division. Studies showed that the first 202 amino acids of FtsK in the N-terminal domain are essential for its role in membrane localization and cell division

(Wang and Lutkenhaus, 1998). The 600 amino acids of the C-terminal domain display homology with SpoIIIE, a protein in *Bacillus subtillis* required for translocation of the chromosome into the prespore during sporulation. The Carboxy-terminal domain is capable of ATP-dependent translocation along DNA, consistent with a role in pumping DNA through a closing septum (Liu *et al.*, 1998; Aussel *et al.*, 2002). Recently, an analysis of molecular diffusion in *ftsK* mutant lacking C-terminal domain contradicted this hypothesis (Goksor *et al.*, 2003). A laser workstation was developed to image diffusion of propidium iodide (PI) in real time by surgical incisions of *E.coli* cell poles. The analysis showed that PI was unable to diffuse from one cell equivalent to another in chain-forming filaments, which were deficient in the carboxy-terminal domain of FtsK, indicating that the cytoplasm of the cell compartments in the FtsKc-deficient cells seemed to be fully separated.

The localization of FtsK depends on the three early division proteins, FtsZ, FtsA and ZipA.

2.2.5 FtsL, FtsB (YgbQ) and FtsQ, form a complex before migrating to the potential division site

FtsL, FtsB and FtsQ of *E.coli* share a similar transmembrane topology: a single cytoplasmic, transmembrane and periplasmic domain. All of these are present at a very low abundance of about 20-40 molecules each per cell. They are essential for cell division, but little is known about their precise functions (Errington *et al.*, 2003).

Localization studies show that their localization depends on FtsZ, FtsA, ZipA and FtsK. Results of genetic analyses for functional interactions suggest that different domains in these proteins are needed for localization in *E. coli* cells: the membrane-spanning segment and the periplasmic domain of FtsL, but not the cytoplasmic domain, are required for localization to the division site; the cytoplasmic domain of FtsQ is necessary for complementation of a null mutant, while the transmembrane segment could be swapped without deleterious effect (Guzman *et al.*, 1997; Chen *et al.*, 1999; Ghigo and Beckwith, 2000; Buddelmeijer and Beckwith, 2002).

These three proteins exhibit some distinct features for localization. In contrast with FtsA/ZipA, FtsB localizes to the division site with FtsL in a codependent fashion, each of them is required for their own stabilization. Recent evidence shows that FtsB, FtsL and FtsQ form a complex *in vivo* before they migrate to the potential division site after FtsK assembly (Buddelmeijer and Beckwith, 2004).

2.2.6 FtsW, a member of the SEDS family of proteins

FtsW of *E. coli* belongs to a large family of polytopic membrane proteins that appear to be present in all bacteria with a peptidoglycan cell wall. This family has been named SEDS, which is derived from shape, elongation, division, and sporulation. Each of the proteins in the SEDS family has 10 transmembrane spans. In *E.coli*, FtsW fixes the SEDS description. The localization studies suggest that FtsW is a late recruit to the division site of *E.coli* and it is required for localization of its cognate FtsI (PBP3) to the septal ring

(Wang *et al.*, 1998; Mercer and Weiss, 2002).

2.2.7 FtsI, an enzyme involved in peptidoglycan synthesis

FtsI, also known as penicillin-binding protein 3, is a cytoplasmic membrane protein with biotopic membrane topologies similar to FtsL, FtsB and FtsQ. It has been estimated that about 100 molecules per cell are present. It is the only *E. coli* cell division protein, other than FtsZ, for which a biochemical function has been defined. Its large periplasmic domain encodes an enzymatic activity, transpeptidase, involved in peptidoglycan synthesis at the division septum. Domain-swapping analysis showed that replacement of the cytoplasmic domain and/or a single membrane-spanning segment resulted in the loss of the ability to support cell division (Guzman *et al.*, 1997). The result from FtsI localization with the presence of β-lactam antibiotics, specific inhibitors for the transpeptidase activity of FtsI, was consistent with this activity (Weiss *et al.*, 1999).

The localization of FtsI to the septal ring requires FtsW, as well as all of the remaining upstream cell division proteins from FtZ to FtsL.

2.2.8 FtsN and AmiC, the last two known proteins for cell division

The *ftsN* gene was isolated as a multicopy suppressor of a temperature-sensitive *ftsA* mutation. Strikingly, its overexpression also suppressed the division defect caused by temperature-sensitive mutations in the *ftsI, ftsQ* and *ftsK* genes. It indicated that FtsN might play a role in stabilization of the septum assembly machinery, or possibly in the

indirect regulation of expressions of some division genes (Rothfield and Justice, 1997).

Domain-swapping analysis showed that the function of FtsN resided in its periplasmic domain since a soluble periplasmic portion was enough to complement the *ftsN*-null mutant (Dai *et al.*, 1996).

For a long time, FtsN was considered to be the last known division protein for the septum assembly in *E. coli.* However, a current report from Bernhardt *et al.* (2003) stated that AmiC, a N-acetylmuramoyl-l-alanine amidase in *E. coli,* replaced FtsN as the last known recruit to the septal ring. AmiC is the first entire periplasm-located protein to be localized at the division site in *E. coli.* Localization study showed that recruitment of AmiC to the ring was mediated by a N-terminal non-amidase targeting domain and required FtsN.

In summary, eleven known division proteins target to the potential division site in a coordinated co-dependent manner, in which they properly organize to form a septum for cell division (Figure 3).

2.3 A defined amount of each division protein is necessary for correct cell division

The majority of known division genes are found within the large *mra* cluster located at 2 min on the *E.coli* genetic map, except *ftsK* at 20.1 min, *ftsB* at 1.2 min and *amiC* at 63.5 min. In 2 min of *mra* cluster, they are clustered with other genes involved in envelope biosynthesis. All of these sixteen genes in 2 min are all transcribed in the same

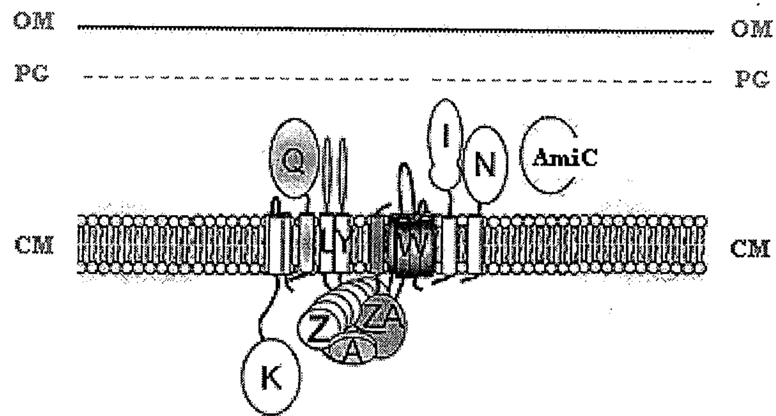


Figure 3. Organization of the cell division apparatus at mid cell

Eleven known division proteins assemble the septum at the potential division site. Their predicted topologies are different.

CM: cytoplasmic membrane OM: outer membrane PG: peptidoglycan

The figure was modified from the picture taken from Errington *et al.*, 2003, Review

direction, with many of the ORFs overlapping or separated by only very short gaps.Expression of these genes is regulated by an extremely complex arrangement of interwoven controls that ensures continuous proper cell growth and division during the *E. coli* life cycle.

The *mra* promoter, at the beginning of the *mra* cluster, is required for full expression of the first nine genes, including the division genes *ftsL, ftsI* and *ftsW*. The transcription of the genes located downstream from these nine genes is also mainly dependent on the P_{mra} promoter, even the expression of the last gene in the cluster, *ftsZ*, is significantly reduced when P_{mra} is repressed (Mengin-Lecreulx *et al.*, 1998). Moreover, multiple promoter sites are present in the ftsQ-A-Z operon, and they also regulate gene expression (Dewar and Dorazi, 2000).

The correct intracellular concentration of the division proteins is extraordinarily important for cell growth. Even small fluctuations in the levels of some essential division proteins can disrupt cell division. For example, overexpression of FtsZ results in minicell formation (Ward and Lutkenhaus, 1985); the proper ratio of FtsZ to FtsA is critical for correct cell growth (Dai and Lutkenhaus, 1992); and overproduction of ZipA abolishes cell division (Hale and de Boer, 1999).

There is no doubt that deletion or inactivation of each division gene leads to blockage of cell division. Some interesting findings showed that some mutants exhibit aberrant cell morphology other than filaments. A report from Dr. Beckwith's lab showed that cells with *ftsL*-null mutant displayed Y-shaped filaments or contained bulges along

the filaments (Guzman *et al.*, 1992). Another study showed that a specific mutation in *ftsA* caused C-shaped cell formation (Gayda *et al.*, 1992).

3. Current conventional methods for division protein localization experiments

The work in this thesis is intended to determine division protein localization in *metK84* filaments, in order to see whether *metK84* filaments have a specific block in the cell division process. To do this, I used methods that are summarized below:

3.1 Green Fluorescent protein (GFP) acts as a marker for protein localization

Green fluorescent protein, GFP, is a spontaneously fluorescent protein isolated from *Aequoriz victori*a, which is a species of jellyfish living in the north Pacific. In jellyfish, its role is to transduce, by energy transfer, the blue chemiluminescence of another protein, aequorin, into the green fluorescent light. When isolated from the jellyfish, the purified protein absorbs ultraviolet light, and then emits it as lower-energy green light (Yang *et al.*, 1996).

The first report from Chalfie demonstrated that expression from the molecular cloning of GFP complementary DNA can be used to monitor gene expression and protein localization in living organisms, such as *Escherichia coli* or eukaryotes (Chalfie *et al.*, 1994). The study has opened a new exciting tool of investigation in cell and molecular biology. Subsequently, GFP has been widely applied in bacteria, yeast, plants, and mammalian cells due to its extreme stability and ease of handling.

GFP, as a reporter, is well introduced into the cell division proteins in *E. coli*. Each of the known division proteins has been studied, by using GFP fusions. This method was used in this work to examine division protein localization in filaments caused by a lack of S-adenosylmethionine. Most of the fusions were inserted in the chromosome and were made available to us by the kindness of Dr. Weiss. One plasmid carried *gfp-ftsK* fusion was also used and described in Materials and Methods section.

3.2 Merodiploid strains constructed by a single-copy gene expression system

As mentioned before, the intracellular concentration of the division proteins must be maintained within defined limits to guarantee the normal division process. One of key strategies for protein localization is to keep the gene expression at a physiological level. A single-copy gene expression system developed from Boyd *et al.* (2000) meets the requirement.

The system transfers the genes from plasmids to the chromosome through bacteriophage lambda derivative, named lambda InCh (for "into the chromosome"). The process requires both homology-dependent and site-specific recombination. Finally, the designed genes integrate into the chromosome at the lambda attachment site with a single-copy expression under the designed promoter coming together from the plasmid. This results in merodiploid strains, which have a wild-type copy of the target gene at the normal chromosomal locus and another copy at the lambda attachment site. Furthermore,

Lambda InCh-derived chromosomal insertions can be readily moved from one strain to another by P1-mediated transduction, resulting in another merodiploid.

Merodiploids were used successfully to demonstrate almost all of the known division protein localization mainly from the labs of Dr. Beckwith and Dr. Weiss.

In this study, I used merodiploids containing different *gfp* fusions to monitor the division protein localization in *metK84* filaments. Each merodiploid carried two copies of the division gene: a wild-type copy located at the normal chromosomal locus with its expression controlled by its normal promoter; and another copy fused to the *gfp* gene and localized at the lambda attachment site. Its expression was under the IPTG-regulatable promoter. FtsK was studied by expression from a plasmid because no chromosomally located *gfp* fusion was available. The wild-type *ftsK* allele was still on its normal site. This meant that the expression of the *gfp* gene fusion was regulated under control of an arabinose-regulatable promoter.

4. The purpose of this study

Previous work from our lab showed that a point mutant in the promoter region of *metK* gene caused the concentration of its product, SAM synthetase, to decrease greatly, so that the SAM pool was low inside the cells. As a result, the cells exhibited a filamentous phenotype under certain growth conditions, in which the SAM level was less than 10% of the wild type, indicating that cell division was blocked in *metK* mutant

background. The question of why cell division was blocked in *metK* mutants was not answered as of yet.

In this study, I am going to answer the following questions:

(1) Does each of the division proteins find its way into the potential division site in *metK* filaments?

(2) If not, which is the first division protein that cannot localize?

Materials and Methods

1. Bacterial strains and plasmids

All of the bacterial strains used in this study were derivatives of *E.coli* K-12

The bacterial strains and plasmids are listed in the Table 2.

2. Media, buffers and solutions

2.1 Recipes for media

The following recipes gave the amount of each component added for 1 liter of liquid medium, which was neutralized to pH7.0 where necessary. Corresponding solid media were made by adding 2% (W/V) bacto-agar.

2.1.1 Luria-Bertani medium (LB)

Bacto-peptone 10 g Yeast extract 5 g NaCl 5 g

2.1.2 Minimal medium (NIV)

$(NH_4)_2SO_4$ 2 g K_2HPO_4 15 g KH_2PO_4 5.25 g.

L-isoleucine 0.5 g L-valine 0.5 g

Sterile $CaCl_2$ (1%,W/V) and $MgSO_4$ (20%, W/V) were added to autoclaved medium before using to a final concentration of 0.004% and 0.08%.

Table 2: Bacterial strains and plasmids

Strains	Relevant genetic marker(s) / feature(s)	Construction	Source / Reference
Strains			
Cu1008	E.coli K-12 *ilv*A		L.S.Williams
MC4100	F⁻ *ara*D139 Δ*lac*U169 *rel*A1 *rps*L150 *thi mot flb*-5301 *deo*C7 *pts*F25 *rbs*R		Laboratory collection from Dr. Jon Beckwith
MEW1	Cu1008 Δ *lac*		Newman *et al.*, 1985
MEW402	NEW1 *metK*84, leucine-requiring	P1 (RG62)×MEW1, screen *serA*⁻	Newman *et al.*, 1998
EC436	MC4100 Δ (λ*attL-lom*)::*bla lacI*^{q} P_{207}-*gfp-ftsI*		Weiss *et al.*, 1999
EC440	MC4100 Δ (λ*att*L-*lom*)::*bla lac*I^q P_{207}-*gfp-ftsN*		From Dr. David Weiss
EC442	MC4100 Δ (λ*att*L-*lom*)::*bla lac*I^q P_{207}-*gfp-ftsQ*		Chen *et al.*, 1999
EC447	MC4100 Δ (λ*attL-lom*)::*bla lacI*^{q} P_{209}-*ftsA-gfp*		Weiss *et al.*, 1999
EC448	MC4100 Δ (λ*attL-lom*)::*bla lacI*^{q} P_{208}-*ftsZ-gfp*		Weiss *et al.*, 1999
EC450	MC4100 Δ (λ*att*L-*lom*)::*bla lac*I^q P_{208}-*zip*A-*gfp*		Weiss *et al.*, 1999
EC791	MC4100 Δ (λ*attL-lom)::bla lacI*^{q} P_{209}-*gfp-ftsW*		Mercer *et al.*, 2002
metK84/ gfp-ftsI	MEW402 Δ (λ*attL-lom*)::*bla lacI*^{q} P_{207}-*gfp-ftsI*	P1 (EC436)×MEW402 → select Amp^r	This study
metK84/ gfp-ftsN	MEW402 Δ (λ*att*L-*lom*)::*bla lac*I^q P_{207}-*gfp-ftsN*	P1 (EC440)×MEW402 → select Amp^r	This study
metK84/ gfp-ftsQ	MEW402 Δ (λ*att*L-*lom*)::*bla lac*I^q P_{207}-*gfp-ftsQ*	P1 (EC442)×MEW402 → select Amp^r	This study
metK84/ ftsA-gfp	MEW402 Δ (λ*attL-lom*)::*bla lacI*^{q} P_{209}-*ftsA-gfp*	P1 (EC447)×MEW402 → select Amp^r	This study
metK84/ ftsZ-gfp	MEW402 Δ (λ*attL-lom*)::*bla lacI*^{q} P_{208}-*ftsZ-gfp*	P1 (EC448)×MEW402 → select Amp^r	This study
metK84/ zipA-gfp	MEW402 Δ(λ*att*L-*lom*)::*bla lac*I^q P_{208}-*zip*A-*gfp*	P1 (EC450)×MEW402 → select Amp^r	This study
metK84/ gfp-ftsW	MEW402 Δ(λ*attL-lom)::bla lacI*^{q} P_{209}-*gfp-ftsW*	P1 (EC791)×MEW402 → select Amp^r	This study
metK84/ gfp-ftsK	MEW402 pBAD *gfp-ftsK*	pF×158 transformed into MEW402 → select Amp^r	This study

Table 2: Bacterial strains and plasmids---- continue

Strains	Relevant genetic marker(s) / feature(s)	Construction	Source / Reference
Cu / *gfp-ftsI*	Cu1008 Δ (λ*attL-lom*)::*bla lacI*^{q} P_{207}-*gfp-ftsI*	P1 (EC436)×Cu1008 → select Amp^r	This study
Cu / *gfp-ftsN*	Cu1008 Δ (λ*att*L-*lom*)::*bla lac*I^q P_{207}-*gfp-ftsN*	P1 (EC440)×Cu1008 → select Amp^r	This study
Cu / *gfp-ftsQ*	Cu1008 Δ (λ*att*L-*lom*)::*bla lac*I^q P_{207}-*gfp-ftsQ*	P1 (EC442)×Cu1008 → select Amp^r	This study
Cu / *ftsA-gfp*	Cu1008 Δ(λ*attL-lom*)::*bla lacI*^{q} P_{209}-*ftsA-gfp*	P1 (EC447)×Cu1008 → select Amp^r	This study
Cu / *ftsZ-gfp*	Cu1008 Δ(λ*attL-lom*)::*bla lacI*^{q} P_{208}-*ftsZ-gfp*	P1 (EC448)×Cu1008 → select Amp^r	This study
Cu / *zipA-gfp*	Cu1008 Δ(λ*att*L-*lom*)::*bla lac*I^q P_{208}-*zip*A-*gfp*	P1 (EC450)×Cu1008 → select Amp^r	This study
Cu / *gfp-ftsW*	Cu1008 Δ(λ*attL-lom)*::*bla lacI*^{q} P_{209}-*gfp-ftsW*	P1 (EC791)×Cu1008 → select Amp^r	This study
Cu/ *gfp-ftsK*	Cu1008 pBAD *gfp-ftsK*	pF×158 transformed into Cu1008 → select Amp^r	This study
Plasmids			
pF×158	PBAD-*gfp-ftsK*		From Dr. David Sherratt

2.1.3 R-top agar for transduction

10g bacto-tryptone	1g yeast extract
8g NaCl	8g bacto-agar

Sterile $CaCl_2$ (1M) and Glucose (20%, W/V) were added to the medium to the final concentration of 2mM and 0.1% separately after autoclaving.

2.1.4 SOC medium for electro-transformation

Bacto-tryptone 20g	Yeast extract 5g	NaCl 10mM
KCl 2.5mM	$MgCl_2$ 10mM	Glucose 20mM

2.1.5 The carbon sources for NIV minimal medium

The carbon sources were sterilized separately from the NIV minimal medium, and then added to the medium where indicated at a final concentration of 0.2% (D-glucose) and 0.5% (glycerol).

2.2 Buffers

2.2.1 SM buffer

SM buffer was used for storage and dilution of bacteriophage λ stocks. Per liter containing:

NaCl	5.8g	$MgSO_4 \cdot 7H_2O$	2g
1M Tris·Cl (pH 7.5)	50ml	2% gelatin solution	5ml

2.2.2 Phosphate-buffered saline (PBS) (pH7.4)

Per liter containing:

NaCl	8g	KCl	0.2g
Na_2HPO4	1.44g	KH_2PO_4	0.24g

2.2.3 Sodium phosphate buffer (pH7.4)

1M	Na_2HPO_4	77.4ml
1M	NaH_2PO_4	22.6ml

Dilute the combined 1M stock solution to 1000ml with distilled H_2O.

2.3 Solutions

2.3.1 Antibiotics

Resistance to ampicillin conferred by the *bla* gene depended on its location. With the gene on the chromosome, 25µg/ml ampicillin allowed maintenance of an antibiotic resistant strain. However, with the many copies of *bla* gene on a plasmid, 200µg/ml ampicillin was used.

2.3.2 Inducer for the expression of various GFP fusion proteins

Expression from the chromosome

All of the GFP fusions were cloned under the control of the *lac* promoter, as described in the localization study from Dr. Weiss' lab. IPTG (Isopropyl-beta-D-

thiogalactopyranoside) was used to induce expression of the GFP fusion proteins. The concentration of IPTG used depended on the strength of each promoter according to Mercer and Weiss (2002). They were:

2.5μM for *ftsZ-gfp*	100μM for *ftsA-gfp*
50μM for *zipA-gfp*	5μM for *ftsQ-gfp*
2.5μM for *gfp-ftsI*	1mM for *gfp-ftsW*
2.5μM for *gfp-ftsN*	

Expression from the plasmid

0.02% arabinose was used for GFP-FtsK expression under the control of paraBAD on the plasmid pF×158.

2.3.3 DAPI (4'-6-Diamidino-2-phenylindole), a DNA staining reagent

DAPI powder (Sigma Co.) was dissolved in PBS buffer to the final concentration 20mg/ml. The aliquots of the stock were kept in the refrigerated and dark place due to its light sensitivity.

2.3.4 Fix solution for microscopy

Per 100 μl fix solution contains:

100 μl 16% paraformaldehyde (DHM Co., EM grade)

0.2μl 50% glutaraldehyde (Sigma Co.)

2.3.5 Cell division inhibitor- aztreonam

Aztreonam was used as a specific cell division inhibitor for FtsI by interacting with its transpeptidase catalytic site. It was stored in –20°C freezer. The stock concentration was 2 mg/ml, and the working concentration was 1μg/ml.

3. Others

Restriction enzymes were purchased from MBI fermentas (Montreal, Canada). Plasmid miniprep Kit was purchased from QIAGEN (Montreal, Canada). They were used to confirm the structure of the plasmid pF×158.

4. P1 phage transduction

P1 phage-mediated transductions were performed as described by using methods current in the laboratory.

5. Growth and harvesting of cells for protein localization

5.1 Preparation of dividing cells

A single colony was grown in LB liquid medium with proper concentration of ampicillin (25μg/ml for the merodiploid strains, 200μg/ml for the strain with the plasmid pF×158) at 30 °C to optical density at $0D_{600}$ of 0.7-1.0. Cells were washed 3 times with NIV medium plus L-leucine 100μg/ml. Then the culture was inoculated 1:200 into fresh NIV medium plus leucine 100μg/ml and grown at 30°C to late log phase of OD_{600} 0.7-

1.0. Cells were washed 3 times again with NIV medium plus 5μg/ml L-leucine and inoculated 1:1000 into fresh NIV medium with leucine 5μg/ml and IPTG. When the optical density was reached to early log phase of OD_{600} 0.2-0.3, a 500μl sample was fixed for protein localization. For Cu/*gfp-ftsK* cells, 0.02% arabinose was added when the OD_{600} was about 0.2, and then cells were fixed after 1-hour further incubation.

5.2 Preparation of filaments

metK84 (MEW402) was a leucine-dependent strain as described earlier (Newman *et al.*, 1998). It required 100μg/ml of L-leucine for normal growth in glucose minimal medium, while it produced filaments after overnight incubation when 5 μg/ml of L-leucine was given.

Growth conditions for merodiploids and the transformant in *metK84* background were similar to those for the dividing cells as described above, except for the last incubation step in NIV medium with 5μg/ml L-leucine. Instead of 5-6 hours incubation for dividing cells, the cultures were incubated in such medium for 16 hours, at which time filaments of a useful length typically formed. Then samples were fixed for protein localization. For *metK84/gfp-ftsK* filaments, 0.02% arabinose was added 1 hour before harvesting.

5.3 Septum formation in the presence of aztreonam

After growth in minimal medium with 5μg/ml L-leucine and IPTG for 10 hours, *metK84* mutants containing various *gfp* fusions became filamentous. Then the mixture of

2× LB with 0.4% glucose was added to dilute the culture 1:2, and 2mg/ml aztreonam was added to a final concentration of 1μg/ml. The cultures were incubated further at 30°C. Filaments were harvested immediately before dilution and at different times after.

6. Fixing of cells and fluorescence microscope methods

6.1 Fixing cells to visualize GFP

A 500μl aliquot of the culture was added directly to 100μl of the fix solution and 20μl of 1 M sodium phosphate (pH7.4). This mixture was incubated at room temperature for 15 minutes, following by incubation on ice for 15 minutes. After centrifugation at low speed of 4,000-5,000rpm for 5 minutes, the pellet was washed 2-3 times in 1-ml of PBS buffer, and resuspended in PBS buffer at a concentration of 50-100μl of PBS per 0.1 OD_{600} unit.

At this point or later, the cells were stable and GFP signal was well protected even after one month. Thus the fixed cells can be kept at 4 °C until needed.

6.2 Staining of DNA with DAPI

To stain DNA, fixed cells were incubated at room temperature in the dark with a mixture of 10μl of 20μg/ml DAPI ((4'-6-Diamidino-2-phenylindole) in 1ml PBS for 5-10 minutes. Then cells were washed 2-3 times with PBS buffer to reduce the background of DAPI, and finally resuspended in PBS buffer at a concentration of 50-100μl PBS/0.1 OD_{600} unit.

6.3 Fluorescence microscopy

Fluorescence micrographs were recorded on a Leica microscope equipped with a 63× Leica oil-objective. The filters used in this study were: a GFP filter for phase contrast and GFP signal; and a DAPI filter for DAPI signal. The exposure times were 0.5 – 1.5 seconds for GFP signal, 0.1 seconds for DAPI signal and 1.0-2.0 seconds for phase image. The images were captured by Hamamatsu Orca C9495 white and black camera and analyzed by the Openlab software for Macintosh.

The experiments of fluorescence microscopy were performed in the BRI (Biotechnology Research Institute). I would like to thank Dr. Whiteway for usage permission and Anne Marcil for technical help. This project could not have been accomplished without their help.

Additionally, the fluorescent microscope in our department is Zeiss Axiophot equipped with a SPOT Insight Color 3.2.0 camera. Unfortunately, they are not suitable for my samples. The reason is that signals from my GFP fusions are very weak, especially from those late division proteins in low abundances in *E.coli* cells, the camera and perhaps the oil objective as well are not sensitive enough to catch GFP signals. The GFP fusions that I could detect in our department were FtsZ-GFP and ZipA-GFP, two early division proteins present in higher numbers of molecules in cells.

7. Plasmid isolation and restriction enzyme digestion

7.1 Plasmid isolation

Plasmid was isolated by QIAgen nimiprep kit following the manufacturer's instructions.

7.2 Restriction enzyme digestion

DNA samples were digested by restriction enzymes from MBI fermentas following the manufacturer's instruction.

7.3 DNA gel electrophoresis analysis

DNA agarose gel electrophoresis analysis was performed as described by Maniatis *et al.* (1989).

8. Electro-transformation

Electro-transformation was carried out by the Gene Pulser (Bio-Rad Co.), following the manufacturer's manual.

9. Construction of strains

9.1 Construction of merodiploids using P1 mediated transduction

All of the strains used in this study, except Cu/*gfp-ftsK* and *metK84/gfp-ftsK*, are merodiploid strains in Cu1008 or *metK84* (MEW402) background: each of them contains two copies of one cell septum-assembling gene. One is the wild type copy, which is located at the original site. Another copy, fused with *gfp*, is located at the λ attachment

site. The wild type strain Cu1008 (*E.coli* K-12 *ilvA*) is used in my study for a comparison with the isogenic *metK84* mutant.

All of the original merodiploid strains were obtained from Dr. Weiss in whose lab the constructions of merodiploids were made, using the λInCh insertion system. In each case, P1 lysates were made on Dr. Weiss's strains and used to infect *Cu1008* and *metK84* (MEW402), selecting transductants on LB plates with ampicillin 25μg/ml. The nature of the transductants was confirmed by examining GFP fluorescence. In this way, the following genes were transferred to Cu1008 and *metK84* (MEW402) respectively:

ftsZ-gfp	from strain EC448
ftsA-gfp	from strain EC447
zipA-gfp	from strain EC450
gfp-ftsQ	from strain EC442
gfp-ftsW	from strain EC791
gfp-ftsI	from strain EC436
gfp-ftsN	from strain EC440

9.2 Construction of *metK84/ gfp-ftsK* using electro-transformation

The plasmid pF×158, which contains *gfp-ftsK* fusion, was obtained from Dr. Sherratt. It was isolated from the strain and transformed into *metK84* (MEW402) cells. The transformants were selected on LB plates with ampicillin 200μg/ml. They were named

metK84/gfp-ftsK (MEW402 pBAD *gfp-ftsK*) and confirmed by monitoring the GFP fusion location via the florescent microscope.

Results

metK is a gene encoding SAM synthetase in *Escherichia coli.* Previous studies in our lab found that a point mutant (A→G), located in the –10 region of the *metK* promoter (*metK84*), causes a decrease in the level of SAM synthetase in cells grown without an external inducer of SAM synthetase, e.g. leucine. In the absence of inducer, the SAM pool is insufficient to maintain growth, and cells exhibit a defect in the cell division (Newman *et al.*, 1998;Wei and Newman, 2001).

The experiments presented here are designed to see whether any part of the septum is made in the filaments. To do this, I investigated which, if any, division proteins localize to the septum. This information might locate the point in septum formation where division breaks down in the *metK* mutant.

The results will be presented in two parts: Part I, the localization of each cell division proteins was monitored by using merodiploid strains that expressed *gfp* fusions to each division gene. The results showed that the early proteins FtsZ, FtsA and ZipA were found in the septum, and the localization frequency of FtsK, an intermediate recruit to the division site, was decreased in the *metK84* filaments. All of the subsequent proteins after FtsK did not localize to the septum. In Part II, the cell division inhibitor, aztreonam, was used to allow all the proteins to accumulate at the septum in the absence of cell division, and showed that if the proteins localized, our methods could detect them.

Part I

Localization of the cell division proteins in the *metK84* mutants

1. The growth conditions for *metK84* and the control strain, Cu1008

1.1 Demonstration that methodology used is adequate

Cu1008 is a wild type of *E.coli* K-12.This strain divides, and locates division proteins to the septum and of course does not form filaments. Thus in a population of cells, those cells which are dividing at any given moment should show division proteins at the septum. I therefore checked that each of the *gfp*-fusions could be seen at mid cell in the dividing cells of Cu1008. This provided a basis of comparison of microscope and image processing for the experiments in *metK84* mutants. It also demonstrated that the overproduction of the division proteins, needed to find their localizations in the cells, did not itself result in abnormal cell morphology. The concentrations of IPTG were different in different *gfp*-fusions as described in Materials and Methods and the same amount of IPTG was used in both strains of Cu1008 and the corresponding merodiploid of *metK84.* If Cu1008 exhibited normal morphology during the growth, it suggested that the extra expression of the division protein from the lambda attachment site did not significantly affect morphology.

For the protein localization, a single colony was grown in LB liquid medium with antibiotics, subcultured into glucose (or glycerol for *gfp-ftsK* fusion expression) minimal

medium with leucine 100μg/ml, and then 5μg/ml. 500μl of aliquots were taken in early log phase for fixation. Details of this experiment are given in Materials and Methods entitled "Growth and harvesting of cells for protein localization".

The GFP images of various *gfp*-fusions localization in Cu1008 showed that the cells exhibited almost normal-length and different *gfp*-fusions bands were localized at mid cell in the majority of the population (Figure 6A, 7A, 8A, 9A, 13A, 14A, 15A, 16A).

1.2 *metK84* is a leucine-requiring strain

Previous work showed that the *metK* mutant displayed a complex morphology in glucose minimal medium with different amounts of leucine (Newman *et al.*, 1998). With a high concentration of leucine (100μg/ml), cells grew normally and cell division occurred as in wild type cells. But when they were incubated with a low concentration of leucine (5μg/ml), the cells did not divide, and formed long filaments with constrictions, indicating incomplete division sites (Figure 4).

This allowed me to routinely prepare filaments from single colonies by incubating in glucose minimal medium with limiting leucine for between 10 and 16 hours. The method for doing this is described in Materials and Methods under "Preparation of filaments ".

2. Nucleoid segregation in *metK84* filaments

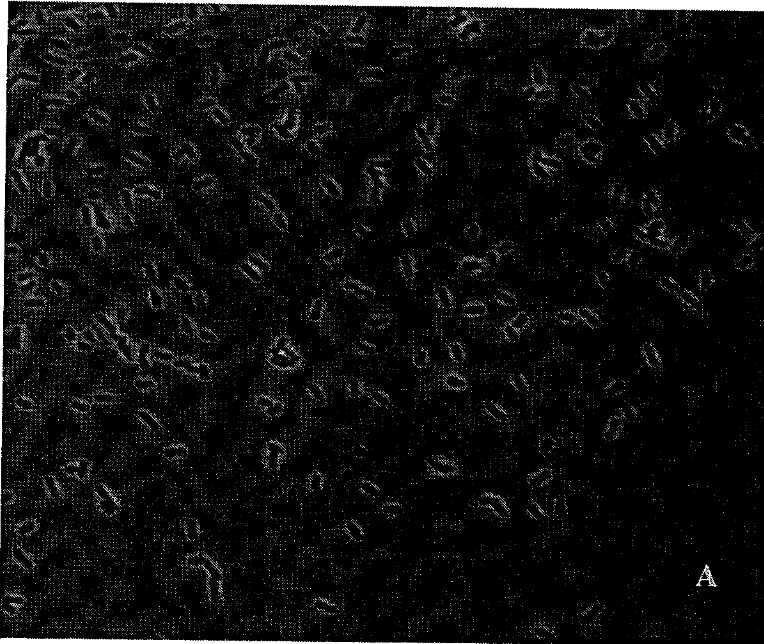
DAPI is a useful tool for DNA staining because it forms fluorescent complexes with natural double-stranded DNA, and this process increases the amount of its blue

fluorescence greatly, permitting a simple visualization of DNA to be seen in the microscope.

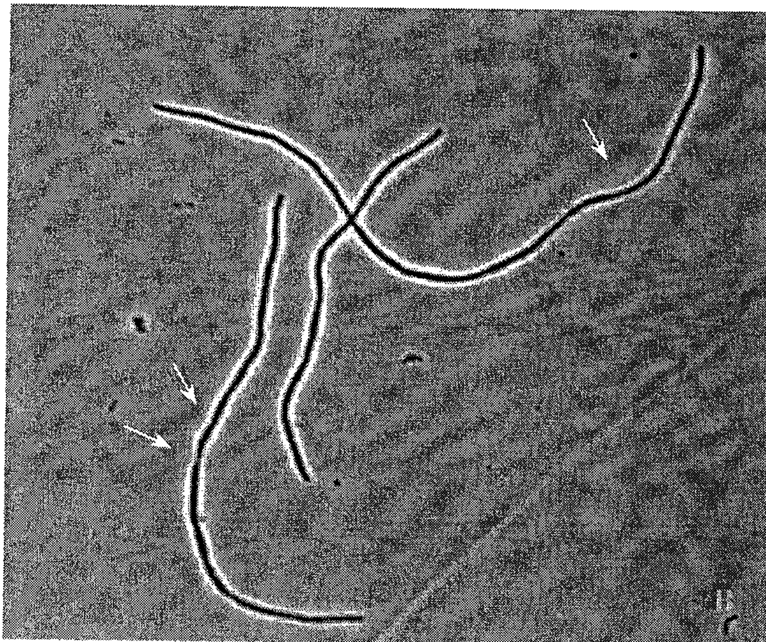
In our study, DAPI stain was used to verify nucleoid segregation in 16-hour *metK84* filaments. Images were recorded in the fluorescent microscope for subsequent localization analysis. 366 filaments were scored for nucleoid segregation. The results were summarized in Table 3. 83% of the cells exhibited even nucleoid distribution throughout the *metK84* filaments (Table 3, Figure 5). 17% of the cells had aberrant nucleoids either highly compacted or extremely diffused, indicating that either the chromosome did not segregate at all, or a part of the filament had partially lysed and contained no DNA (Figure 5).

These results of nucleoid segregation show that DNA replication and chromosome segregation continue in the majority of *metK84* filaments, suggesting that the block of cell division is not caused by problems in DNA segregation. This is in agreement with the previous work in our lab (Newman *et al.*, 1998).

In this study, only the filaments with even nucleoid segregation were scored for localization of the division proteins.



Minimal medium with leucine 100μg/ml



Minimal medium with leucine 5μg/ml

Figure 4. ***metK84*** **is a leucine-requiring strain**

metK84 cells showed different morphology in glucose minimal medium with different concentration of leucine. The white arrows in Figure B point to incomplete division sites along the filaments.

Table 3. Nucleoid Segregation in *metK84* filaments

No. of cells scored	Average cell length ±SD (μm)	Nucleoid Segregation			
		Properly	%	Aberrant	%
366	21.1±13.0	303	83%	63	17%

a. The cells were subcultured from glucose minimal medium with leucine 100μg/ml to glucose minimal medium with leucine 5μg/ml. After 16hours of incubation at 30°C, the cells were collected and fixed for the experiment.

b. The length of the filaments was measured using NIH Image software (http://rsb.nih.gov/nih-image/).

c. SD, standard deviation. SD reflects variation in the length of each filament.

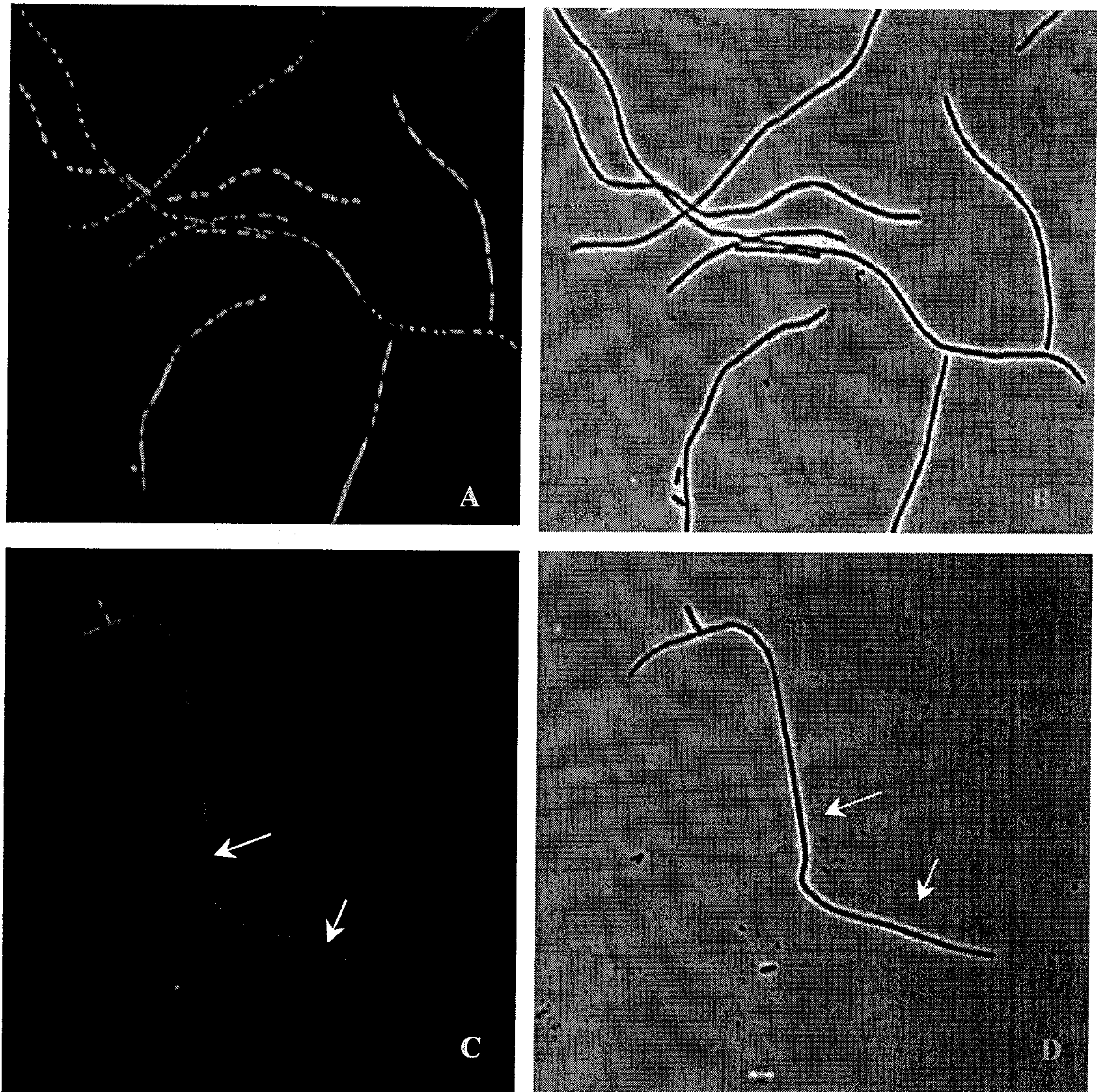


Figure 5. Nucleoid segregation in *metK84* filaments

16-hour m*etK84* culture was fixed to score nucleoid segregation: A and C, DAPI images; B and D, corresponding phase-contrast images.

The majority of filaments displayed regular nucleoid segregation as seen in Figure A, while a few filaments had aberrant nucleoids as seen in Figure C. White arrows in C point to areas that lack nucleoids. White arrows in D point to corresponding parts in the phase-contrast image.

3. Localization of early division proteins in *metK84* filaments

In *Escherichia coli*, a set of at least eleven proteins is required for cell division septum: FfsZ, FtsA, ZipA, FtsK, FtsQ, FtsL, FtsB, FtsW, FtsI (PBP3), FtsN, and AmiC. All of these proteins localize to the constriction site in a defined order. FtsZ is the first protein assembling at the septum, polymerizing to form the Z ring. ZipA and FtsA interact directly with FtsZ, and both localize to the division site after FtsZ and dependent on it, but independent of each other (Hale and de Boer, 1999; Pichoff and Lutkenhaus, 2002). The remaining membrane-bound division proteins are recruited in a linear pathway: FtsK is the first after FtsA/ZipA, then FtsQ → FtsL/FtsB → FtsW → FtsI → FtsN→AmiC. AmiC is the last protein known to be recuited, and its localization requires FtsN, which was formerly considered the last protein (Bernhardt and de Boer, 2003).

It is known that when any one of the cell division proteins is non-functional or absent, cells display a problem in division apparatus, leading to form the filaments. This is indeed how the genes were originally described, *fts* being filamentation temperature sensitive. Therefore, we speculated that the problem in cell division in *metK84* filaments might be due to the failure of one or more than one division protein to localize to the septum.

In order to test this hypothesis, we constructed merodiploid strains in the *metK84* background for each of the division genes and monitored to see whether the GFP, which fused to it, localized to the septum in *metK84* filaments.

3.1 Constructions of merodiploid strains

Various original *gfp-fts* fusions were obtained from Dr. David Weiss, a professor in University of Iowa experienced in the study of cell division proteins in *E.coli.*

We constructed merodiploid strains of *metK84* via P1 phage transduction, selecting for an ampicillin resistance. This resulted in the integration of the *gfp-fts* fusion into the chromosome in single-copy at the lambda attachment site. Expression of the *gfp-fts* fusion was under the control of a weak IPTG-regulated promoter. A wild-type copy of the corresponding *fts* gene located its normal chromosomal locus and its expression was under the control of its native promoter.

The concentration of IPTG used was different for the various promoters. It ranged from 2.5μM to 1.0mM (Mercer and Weiss, 2002).

3.2 Localization of the first division protein, FtsZ, in the *metK84* filaments

FtsZ is the first protein localizing at the future division site. After undergoing GTP-dependent polymerization, FtsZ is assembled into polymers, and then FtsZ polymers form a ring-like structure at the site of cell constriction, supplying a frame structure for all of the downstream division proteins as discussed in a recent review (Errington *et al.*, 2003).

The merodiploids, Cu / *ftsZ-gfp* and *metK84*/ *ftsZ-gfp*, were used to determine FtsZ localization in 16-hour filaments in *metK84*, and in early log phase of Cu1008 cells.

Bright bands of fluorescence at mid cell were seen in most of the parent cells, Cu/*ftsZ-gfp* (>90%, Figure 6A). This demonstrated that the chemical fixing procedure and the microscope did not disrupt the fluorescence signal coming from FtsZ-GFP. Since the cells were of the typical length of wild type *E.coli*, I conclude that the additional copy of FtsZ induced by 2.5μM IPTG did not itself abolish cell division. The same patterns of FtsZ-GFP bands were also observed in 16-hour *metK84* filaments. The DAPI image indicated that the bands localized between the segregated nucleoids, the right place for potential division sites. Each of *metK84* filaments contained multiple FtsZ-GFP bands, and more than 90% displayed regular distribution of the bands (Figure 6C). The spacing of the rings was 3.8μm, which falls within the range of regular distribution between 3.0-12.0μm as reported by the localization studies (Chen *et al.*, 1999; Weiss *et al.*, 1999; Chen and Beckwith, 2001; Mercer and Weiss, 2002).

It is clear from this experiment that FtsZ localizes to the potential division site in *metK84* filaments. The results for FtsZ are summarized in Figure 6 and Table 4.

3.3 Localization of FtsA and ZipA, two independent division proteins, in the *metK84* filaments

FtsA and ZipA interact directly with the Z ring. Both of them localize independently to the division site but require FtsZ to localize. Either one is capable of supporting formation and stabilization of Z rings (Pichoff and Lutkenhaus, 2002).

metK84/ ftsA-gfp and *metK84/ zipA-gfp* were used to check their localization, and were again compared to the corresponding merodiploid strains in the wild type background, Cu1008.

3.3.1 FtsA localization in the *metK84* filaments

FtsA localization was conducted in an experiment with the same protocol as with FtsZ, using a higher concentration of IPTG 100μM.

More than 90% Cu/*ftsA-gfp* exhibited FtsA-GFP bright bands at the mid cell. All of the cells were of normal length (Figure 7A). Thus FtsA overproduction was also not harmful to the cells. Similar FtsA-GFP bands were observed in 16-hour *metK84* filaments. DAPI indicated that the bands localized between the segregated nucleoids, the right place for the potential division site. Each of the *metK84* filaments displayed multiple FtsA-GFP bands (Figure 7C). The spacing of the rings was 3.2μm.

Based on these data, division protein FtsA localizes to the potential division site after FtsZ. The results are summarized in Table 4; the GFP and DAPI images are in Figure7.

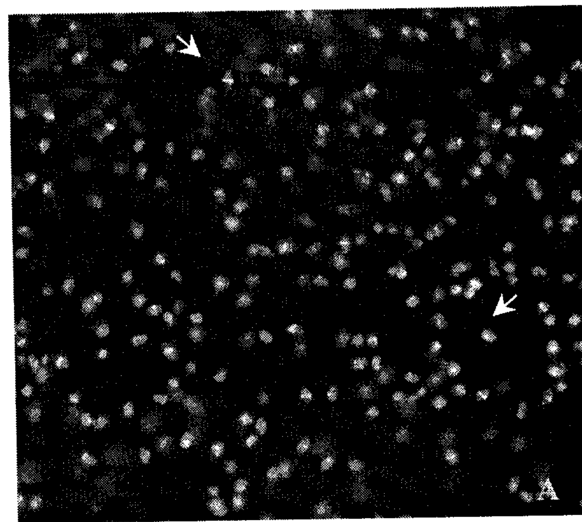
3.3.2 Localization of ZipA, the membrane anchor for Z ring, in the *metK84* filaments

ZipA differs from FtsZ and FtsA in unusual membrane topology in which the N-terminal membrane anchor is followed by a large cytoplasmic portion. ZipA interacts with FtsZ directly via its C-terminal domain and binds the FtsZ ring to the membrane through its large cytoplasmic domain (Errington *et al.*, 2003).

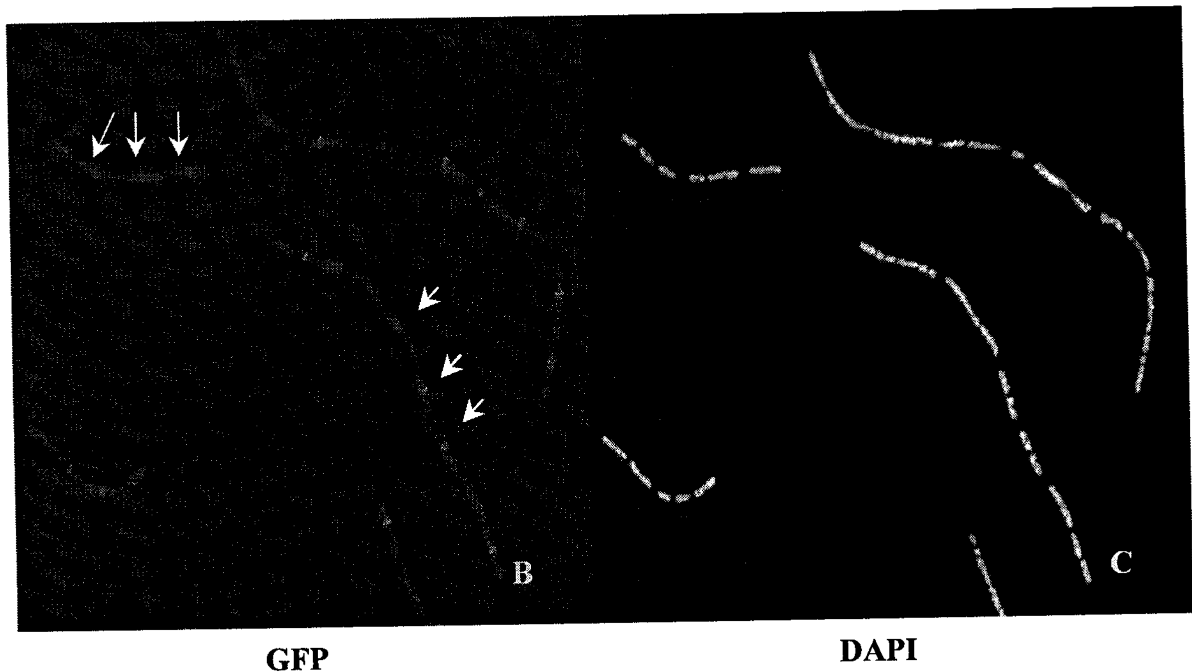
In a similar experiment, this time with 50μM IPTG, ZipA-GFP localization was studied in 16-hour *metK84* filaments and early log phase of Cu1008 cells.

The fluorescent images showed that more than 90% of Cu/*zipA-gfp* cells exhibited ZipA-GFP bright bands at the division site. All of the cells were of normal length (Figure 8A). The same ZipA-GFP bands were observed in 16-hour *metK84* filaments. 100% of *metK84* filaments displayed the ZipA-GFP bands with the regular bands distribution (Figure 8C). The spacing of the rings (the last column of Table 4) indicated that the frequency of ZipA rings was almost the same as the other two early division proteins, FtsZ and FtsA. So ZipA localizes to the potential division site after FtsZ.

In short, the early three division proteins, FtsZ, FtsA and ZipA, localize into the potential division site in *metK84* filaments with a regular distribution. All of them showed similar localization frequencies.



GFP for Cu/*ftsZ-gfp*



GFP **DAPI**

Figure 6. FtsZ localizes to potential division sites in *metK84* filaments

A. Localization of FtsZ-GFP in wild type background (Cu1008)

B – C. Localization of FtsZ-GFP in *metK84* filaments

The *metK84/ftsZ-gfp* culture was collected for viewing with the microscope after 16 hours growth at 30 °C. The Cu/ *ftsZ-gfp* culture (OD_{600} of 0.2-0.3) was collected after 3-4 hours at 30 °C. White arrows point to the localization of FtsZ-GFP bands.

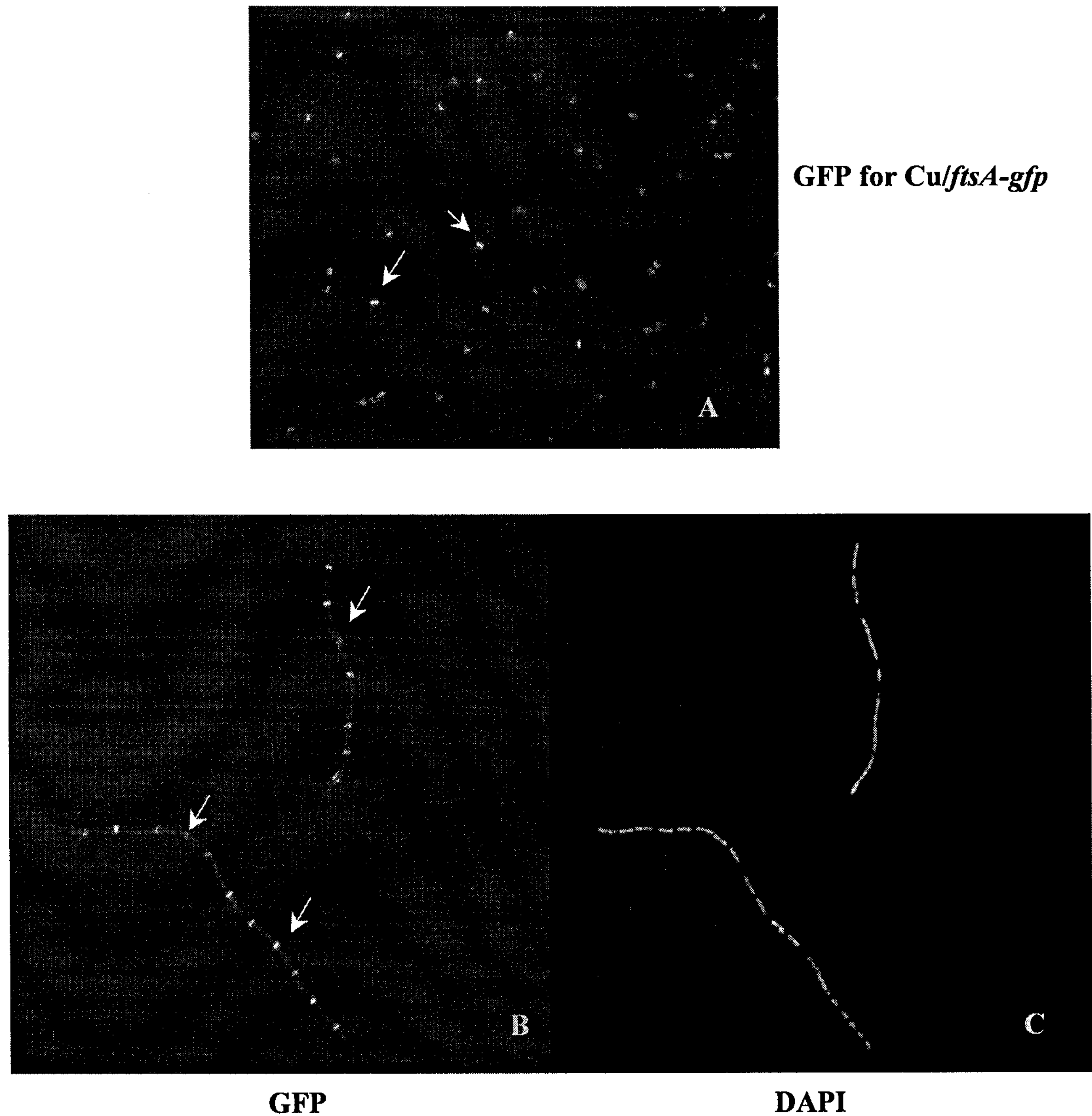


Figure 7. FtsA localizes to potential division sites in *metK84* filaments

A. Localization of FtsA-GFP in wild type background (Cu1008)

B – C. Localization of FtsA-GFP in *metK84* filaments

The *metK84/ftsA-gfp* filaments were collected after 16 hours at 30 °C for examination. The Cu/ *ftsA-gfp* culture (OD_{600} of 0.2-0.3) was collected after 3-4 hours at 30 °C. White arrows point to the localization of FtsA-GFP bands.

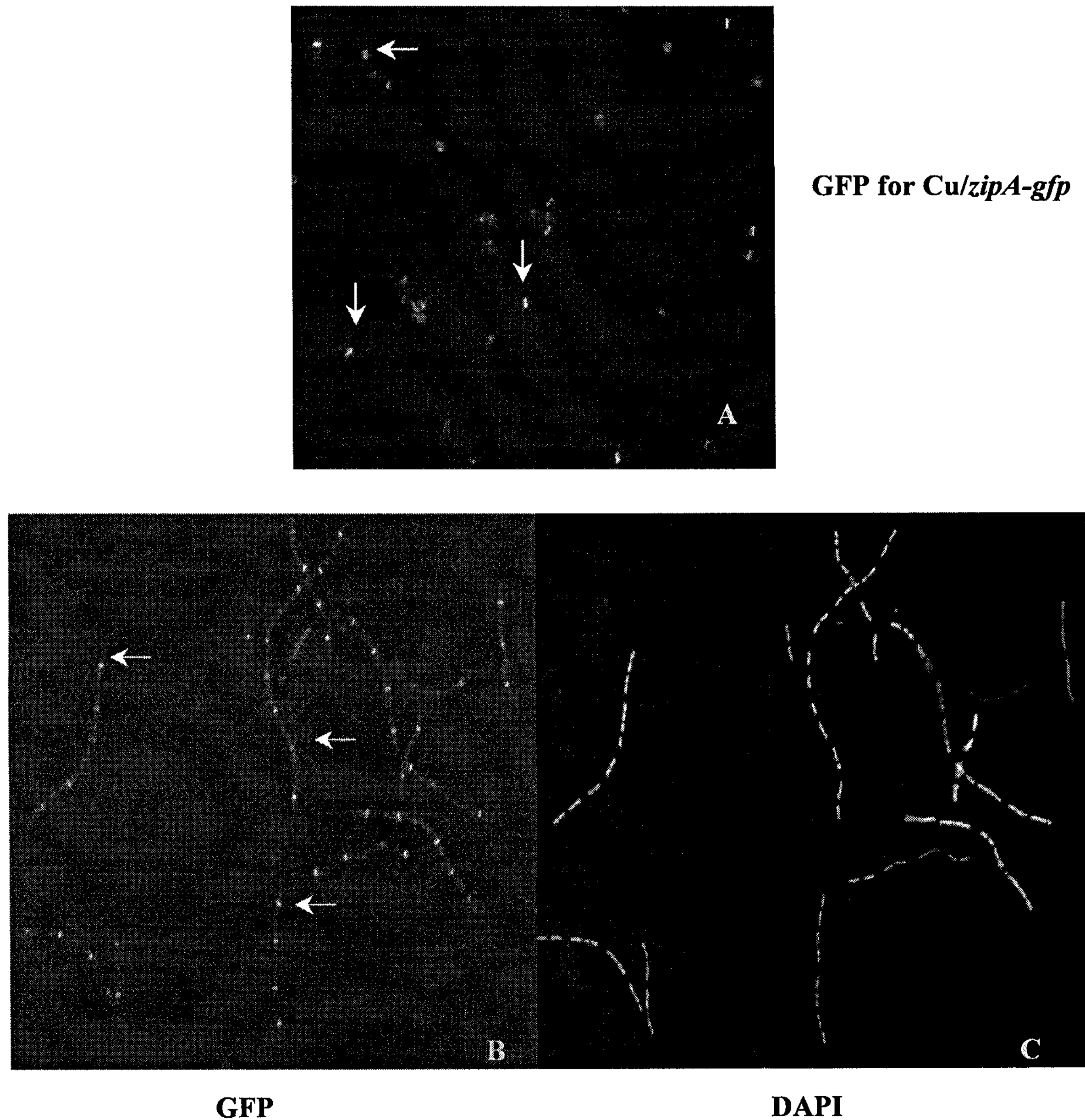


Figure 8. ZipA localizes to potential division sites in *metK84* filaments

A. Localization of ZipA-GFP in wild type background (Cu1008)

B – C. Localization of ZipA-GFP in *metK84* filaments

The *metK84/zipA-gfp* filaments were collected after 16 hours at 30 °C for examination. The Cu/ *zipA-gfp* culture (OD_{600} of 0.2-0.3) was collected after 3-4 hours at 30 °C. White arrows point to the localization of ZipA-GFP bands.

Table 4. Localization frequencies of GFP fusions with early division proteins in *metK84* filaments

GFP fusion	Total no. of cells scored	Total no. of rings	Average cell length ±SD (μm)	% of cells with rings	Spacing of rings (μm / ring)
FtsZ-GFP	154	844	20.8±12.4	100	3.8
FtsA-GFP	76	465	19.8±9.1	100	3.2
ZipA-GFP	190	857	13.0±6.2	100	2.9

a. The16-hour *metK84* filaments were collected and fixed for the experiment as described in Materials and Methods.

b. The length of the filaments was measured using NIH Image software (http://rsb.nih.gov/nih-image/).

c. SD, standard deviation. SD reflects variation in the length of each filament.

d. The spacing is a measure of the frequency of rings per unit cell mass and is calculated by dividing the total number of rings (column3) into the total length of filaments scored (column 2 multiplied with column 4). The spacing is inversely proportional to the frequency of rings.

4. FtsK localization in *metK84* filaments

FtsK is a large protein with several distinct roles in the middle stages of cell division. Its large soluble cytoplasmic domain is required for chromosome segregation, while the N-terminal membrane domain containing four membrane spans is essential for cell division in *E.coli* (Liu *et al.*, 1998; Errington *et al.*, 2003).

In this study, we used a plasmid to express GFP-FtsK instead of constructing the corresponding merodiploid. This is because Dr. Weiss had not constructed the merodiploid of *ftsK*, and advised us that his method for doing so using the lambda Inch system would not work well for this large plasmid.

4.1 Expression of GFP-FtsK fusion under a plasmid-carried pBAD promoter

4.1.1 Plasmid confirmation by enzyme digestion analysis

We obtained plasmid pF×158 from Dr. David J. Sherratt, University of Edinburgh and verifed its structure by extracting the plasmid and digesting it with EcoRI and XbaI to let *gfp-ftsK* (4.7kb) be released from the vector (4.9kb). The HindIII digestion of the plasmid was used to check the insert direction. DNA agarose gel electrophoresis analysis showed the construction was what was expected (Data not shown).

Plasmid pF×158 was transformed into *metK84 and Cu1008* separately, and then Amp^r transformants were selected. They were named *metK84/gfp-ftsK* and Cu/*gfp-ftsK* and used for FtsK localization.

Plasmid extraction, restriction enzyme digestion, DNA gel electrophoresis analysis and

electro-transformation were carried out as described in Materials and Methods section.

4.1.2 Growth condition for *metK84* containing *gfp-ftsK*

While other experiments were done in glucose minimal medium, in this case 0.5% glycerol was used as a carbon source in minimal medium since GFP-FtsK expression from the P_{BAD} promoter would be repressed by glucose. 0.02% arabinose was added to induce GFP-FtsK expression.

For this experiment, strain *metK84/gfp-ftsK* was incubated in minimal medium with glycerol and leucine 5μg/ml for 9 hours to form filaments. Then 0.02% arabinose was added to the culture for 60 minutes further incubation to induce GFP-FtsK expression from the plasmid.

Strain Cu/ *gfp-ftsK* was grown in minimal medium with glycerol and leucine 5μg/ml until it reached OD_{600} of 0.2, at which point, 0.02% arabinose was added and incubation continued for 60 minutes, the cells were collected, fixed and examined microscopically as before.

4.2 Partial localization of FtsK in *metK84* filaments

Unlike the early division proteins FtsZ, FtsA and ZipA, GFP-FtsK showed only partial localization in *metK84* filaments. GFP-FtsK fluorescent bands could not be seen in each of the filaments. 61.2% filaments showed GFP-FtsK bands while 38.8% filaments showed no fluorescent band at all (Table 5). The distribution of GFP-FtsK bands was also

different. Some filaments showed multiple GFP-FtsK bands, while others had only one or none, indicating that they missed the potential localization of FtsK between the fluorescent bands (Figure 9). The spacing of the rings for GFP-FtsK was coherent to the phenomena. For three early division proteins, the spacing of the rings was within the range of 3.0-4.0μm, while the spacing of FtsK ring was10.7μm, which is above the normal distance (Table 5).

Bright bands of fluorescence at mid cell were seen in Cu/*gfp-ftsK* cells (>80%, Figure 9A). All of the cells showed normal length. This indicated two things: First, the additional copy of FtsK did not abolish cell division. Second, since GFP-FtsK bands were observed in majority of dividing cells, Cu/*gfp-ftsK*, nothing in the precedure prevents us from seeing GFP-FtsK bands in *metK84/gfp-ftsK* filaments if they had been there.

In summary, FtsK bands can be seen in *metK84* filaments, but not in all filaments and not evenly distributed. This is quite different from the 3 preceding proteins.

4.3 A change in filamentation morphology observed in glycerol minimal medium

As described previously, *metK84* formed long filaments in glucose minimal medium with a low concentration of leucine. This had not been studied with any other carbon source. However the only clone of *gfp-ftsK* available when I started this work had the *gfp-ftsK* fusion under control of the pBAD promoter. Since pBAD promoter cannot be activated in glucose minimal medium, I grew the *metK84/gfp-ftsK* strain instead with 0.5% glycerol as a sole carbon source. To my surprise, the cells did not become

completely filamentous (Figure 10). Many more single cells were seen under the microscope with relatively few filaments, in contrast to its parental strain *(metK84)* grown in glucose minimal medium in which a majority of cells formed filaments.

In order to find out whether the relative abundance of single cells was caused by either overexpression of FtsK or from growth in glycerol, I grew the parental strain *metK84* in minimal medium supplemented with glycerol. After 16 hours incubation at 30°C, about 35% of the unit of the culture was single cells; after 24 hours, the percentage increased to 50%; and 36 hours worth of culturing yielded a single cell majority along with a few long filaments. However, when *metK84* cells were grown in glucose minimal medium, the percentage of single cells was 10% from a 16-hour culture and 5% from a 24-hour one. After 36 hours, almost all of the cells formed long filaments, and some of them were lysed. This was also true for *metK84/gfp-ftsK* in glucose minimal medium. The results are summarized in Table 6.

Additionally, the effect of FtsK overexpression on *metK84/gfp-ftsK* strain was tested in glycerol minimal medium. In the FtsK localization experiment discussed earlier, I induced with 0.02%arabinose for only 1 hour prior to examining the cells. When I increased this to 2 hours and 16 hours, the same unexpectedly high proportion of single cells was evident. Moreover, the GFP images were so bright, that I could not detect localization of GFP-FtsK in the septum (Data not shown).

In short, the high percentage of single cells in *metK84/gfp-ftsK* was due to the carbon source, glycerol. The *metK84* was not sensitive to the high level of FtsK since no morphology change occurred in *metK84/gfp-ftsK* even after 16 hours of inducing.

4.4. Further investigations on the presence of single cells in glycerol minimal medium

Previous studies in our lab found that *metK84* cells lose their requirement for leucine easily. They can do this by reverting to $metK^{+}$, i.e. increasing *metK* expression due to an intragenic event. They can also do this if they sustain a second mutation in the *lrp* gene, since the *metK lrp* double mutant has increased *metK* expression (Tuan *et al.*, 1990). Could revertants of either kind accumulate in glycerol minimal medium? In order to find the answer, I compared the growth of *metK84* on different plates.

After growth in glucose or glycerol minimal medium with 5μg/ml leucine for 16, 24 and 36 hours, an appropriate dilution of *metK84* cells was plated on minimal medium plates with no additions, to find leucine-independent cells, with leucine 100μg/ml on which all cells should grow, and with leucine 100μg/ml and γ-glutamyl methyl ester (GGME) 500μg/ml on which only *metK* mutants grow (Greene *et al.*, 1973; Newman *et al.*, 1998). I also plated with serine as the sole carbon source, to count the *lrp* mutants.

The distribution pattern of colonies on the plates showed that more than 50% cells in both glucose or glycerol minimal media were still *metK* mutants because they grew on the GGME plates, while approximately 20% of the cells in glucose minimal medium were not leucine-dependent, but this ratio is over 50% in glycerol minimal medium. Less

than 10% of the cells grew on the plates with serine as a sole carbon source, which indicated that most were not *lrp* mutants (Figure 11, 12).

Based on the data in Figure 11, 12, the majority of those cells in glycerol minimal medium were leucine-independent, but still contained the *metK* mutation. Since *lrp* mutants accounted for 10% less after 36 hours incubation, leucine-independent cells contained *metK* mutants other than *lrp*.

These results are very preliminary and lacking in detail. However they suggest that most of the cells even in glycerol are *metK* mutants, and very few have acquired a deficiency of *lrp*. I did not study this further, preferring to try to clone the gene in such a way as to permit *gfp-ftsK* expression in glucose minimal medium.

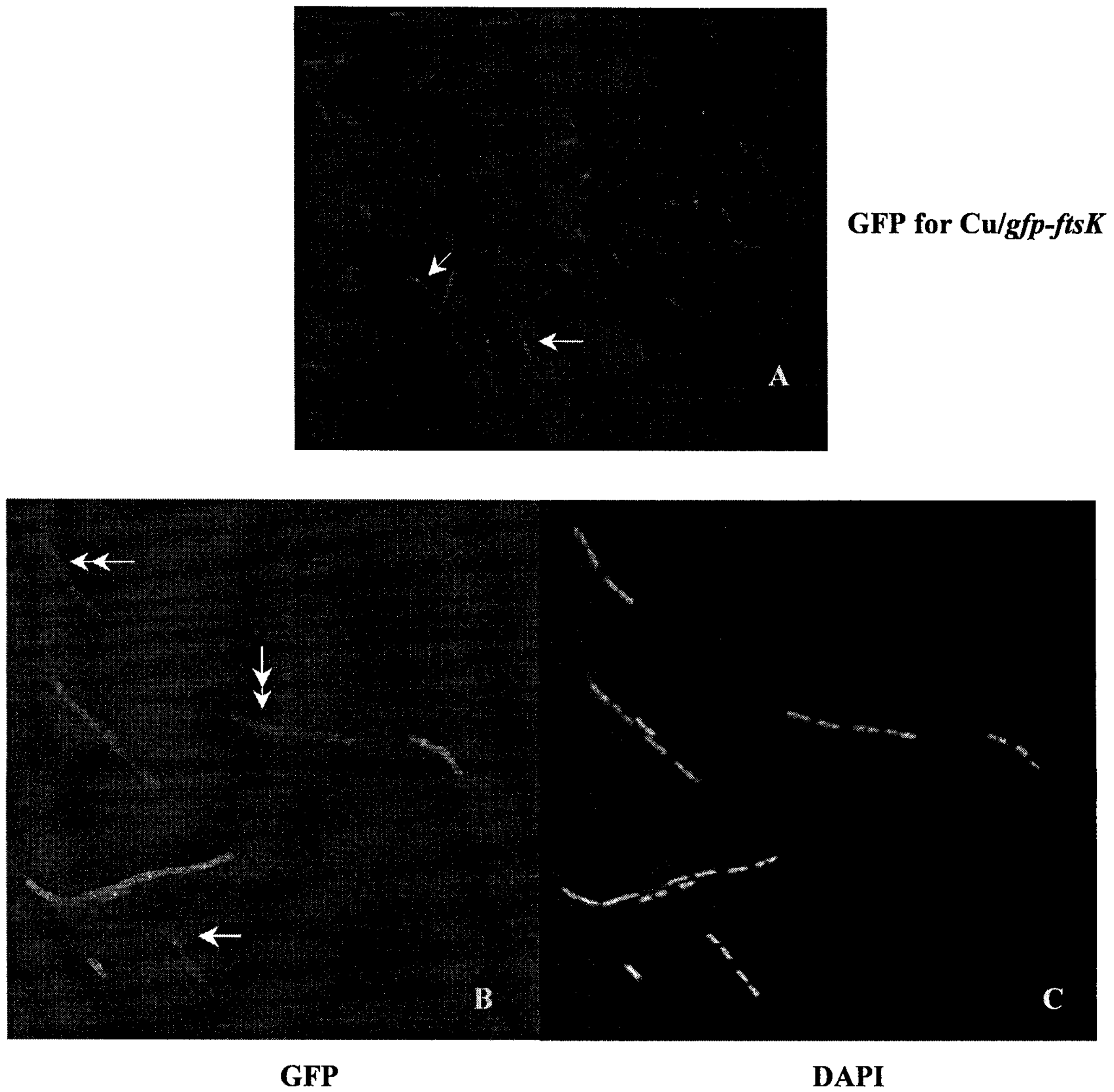


Figure 9. FtsK partially localizes to potential division sites in *metK84* filaments

A. Localization of GFP-FtsK in wild type background (Cu1008)

B – C. Localization of GFP-FtsK in *metK84* filaments

Both *metK84/gfp-ftsK* and Cu/ *gfp-ftsK* cells were collected after having been induced for 60 minutes in 0.02% arabinose. White arrows point to GFP-FtsK bands, double white arrows point to the filaments missing GFP-FtsK bands.

Table 5. Localization frequency of GFP-FtsK in *metK84* filaments

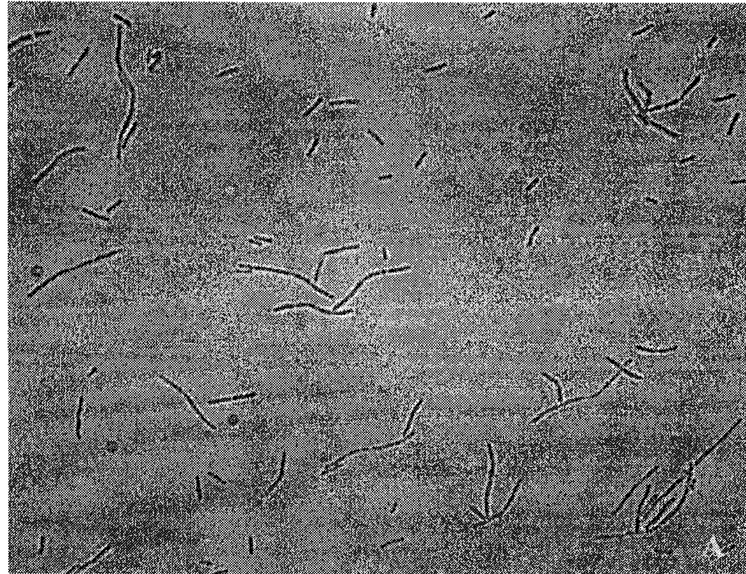
GFP fusion	Total no. of cell scored	Total no. of rings	Average cell length±SD(μm)	% of cells with rings	Spacing of rings(μm / ring)
GFP-FtsK	116	115	10.6±4.4	61.2	10.7

a. After having been induced with 0.02% arabinose, 10-hour *metK84* filaments were collected and fixed for the experiment as described in Materials and Methods.

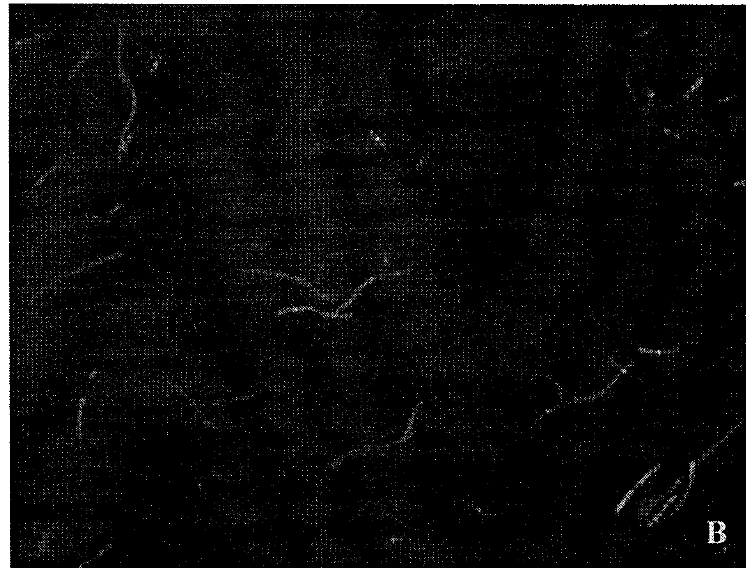
b. The length of the filaments was measured using NIH Image software (http://rsb.nih.gov/nih-image/).

c. SD, standard deviation. SD reflects variation in the length of each filament.

d. The spacing is a measure of the frequency of rings per unit cell mass and is calculated by dividing the total number of rings (column3) into the total length of filaments scored (column 2 multiplied with column 4). The spacing is inversely proportional to the frequency of rings.



Phase-contrast



GFP

Figure 10. Cell morphology of *metK84/gfp-ftsK* grown with glycerol

metK84/gfp-ftsK cells were grown in glycerol minimal medium at 30 °C for 10 hours. About 50% single cells could be seen under the microscope.

A. Phase- contrast image for *metK84/gfp-ftsK*; B. GFP image for *metK84/gfp-ftsK*.

Table 6. **Morphology of *metK84* cells grown in minimal medium with different carbon sources**

Strains	Incubation time (hours)	Glucose minimal medium	Glycerol minimal medium
metK84	16	10% single cells, 90% filaments with 8-50μm length	35% single cells, 65% filaments with 8-50μm length
	24	Only 5% single cells, most filaments were 30-100μm long, and a few of them were lysed	50% single cells, 50% filaments. Most filaments were 8 -50μm long
	36	Very long filaments with 200 -400μm length, some of them were lysed. A few single cells were seen	A majority of single cells (>80%) mixed with long filaments (20-400μm)
metK84/gfp-ftsK	16	15% single cells, 85% filaments with 8-30μm length	50% single cells, 50% filaments with 8-30μm length
	24	15% single cells, 85% filaments with 8-100μm long	80% single cells, 20% filaments with 8-100μm long
	36	Very long filaments with 200-400μm length, 20% single cells	>95% single cells with some very long filaments

a. The data were based on two individual experiments. **b.** 5μg/ml leucine was added in both minimal media.

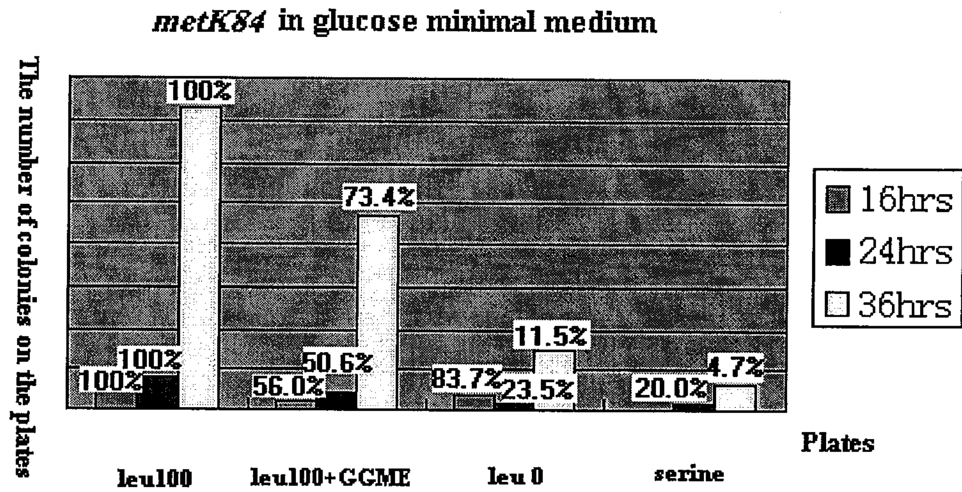


Figure 11. The distribution of cells grown in glucose on different plates

a. The *metK84* culture grown in glucose minimal medium at different incubation times (16,24 and 36 hours), then was plated on various minimal medium plates (descriptions seen in the text).

b. The distribution pattern was based on two individual experiments.

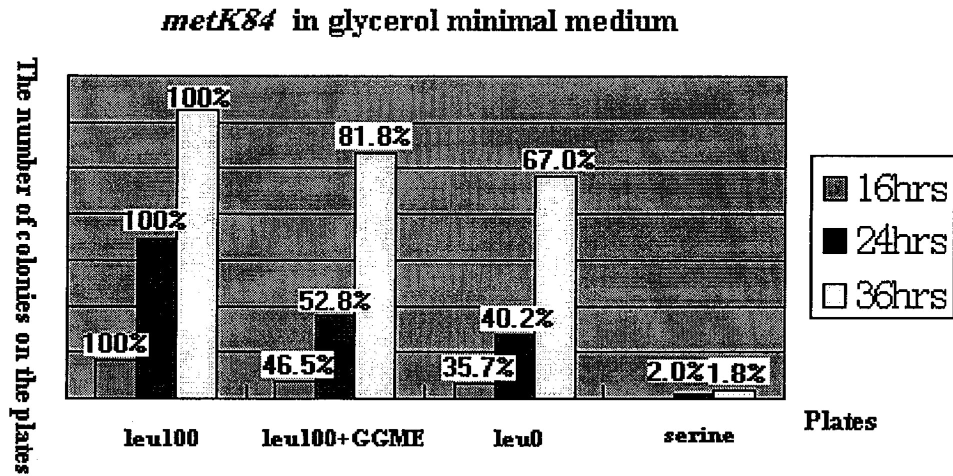


Figure 12. The distribution of cells grown in glycerol on different plates

a. The *metK84* culture grown in glycerol minimal medium at different incubation times (16, 24 and 36 hours), then was plated on various minimal medium plates (descriptions seen in the text).

b. The distribution pattern was based on two individual experiments.

5. Late division proteins, FtsQ, FtsW, FtsI and FtsN, fail to be recruited to the septum in *metK84* filaments

All of the late proteins involved in cell division have at least one membrane span and a substantial extracellular domain. A large periplasmic domain in bitopic proteins of FtsI, FtsQ and FtsN suggests that these proteins participate in division-related functions that occur outside the cytoplasmic membrane (Rothfield and Justice, 1997). Currently, FtsI is the only one of the late proteins with a defined biochemical function: Its periplasmic domain encodes a transpeptidase required for synthesis of peptidoglycan in the division septum (Guzman *et al.*, 1997; Weiss et *al.*, 1999).

Merodiploid strains with various *gfp* fusions of late division proteins in either *metK84* or wild type background were used to demonstrate their localization in *metK84* filaments.

5.1 Localization of FtsQ in *metK84* filaments

For FtsQ localization, I used 5μM IPTG to induce *gfp-ftsQ* in Cu/*gfp-ftsQ* and *metK84*/*gfp-ftsQ* cells respectively.

The majority of Cu/*gfp-ftsQ* cells contained GFP-FtsQ bands at mid cell, and the cells appeared normal (Figure 13A). In contrast with the results in Cu1008 background, most of the *metK84* filaments showed no GFP bands. The GFP-FtsQ bands occasionally observed in filaments, looked very faint compared with ones in Cu/*gfp-ftsQ* cells (Figure 13C). Thus, the localization frequency of FtsQ was greatly reduced in *metK84* filaments (Figure 13, Table 7).

5.2 Localization of FtsW in *metK84* filaments

1mM IPTG was used to examine GFP-FtsW localization. In the GFP images for GFP-FtsW localization, the majority of Cu/*gfp-ftsW* cells exhibited GFP-FtsW bands at the dividing site. All of the cells were of normal length (Figure 14A), indicating that the GFP-FtsW fusion had the right function and that the experimental procedure did not disrupt it under the conditions used. However, in *metK84/gfp-ftsW* filaments, the GFP-FtsW band was rarely seen. The GFP-FtsW bands were absent from 90% of *metK84/gfp-ftsW* filaments. Thus, the localization frequency of FtsW decreased dramatically in *metK84* filaments (Figure 14, Table 7).

5.3 Localization of FtsI in *metK84* filaments

This time 2.5μM IPTG was used to induce *gfp-ftsI* expression from λatt on the chromosome. GFP-FtsI also localized normally in the wild type background. More than 50% of Cu/*gfp-ftsI* cells showed GFP-FtsI bands at mid cell, and all of the cells were of normal length (Figure 15A). The GFP-FtsI bands were rarely seen along the *metK84/gfp-ftsI* filaments, and absent in the majority of filaments (Figure 15, Table 7).

5.4 Localization of the last division protein, FtsN, in *metK84* filaments

2.5μM IPTG was used to induce *gfp-ftsN* expression in Cu/*gfp-ftsN* and *metK84/gfp-tsN* respectively. The localization results are similar to other late division proteins. GFP-FtsN bands were displayed in the majority of Cu/*gfp-ftsN* cells (Figure 16A). However,

GFP-FtsN bands were rarely observed in the *metK84/gfp-ftsN* filaments (Figure 16, Table 7). So GFP-FtsN, like other late proteins, failed to localize to the septum in *metK84* filaments.

In summary, GFP fusions to various late division proteins, FtsQ, FtsW, FtsI and FtsN, rarely showed fluorescence at potential division sites. Occasionally, I observed faint fluorescent bands in a few *metK84* filaments, but each of them contained only one or two rings. Calculations of the spacing between rings (last column of Table 7) showed that the distance between adjacent rings were 20-25 folds larger than that seen with early division proteins, indicating that localization of FtsQ, FtsW, FtsI and FtsN is greatly reduced in *metK84* filaments. I conclude that late division proteins fail to congregate at the septum in *metK84* filaments.

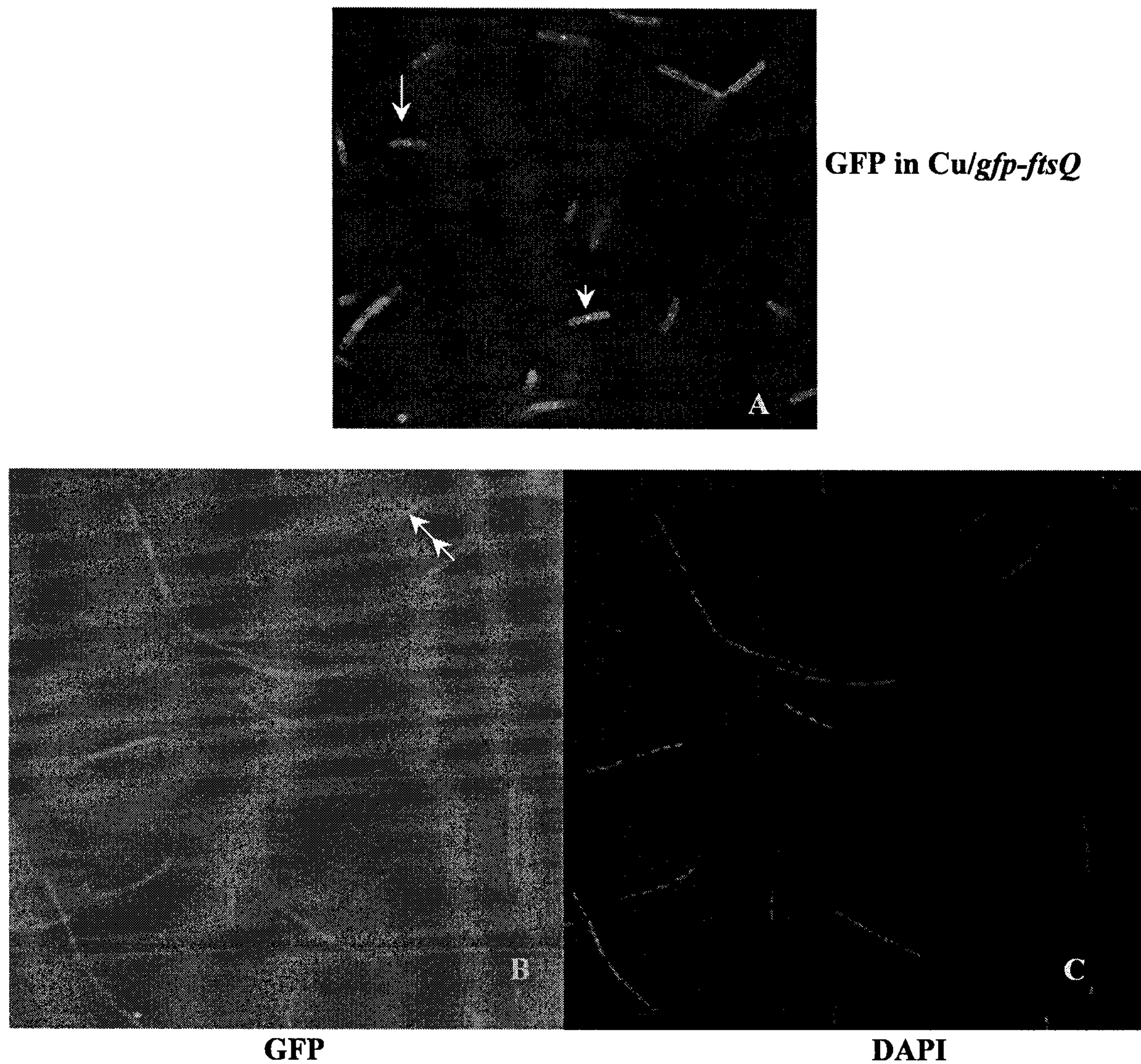


Figure 13. Localization of division protein FtsQ in *metK84* filaments

A. Localization of GFP-FtsQ in wild type background (Cu1008)

B – C. Localization of GFP-FtsQ in the metK84 filaments

The 16-hour *metK84/ gfp-ftsQ* culture at 30°C was collected for microscopy. The culture of Cu/ *gfp-ftsQ* was collected after 3-4 hours growing at 30°C. White arrows in A point to the bright bands in Cu while the white double arrow in B points to the faint band of GFP-FtsQ.

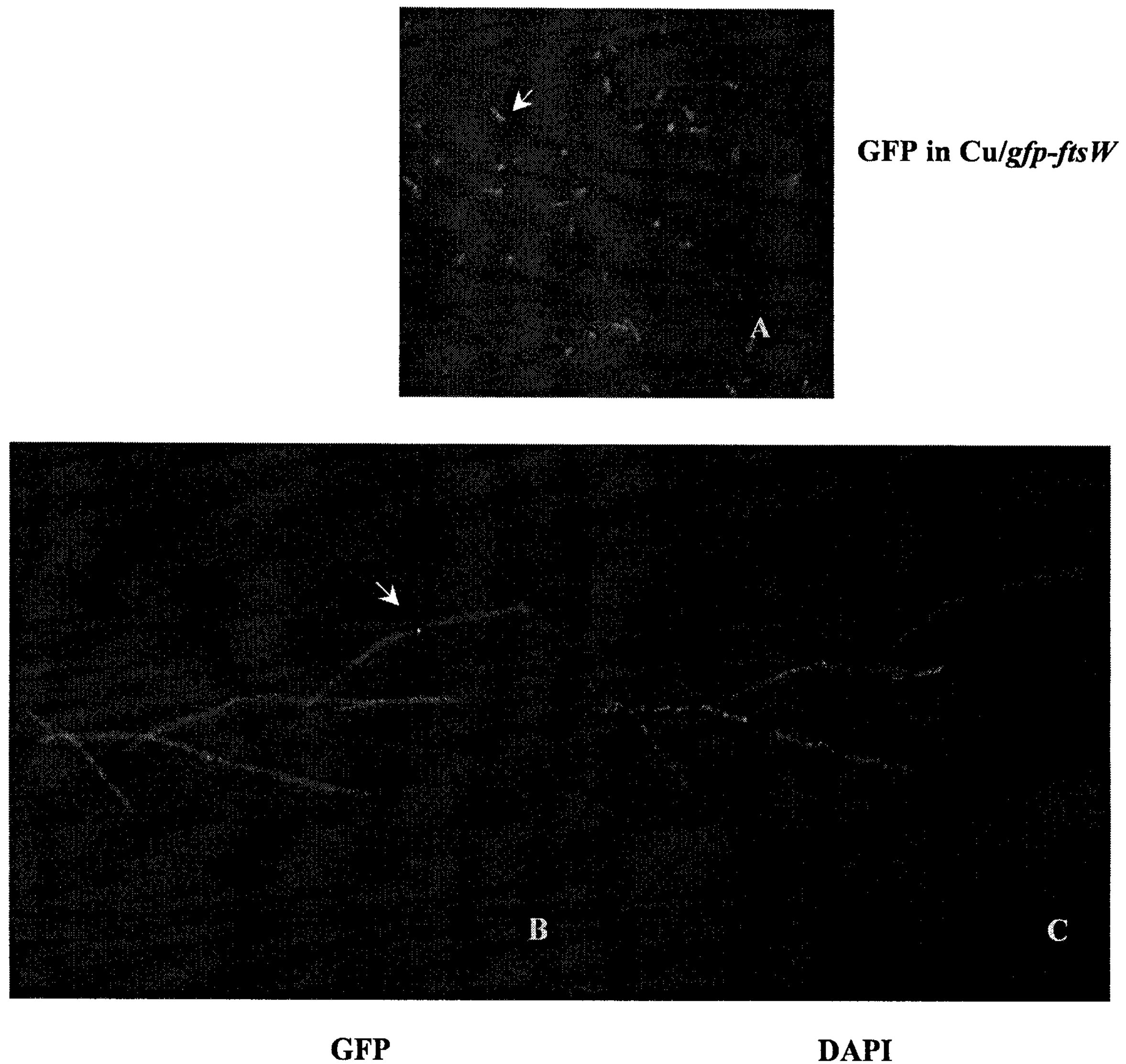


Figure 14. Localization of division protein FtsW in *metK84* filaments

A. Localization of GFP-FtsW in wild type background (Cu1008)

B – C. Localization of GFP-FtsW in *metK84* filaments

The *metK84/ gfp-ftsW* filaments were collected after 16 hours at 30°C. Cu/ *gfp-ftsW* cells with OD_{600} of 0.2-0.3 were collected after 3-4 hours growing at 30°C. The white arrows point to the localization of GFP-FtsW. GFP-FtsW rings were absent from most *metK84* filaments.

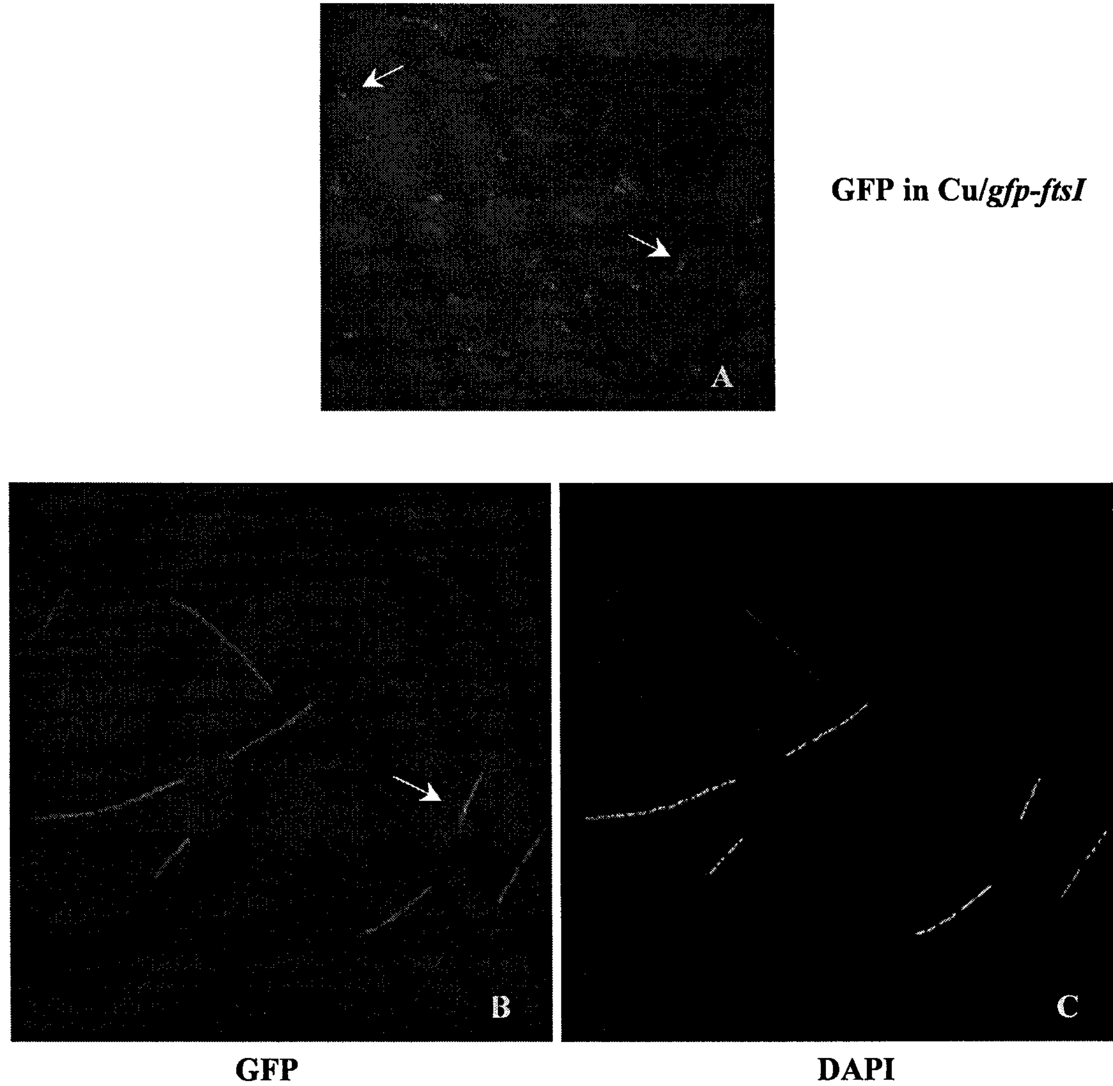


Figure 15. Localization of division protein FtsI in *metK84* filaments

A. Localization of GFP-FtsI in wild type background (Cu1008)

B – D. Localization of GFP-FtsI in *metK84* filaments

The16-hour *metK84/ gfp-ftsI* filaments at 30°C were collected for microscopy. Cu/ *gfp-ftsI* cells were collected when the OD_{600} reached 0.2-0.3. White arrows in A and B point to the localization of GFP-FtsI.

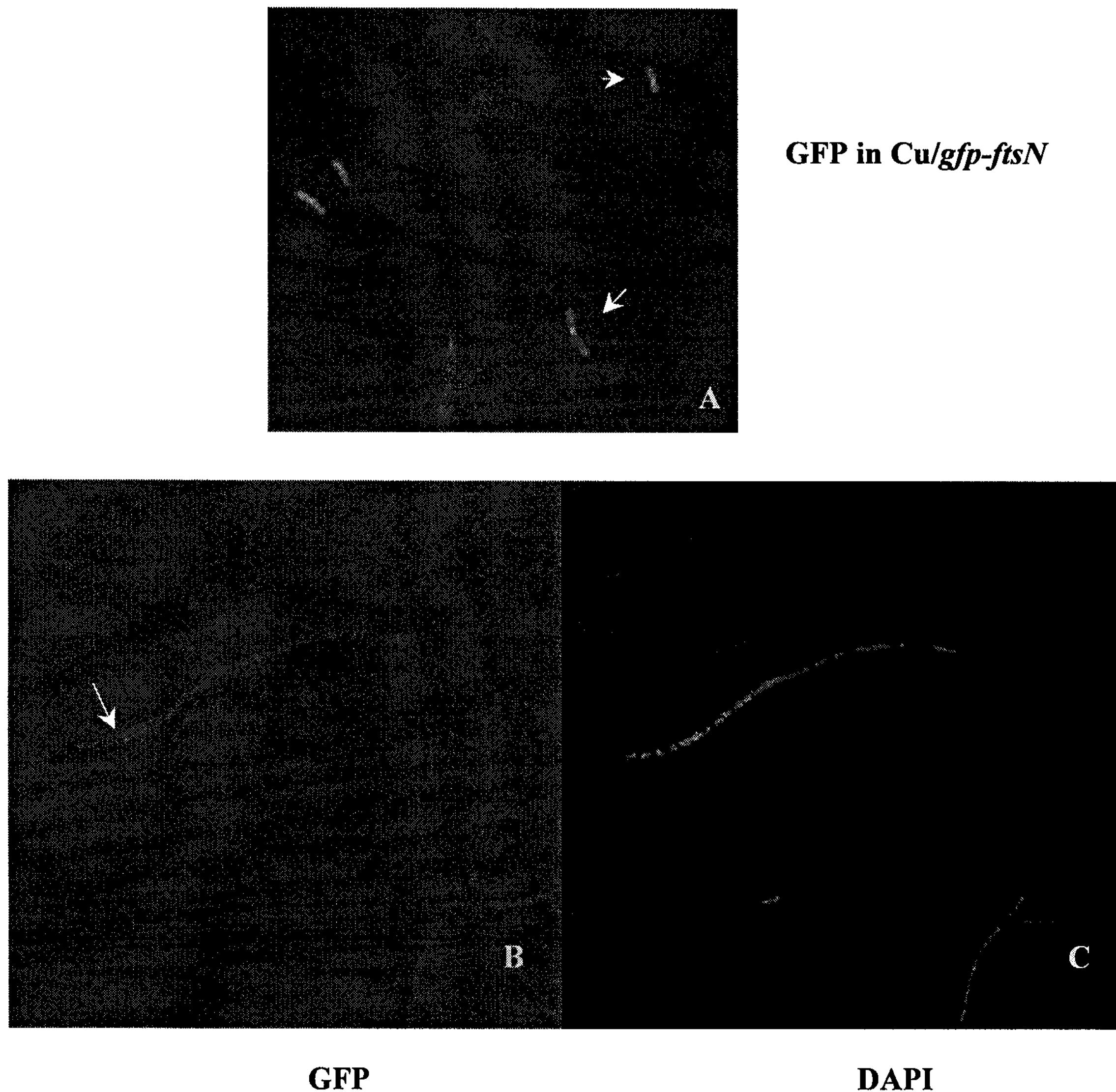


Figure 16. Localization of division protein FtsN in *metK84* filaments

A. Localization of GFP-FtsN in wild type background (Cu1008)

B – D. Localization of GFP-FtsN in *metK84* filaments

The *metK84/ gfp-ftsN* filaments were harvested after 16 hours incubation at 30°C. For Cu/ *gfp-ftsN*, the culture with OD_{600} of 0.2-0.3 was collected after 3-4 hours growing at 30°C. The white arrows in A and B point to the localization of GFP-FtsN. GFP-FtsN rings were absent in most *metK84* filaments.

Table 7. Localization frequencies of GFP fusions with late division proteins in *metK84* filaments

GFP fusion	Total no. of cells scored	Total no. of rings	Average cell length ±SD (μm)	% of cells with rings	Spacing of rings (μm / ring)
GFP-FtsQ	113	20	22.8±12.3	16.0	128.8
GFP-FtsW	69	8	17.3±8.4	11.6	149.2
GFP-FtsI	44	7	21.7±12.5	16.0	136.4
GFP-FtsN	40	6	17.2±10.2	15.0	114.7

a. The 16-hour *metK84* filaments were collected and fixed for the experiment as described in Materials and Methods.

b. The length of the filaments was measured using NIH Image software (http://rsb.nih.gov/nih-image/).

c. SD, standard deviation. SD reflects variation in the length of each filament.

d. The spacing is a measure of the frequency of rings per unit cell mass and is calculated by dividing the total number of rings (column3) into the total length of filaments scored (column 2 multiplied with column 4). The spacing is inversely proportional to the frequency of rings.

Part II

Localization Recovery for late division proteins, FtsK, FtsQ, FtsW, FtsI and FtsN, in *metK84* background

The results in Part I show that early division proteins, FtsZ, FtsA and ZipA, localize into the potential cell division site. The data of localization frequencies confirmed their localization: the spacing between rings is within the range of 3.0-4.0μm, which is a expected range for division protein localization reported from various laboratories (Chen *et al*, 1999; Chen and Beckwith, 2001; Mercer and Weiss, 2002). Localization frequency decreased sharply with the later proteins; GFP fusions with late division proteins were rarely observed in *metK84* filaments, whereas some FtsK could be seen in the setpum.

Do late division proteins fail to target to the septum in *metK84* filaments? Might our expermental procedures be not sensitive enough to detect these unabundant GFP fusions at the septum in *metK84* filaments? We took advantage of the properties of aztreonam to answer these questions, a method described in the work of Dr. Weiss (Arends *et al.*, 2004). Aztreonam permits the localization of all the division proteins to the septum, but does not allow cell division because it is a specific inhibitor for FtsI by disrupting its transpeptidase activity. After incubation of cells or filaments with aztreonam in otherwise permissive conditions, one should be able to see any division proteins that are present in the septum. My experiments indicate that this is indeed the case.

1. The growth conditions for Merodiploids/ transformants with aztreonam in *metK84* background

For these experiments, I allowed filaments to be formed during incubation with limited leucine and took samples for examination, while continuing the incubation with aztreonam and nonlimited leucine. This permits the cells to complete septum formation if they are able to do so. The details of the experiment are presented in Materials and Methods under the heading "septum formation in the presence of aztreonam".

After 10 hours incubation, a sample was fixed to show the state of the filaments prior to the aztreonam treatment. Then 5ml of the culture was transferred to a flask containing 5ml of 2×LB medium plus 0.2% Glucose, and at this time aztreonam was added to the flask. Cultures were then collected at different times after adding aztreonam.

2. Aztreonam treatment does not interrupt the assembly of division septum

2.1 Aztreonam blocks cell division by interacting with division protein FtsI

FtsI, one of the late division proteins, is a transpeptidase required for cross-linking of the peptidoglycan cell wall during division. Aztreonam forms a covalent adduct with a serine residue in the transpeptidase catalytic site and thus prevents synthesis of septal peptidoglycan. This causes a defect in cell division.

2.2 Aztreonam does not affect the presence of early division proteins in *metK84* filaments

Arends *et al.*(2004) had demonstrated that the presence of aztreonam does not prevent the recruitment of FtsI, nor does it prevent subsequent localization of FtsN. Filaments obtained by aztreonam treatment are due to the inactivation of FtsI.

In part I, I demonstrated that FtsZ, FtsA and ZipA localize in the potential site in *metK84* filaments. Is their localization in *metK84* background destroyed if we transfer our filaments to a medium containing aztreonam?

In order to test this, *metK84/ftsA-gfp* was used as a demonstration to check FtsA-GFP localization in the presence of aztreonam. After a 10 hour incubation at 30 °C with IPTG, *metK84/ftsA-gfp* filaments were transferred to LB medium plus aztreonam, at which point 500μl of the culture was fixed starting at T = 0min. Later filaments were fixed at T=60 minutes. This was to show the effect of aztreonam on FtsA localization.

Figure 17 indicated that FtsA localization was not abolished after adding aztreonam. The filaments displayed the regular FtsA-GFP distribution in the cells before and after adding aztreonam.

So I conclude that aztreonam does not affect the localization of early division proteins when *metK84* filaments are put back to the rich medium.

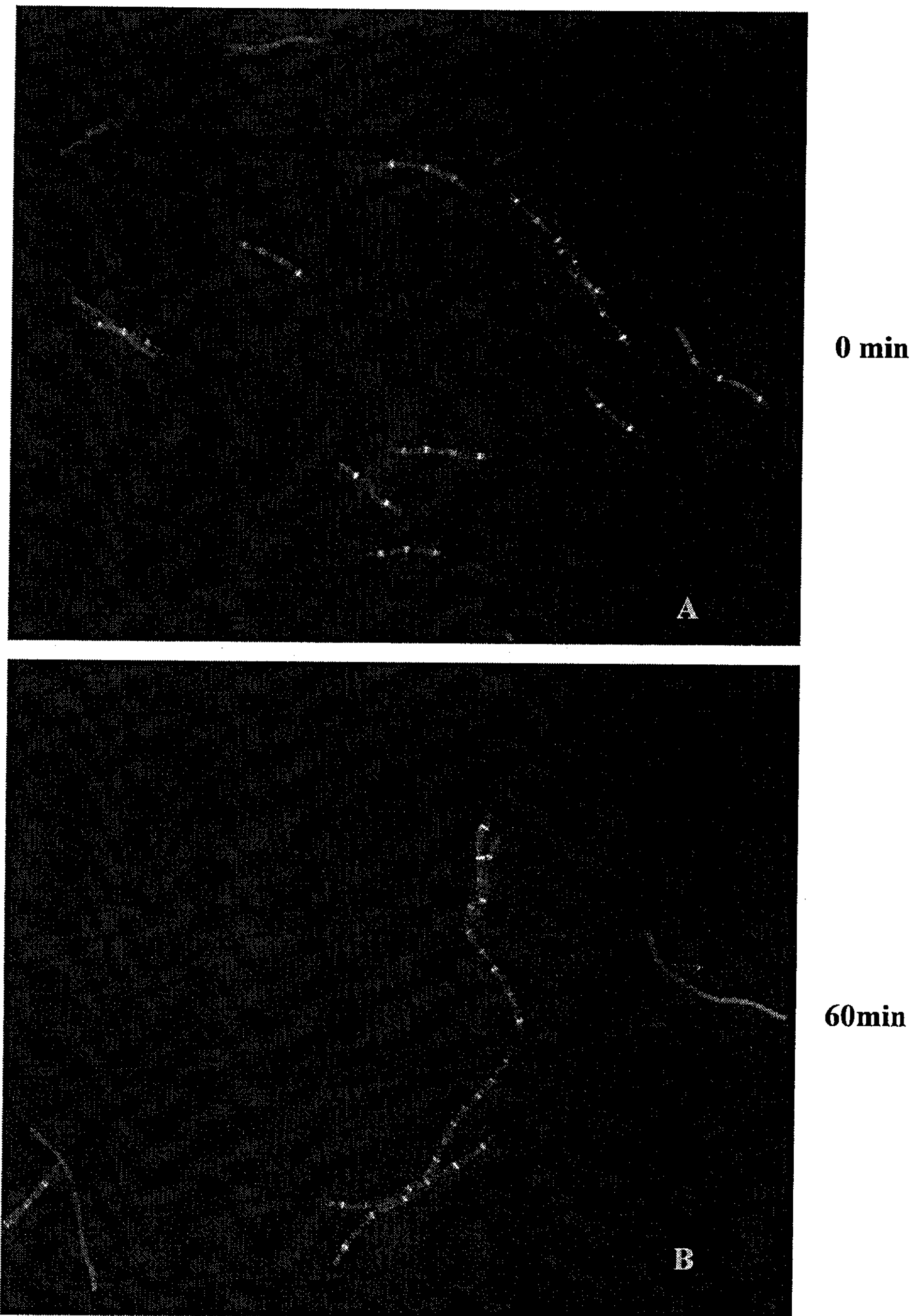


Figure 17. Localization of FtsA in *metK84* filaments with aztreonam treatment

A. Localization of FtsA-GFP in *metK84* filaments before adding aztreonam.

B. Localization of FtsA-GFP in metK84 filaments after adding aztreonam.

FtsA-GFP bands were detected at 0 and 60 minutes with the presence of aztreonam in the rich medium.

3. Localization recovery for late division proteins

The *metK84* mutant exhibits normal growth in rich medium, indicating that all division proteins localize at mid cell. Previous study in our lab found that if *metK84* filaments were put back into the rich medium, the high concentration of leucine in LB increased the SAM level and the filaments could produce single cells. So I caused *metK84* cells to be filamentous in glucose or glycerol minimal medium, and then switched the medium to LB with aztreonam. In this case, *metK84* cells kept the filamentous morphology because of inactivity of peptidoglycan synthesis, while division proteins could still accumulate at the potential division site. We could thus determine whether the late division proteins not seen in the SAM-limited growth condition could still localize when SAM was not limiting, and while cell division was inhibited by aztreonam. Demonstrating their assembly in *metK84* filaments in LB medium with aztreonam could suggest that their rare observation in *metK84* filaments of minimal medium incubation is due to their aberrant function, and not due to inadequate detection methods.

3.1 Localization of FtsK in *metK84* filaments in the presence of aztreonam

GFP-FtsK did not localize well in *metK84/gfp-ftsK* filaments grown in glycerol minimal medium. Only 61.2% filaments contained GFP-FtsK rings, while 38.8% filaments were absent of GFP-FtsK bands.

After transferal of *metK84/gfp-ftsK* filaments into the rich medium with aztreoanm, the samples from 90 and 120-minute incubations were fixed to check the localization frequency. The GFP images taken at different times (Figure18) showed that 100% filaments from 90 and 120 minutes contained the GFP-FtsK bands. The spacing of the rings in the last column of Table 8 indicates an increase in GFP-FtsK localization frequency: 5.9μm in 90 minutes and 5.7μm in 120 minutes.

3.2 Localization of FtsQ in *metK84* filaments in the presence of aztreonam

There were rare GFP-FtsQ bands observed in 10 hours of *metK84 /gfp-ftsQ* filaments grown in glucose minimal medium. After continuous incubation in rich medium with aztreonam for 60 and 80 minutes, the percentage of the filaments containing rings increased from beginning of 21.9% to 89.2% and 90.5%, separately, while the spacing between GFP-FtsQ bands was reduced to 1/7 of the value in the original filaments (Figure 19, Table 8).

3.3 Localization of FtsW in *metK84* filaments in the presence of aztreonam

Only 11.1% metK84/*gfp-ftsW* filaments had one GFP-FtsW band after a 10-hour incubation in glucose minimal medium. The spacing of GFP-FtsW bands was 101.7μm. After the filaments were transferred into the rich medium with aztreonam and incubated 60 and 90 minutes, more and more GFP-FtsW bands could be visualized in *metK84/gfp-ftsW* filaments, indicating a reduction in the spacing of the rings to 7.6μm in 60 and to 5.9

μm in 90 min. Thus, the GFP-FtsW also localized well in *metK84* filaments (Figure 20, Table 8).

3.4 Localization of FtsI in *metK84* filaments in the presence of aztreonam

GFP-FtsI bands were rarely observed in 10-hour *metK84 /gfp-ftsI* filaments grown in glucose minimal medium. This is the same localization defect as I previously detected in the 16-hour filaments. When I put the filaments into the rich medium with aztreonam, GFP-FtsI localized to at least one potential division site in nearly each filament of 91.1% in 60 minutes and 100% in 180 minutes, while only 27.4% of filaments showed GFP-FtsI bands in the 10-hour culture. The spacing of GFP-FtsI decreased greatly: in contrast with 36.0μm in the 10-hour filaments, it was 9.0μm in 60 minutes and 8.0μm in 180minutes, which were approximately 3.5-fold shorter (Figure 21, Table 8)

3.5 Localization of FtsN in *metK84* filaments in the presence of aztreonam

One of the last known division proteins, FtsN, also failed to be recruited to the potential division site in the *metK84* filaments grown in glucose minimal medium. The 10-hour filaments displayed the rare GFP-FtsN bands and their spacing was 59.0μm. After the filaments were transferred into the rich medium with aztreonam, they showed more and more GFP-FtsN bands: after 60 minutes with a spacing of 9.6μm, while after 180 minutes, the spacing decreased to 8.1μm (Figure 22, Table 8).

In summary, late division proteins returned to the septum in LB medium with aztreonam, and their localization frequencies increased greatly, as compared to the filaments in glucose or glycerol minimal medium examined in PartI.

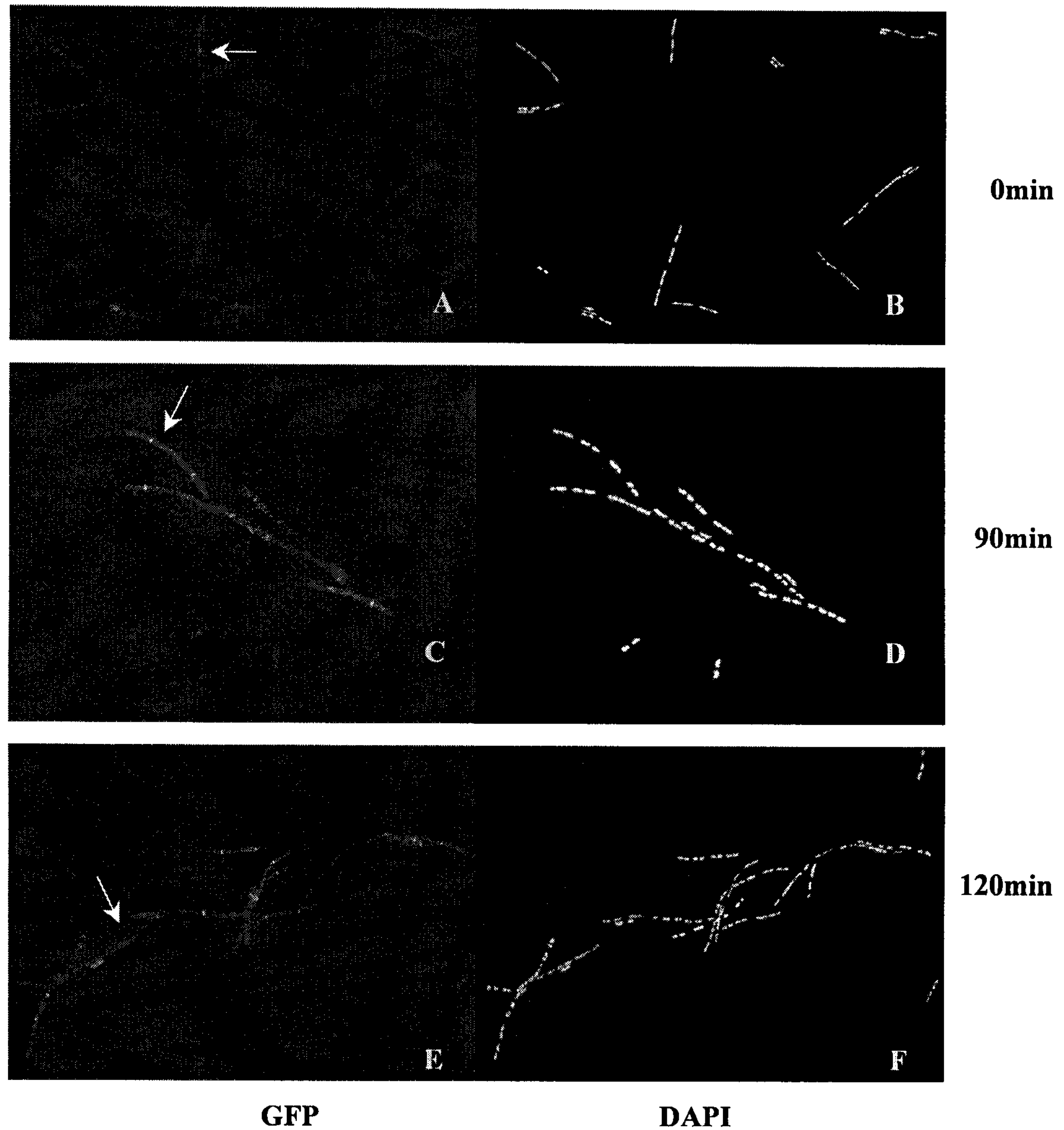


Figure 18. Localization recovery for FtsK in *metK84* filaments

A-B. Localization of GFP-FtsK in *metK84/gfp-ftsK* at 0 min

C-D. Localization of GFP-FtsK in *metK84/gfp-ftsK* at 90min

E-F. Localization of GFP-FtsK in *metK84/gfp-ftsK* at 120min

The 10-hour *metK84/gfp-ftsK* filaments were incubated in the rich medium with aztreonam after different time. White arrows point to the GFP-FtsK bands.

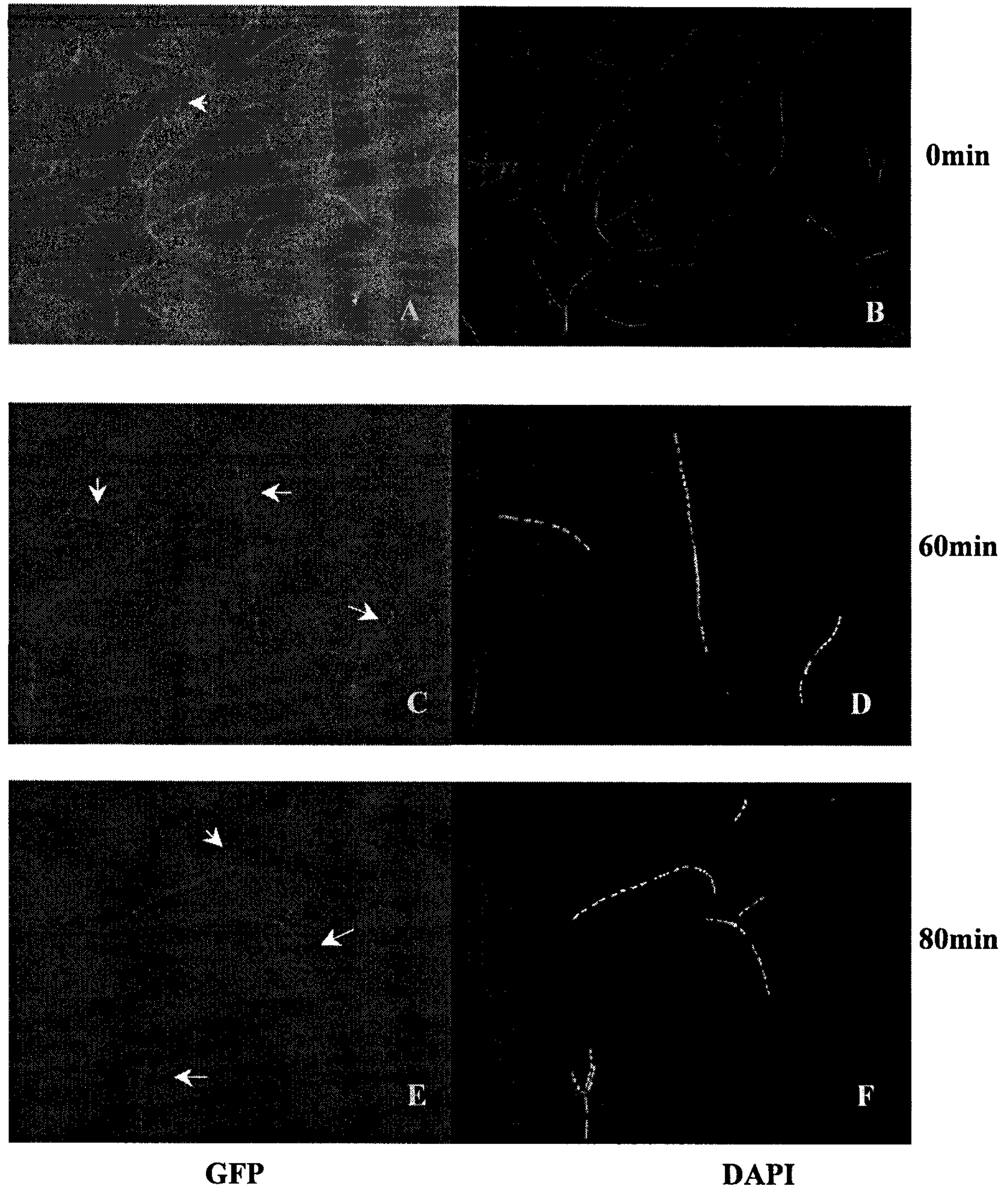


Figure 19. Localization recovery for FtsQ in *metK84* filaments

A-B. Localization of GFP-FtsQ in *metK84/gfp-ftsQ* at 0 min

C-D. Localization of GFP-FtsQ in *metK84/gfp-ftsQ* at 60min

E-F. Localization of GFP-FtsQ in *metK84/gfp-ftsQ* at 80min

The 10-hour *metK84/gfp-ftsQ* filaments were incubated in the rich medium with aztreonam after different time. White arrows point to the GFP-FtsQ bands.

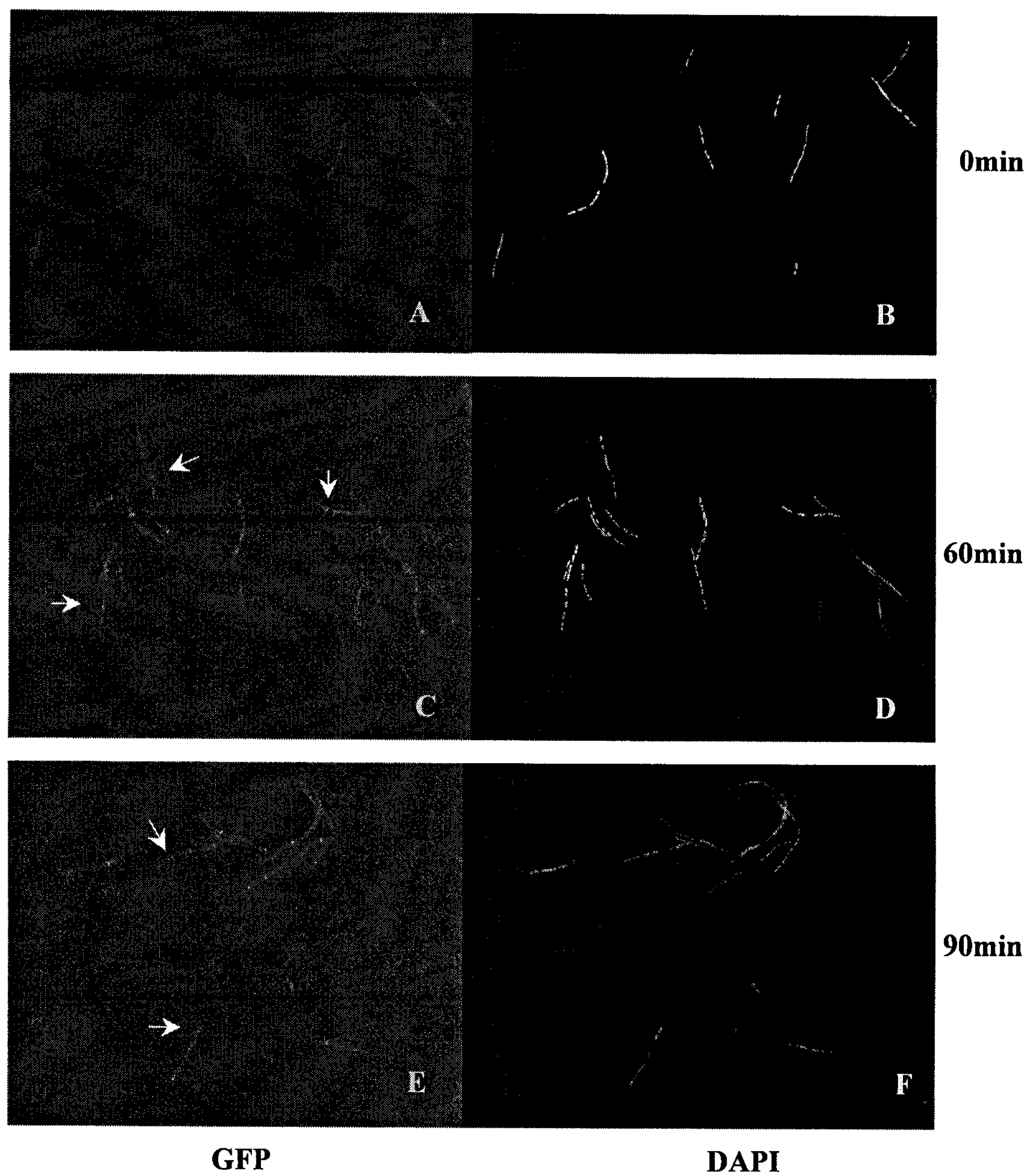


Figure 20. Localization recovery for FtsW in *metK84* filaments

A-B. Localization of GFP-FtsW in *metK84/gfp-ftsW* at 0 min

C-D. Localization of GFP-FtsW in *metK84/gfp-ftsW* at 60min

E-F. Localization of GFP-FtsW in *metK84/gfp-ftsW* at 90min

The 10-hour *metK84/gfp-ftsW* filaments were incubated in the rich medium with aztreonam after different time. White arrows point to the GFP-FtsW bands.

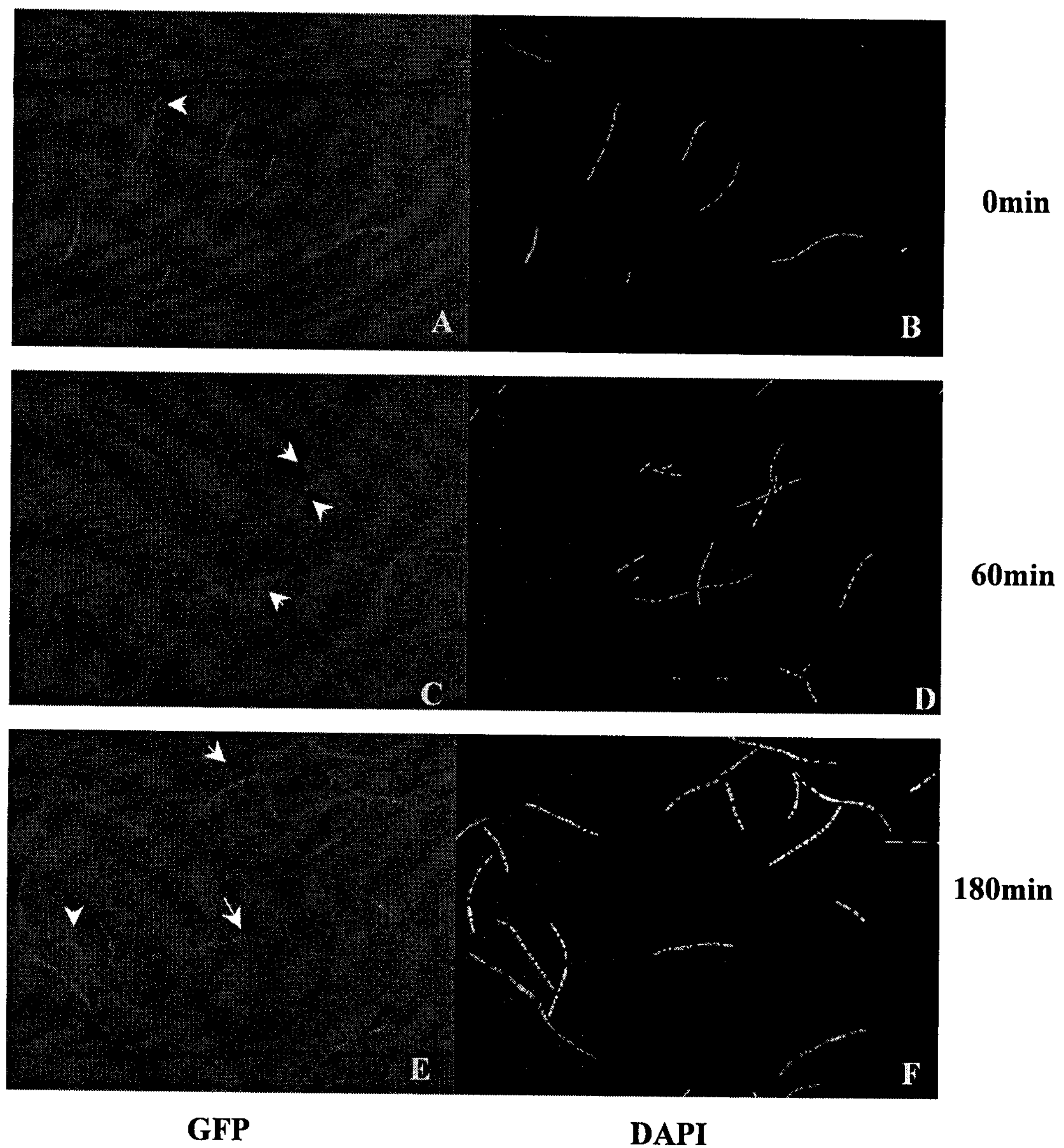


Figure 21. Localization recovery for FtsI in *metK84* filaments

A-B. Localization of GFP-FtsI in *metK84/gfp-ftsI* at 0 min

C-D. Localization of GFP-FtsI in *metK84/gfp-ftsI* at 60min

E-F. Localization of GFP-FtsI in *metK84/gfp-ftsI* at 180min

The 10-hour *metK84/gfp-ftsI* filaments were incubated in the rich medium with aztreonam after different time. White arrows point to the GFP-FtsI bands.

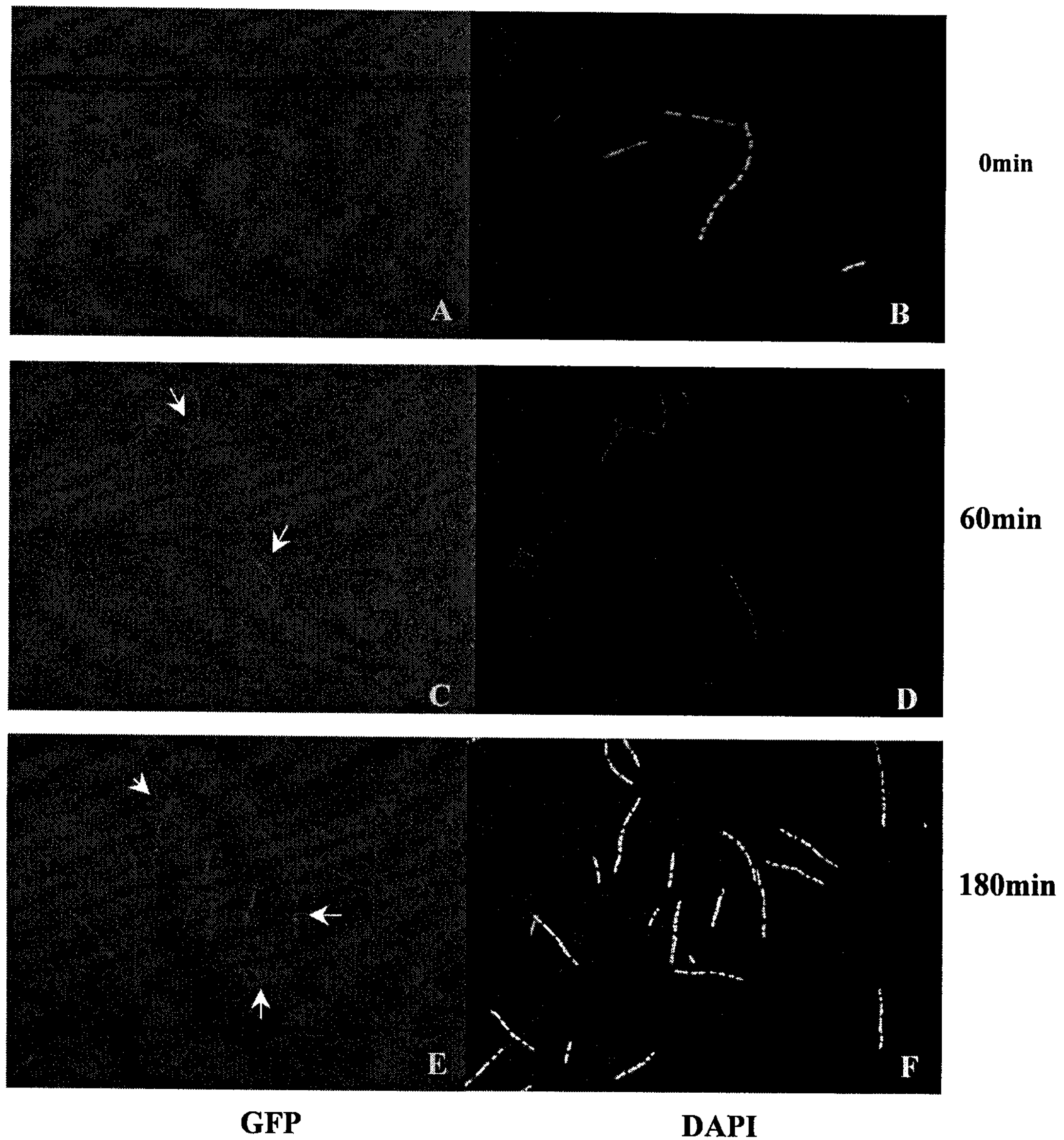


Figure 22. Localization recovery for FtsN in *metK84* filaments

A-B. Localization of GFP-FtsN in *metK84/gfp-ftsN* at 0 min

C-D. Localization of GFP-FtsN in *metK84/gfp-ftsN* at 60min

E-F. Localization of GFP-FtsN in *metK84/gfp-ftsN* at 180min

The 10-hour *metK84/gfp-ftsN* filaments were incubated in the rich medium with aztreonam after different time. White arrows point to the GFP-FtsN bands.

Table 8. Localization frequencies of GFP fusions with late division proteins in *metK84* filaments with aztreonam treatment

GFP fusion	Different times for Aztreonam treatment (min)	Total no. of cells scored	Total no. of rings	Average cell length ±SD (μm)	% of cells with rings	Spacing of rings (μm/ring)
GFP-FtsK	0	116	115	10.6±4.4	61.2	10.7
	90	61	124	11.9±4.9	100	5.9
	120	85	240	16.2±8.0	100	5.7
GFP-FtsQ	0	96	21	10.4±4.8	21.9	47.5
	60	37	80	16.4±8.6	89.2	7.6
	80	21	67	21.4±13.2	90.5	6.7
GFP-FtsW	0	18	2	11.3±4.7	11.1	101.7
	60	58	93	12.2±4.1	89.7	7.6
	90	41	109	15.7±10.7	97.5	5.9
GFP-FtsI	0	84	28	12.0±6.2	27.4	36.0
	60	101	136	12.1±5.3	91.1	9.0
	180	77	182	18.8±8.5	100	8.0
GFP-FtsN	0	96	20	12.3±9.0	11.5	59.0
	60	32	44	13.1±6.8	96.9	9.6
	180	85	139	13.3±7.3	98.8	8.1

a. The *metK84* filaments with various *gfp* fusion genes were collected at different times after aztreonam treatment and fixed as described in Materials and Methods.

b. The length of the filaments was measured using NIH Image software (http://rsb.nih.gov/nih-image/).

c. SD, standard deviation. SD reflects variation in the length of each filament.

d. The spacing is a measure of the frequency of rings per unit cell mass and is calculated by dividing the total number of rings (column4) into the total length of filaments scored (column 3 multiplied with column 5). The spacing is inversely proportional to the frequency of rings.

Discussion

A strain deficient in S-adenosylmethionine is known to make filaments and thus cannot divide. However the point at which cell division stops is not yet known (Newman *et al.*, 1998). The work in this thesis was performed to determine which of the division proteins, if any, could find their way into the septum in these filaments. I tested eight of the division proteins, each of which was fused individually to GFP and found a consistent pattern. Early division proteins, FtsZ, FtsA and ZipA, were able to establish themselves in the septum, while the late proteins were not. This conclusion is based on a set of experiments that showed whether or not each *gfp* fusion could be found in the *metK84* filaments. A failure to locate them could be due in part to technical problems in detecting proteins present in low abundances. I therefore verified, with aztreonam-treated filaments, that if indeed these proteins exist at mid cell, the methods used in this work would detect them.

In this discussion, I will summarize the results of eight protein localization in *metK84* filaments and explain the findings for gfp-fusions in the Cu1008 and its derivative strain, *metK84*; and will outline the potential methlyation targets involved in cell division based on my results.

1. Localization of division proteins in *metK84* filaments.

1.1 Three early division proteins localize into the potential division sites

The GFP images of FtsZ, FtsA and ZipA showed that GFP bands were localized regularly at the potential division site in each of the *metK84* filaments — the rings being spaced regularly along the filaments (Figure 6, 7, 8). In studies such as this, many investigators use a measure of the frequency of rings per unit of cell mass, which they call "spacing" (Chen *et al.*, 1999; Weiss *et al.*, 1999; Chen and Beckwith, 2001; Mercer and Weiss, 2002). This is calculated by dividing the total number of rings into the total length of cells or filaments scored. The spacing of rings is inversely proportional to the frequency of rings. For instance, the higher the value of the spacing, the lower frequency of the rings.

In properly located rings, the spacing of rings is between 3.0-4.0 μm. As can be seen in Figures 6B, 7B and 8B, the values for FtsZ, FtsA and ZipA fall into that range. Thus, one cannot cast doubt on the idea that FtsZ, FtsA and ZipA are properly recruited to the division septum in *metK84* filaments.

1.2 All late division proteins, from FtsQ to FtsN, failed to assemble the septum.

The late division proteins were rarely localized to the septal rings. GFP bands were rarely seen, about 40-fold less frequently that the early proteins. Consistent with this is a calculated spacing above 100μm, as compared to the 3.0-4.0μm for the early proteins, decreased up to 40 times in 16-hour *metK84* filaments. I conclude that all the late division

proteins tested: FtsQ, FtsW, FtsI and FtsN were absent from the division septum in *metK84* filaments.

1.3 Morphology of *metK84* filaments.

During cell division in *E.coli*, assembled division proteins coordinately and gradually organize the annular constriction of the cytoplasmic membrane, the rigid peptidoglycan layer and the cell wall, which leads to invagination of the cytoplasmic membrane and a coupled synthesis of the cell wall at the potential division sites (Errington *et al.*, 2003). Therefore, visible constriction is the last step in septation before septum closure and cell separation. Buddeleijer and Beckwith (2002) suggested that it is not until all the cell division proteins are localized at mid cell that the initiation of cell division (invagination) is triggered. That is, the filaments, if caused by depletion of null mutants for early- or late-recruited cell division proteins, should appear having smooth morphology with no visible constriction.

In *metK84* filaments, I observed some constrictions along the filaments (Figure 4). The possible explanation is that the visible indentations result from residual activity of any late division protein, whose localization is defective largely when SAM level falls below a certain critical threshold. Earlier studies on the filaments using temperature-sensitive mutants also observed visible constrictions along the filaments. It is thought that the explanation for such morphology is a result of the leakiness of these mutants in

nonpermissive temperature (Taschner *et al.*, 1988; Carson *et al.*, 1991; Addinall *et al.*, 1996; Yu *et al.*, 1998; Buddeleijer and Beckwith, 2002).

1.4 Synthesis of septa in aztreonam-treated filaments

The localization experiments described in this thesis require observations at the limit of the possibilities of the microscope used. The proteins studied are not present in high concentrations. FtsZ occurs at 10.000 to 20.000 molecules per cell, and FtsA and ZipA are 10-100-fold less abundant than FtsZ. However, all late division proteins are found at much lower concentrations, about 20-100 molecules per cell. Failure to see them in rings could therefore be due to experimental difficulties rather than their actual absence.

The results from the aztreonam experiment demonstrated that the low localization frequencies of late division proteins in *metK84* filaments were not in fact due to the weak GFP signal from these fusions. The *metK84* filaments are produced when leucine, thus SAM, is limiting. Therefore increasing SAM level would allow the septum to be completed for cell division. To make this easier to analyze, we took advantage of a technique introduced by Dr. Weiss, increasing SAM levels in order to restore growth, but also adding aztreonam, the compound that binds to FtsI to prevent cell division. If this microscope could detect the late proteins, they should be visible in aztreonam-treated cells. And indeed they were. In contrast with the filaments in glucose minimal medium, nearly 100% of *metK84* filaments contained at least one GFP band for each division proteins in rich medium with aztreonam. There was a remarkable increase in localization

frequencies for each division protein's localization in such growth condition. Therefore, I conclude that late proteins cannot localize at the septum in *metK84* filaments.

The data for protein localization are consistent with the accepted order of cell division process. In *E.coli,* there is a linear dependency pathway for division protein assembly in the septum. FtsZ migrates to the potential division site after its polymerization; FtsA and ZipA bind directly to FtsZ, but independently of each other; and then subsequential proteins localize to the septum in this order: FtsK, FtsQ, FtsL/FtsB, FtsW, FtsI, FtsN and finally AmiC. In my experiments, FtsQ was not observed at the potential division site in *metK84* filaments. One would not expect that any protein, which assembled later than FtsQ, would be present at the division site. And in fact, all division proteins dependent on FtsQ did fail to be recruited to the septum in *metK84* filaments.

1.5 Effects of a specific fts fusion: FtsQ

I examined all of the fusion proteins in the parental strain, Cu1008, as well as its derivative *metK84*. The data also indicated that less abundant proteins are detectable in our system. In these cells, expression of GFP-FtsQ was strikingly different from GFP fused to the other proteins. Cu/*gfp-ftsQ* cells were longer and brighter than Cu1008 carrying other fusions like the *gfp-ftsI*. The strong GFP signal indicated that GFP-FtsQ localized at the division sites in Cu1008, while other GFP-FtsQ fusion molecules spread throughout the membrane, as is expected for membrane proteins. FtsQ overproduction in glucose minimal medium has been shown to have profound effects on the *E.coli* cells in

other systems. Dr. Beckwith and his colleagues showed that cells formed multiseptate filaments when the *ftsQ* gene products was overexpressed up to 70-fold in minimal medium (Carson *et al.*, 1991). I did not quantify the FtsQ overexpression in our system in this study. Chen *et al.* (1999) reported that the level of GFP-FtsQ was about 10-15 times that of wild-type FtsQ in the presence of 5μM IPTG as an inducer of the gfp fusion. As I used the same *gfp-ftsQ* fusion and IPTG concentration, my estimation was that there would be close to the same amount of GFP-FtsQ molecules present in Cu/*gfp-ftsQ* cells, an amount not high enough to abolish cell division in minimal medium compared to 70-fold overexpression.

Since, GFP fusions to FtsQ, FtsI and FtsN were under control of the same promoter, one might suppose that they were expressed to a similar extent in the same external conditions. My doubling of the IPTG concentration for *gfp-ftsI* and *gfp-ftsN* fusions, a concentration equivelent to the one used for *gfp-ftsQ*, showed no increase in cell length in Cu/*gfp-ftsI* and Cu/*gfp-ftsN* cells (data not shown). It seems that the effect of ftsQ is inherent in the FtsQ structure. The unusual behavior of FtsQ in cell phenotype has also been reported before. The *ftsQ* mutants, different from *ftsZ and ftsA*, showed a particular increase in average cell length at the permissive temperature when cells grown in glucose minimal medium (Taschner *et al.*, 1988).

Additionally, though overexpressed, neither FtsN nor FtsI increased the cell length in Cu1008. GFP-FtsN gave a much brighter image than did GFP-FtsI with the same level of inducer (Figure 15A, 16A). Since both of Cu/*gfp-ftsI* and Cu/*gfp-ftsN* cells were grown at

the same inducer concentration (IPTG 2.5μM), and the gfp fusions were expressed from the same promoter (P_{lac207}), the total GFP fusion concentrations should be the same. However if the proportion of one protein integrated into the entire membrane is larger than at the septum, the resulting cell would be much brighter in the entire membrane. It seems then that FtsN must integrate into the membrane to a greater extent than FtsI, which gives a weaker GFP signal in the entire membrane.

Nonetheless, since the inducible level of GFP-FtsQ and GFP-FtsN did not abolish their localization in the potential division site, I used the same concentration of IPTG as for Cu1008 merodiploids to induce *gfp-ftsQ* and *gfp-ftsN* expression in *metK84* cells.

1.6 FtsK localization is uncertain

Since the three early division proteins localize and later ones do not in *metK84* filaments, it seems that the block in septum assembly must be at ftsK or FtsQ. I have shown that FtsQ does not localize. I did try to study FtsK localization in *metK84* filaments.

Unfortunately the results of FtsK localization are not clear. The spacing of FtsK rings was 10.7μm, indicating that the localization frequency was two-fold lower than early division proteins (i.e. FtsK rings occurred at about one half the frequency of the 3 early proteins but much more frequently than the late proteins). This indicated that FtsK could enter the rings to some extent. FtsK localization results showed that only 61.2% *metK84/gfp-ftsK* filaments contained one or multiple GFP-FtsK bands, while at least 30%

of the population did not have GFP-FtsK localization at all (Figure 9, Table 5). However, when a division protein is properly recruited to the septum in other kinds of filaments, the protein localizes in 100% of filaments and in at least one potential division site even with a spacing of 10-13μm between rings (Chen and Beckwith, 2001). At this time, therefore, I cannot make a definite conclusion for FtsK localization in *metK84* filaments.

This was further complicated by the fact that we had *ftsK* cloned only into a pBR322 derivative plasmid and its expression was under control of the pBAD promoter. This necessitated growing cells in glycerol that altered the state of the cells markedly (see below).

1.6.1. Filamentation due to SAM deficiency is affected by the carbon source supplied

During the localization study for GFP-FtsK in *metK84* filaments, about 50% of its population were single cells. At first glance, it seemed that the high level of FtsK in *metK84/gfp-ftsK* enabled the *metK84* cells to overcome filamentation. However the parental strain, *metK84*, also made single cells in glycerol grown cultures. Therefore, the presence of single cells in the *metK84/gfp-ftsK* culture did not depend on overproduction of FtsK. One would suppose that the FtsK-GFP localized itself better in glycerol minimal medium since so many cells were able to divide. This has not been studied in enough detail to allow any conclusion.

1.6.2 Other ways to decide FtsK localization in future studies of *metK84* filaments

To avoid having to grow *metK84* cells in glycerol minimal medium, I would like to clone *gfp-ftsK* into a vector with a promoter inducible in the presence of glucose, like the pLtet0-1 that contains an anhydrotetracycline-inducible promoter. With this construction, the localization of FtsK in *metK84* cells can be tested in glucose minimal medium, in which *metK84* produces filaments. This should give a clearer answer for FtsK localization.

2. Potential methylation targets involved in cell division

The experiments presented here are from a part of a project aimed to demonstrate the existence of a methylation reaction involved in cell division in *E.coli*, as was deduced from the fact that cell division in *E.coli* was blocked by SAM starvation (Newman *et al.*, 1998).

SAM is a major methyl donor in *E.coli*. The methylation targets range from RNA and DNA to proteins. SAM-dependent methylation reactions play significant roles in controlling and regulating a variety of cellular function (Hughes *et al.*, 1987). Carion *et al.*(1999) showed that the MraW protein catalyzed SAM-dependent methylation of 2 unidentified proteins. It is worth noting that the *mraW* gene is located at the upstream start of the 2 min cluster on the *E.coli* map, where most of the cell division genes are present. But unfortunately, no SAM-dependent methylation reactions involved in cell division have been found until now.

My results here demonstrate that defects in cell division in the *metK84*, a mutant starving for SAM, was due to the absence of late division proteins at the potential division site. By surveying known SAM-dependent methylation reactions, identifying several potential SAM-dependent methylation targets required for cell division might become possible:

(1) The first potential target is FtsK. If FtsK is absent in *metK84* filaments, it may need methylation before being recruited to the septum; or if FtsK is partially localized in *metK84* filaments, methylation may be required in order for it to bring late proteins to the septum.

(2) The second targets are either FtsA or ZipA. They might need methylation, which allows the downstream division proteins to bind to the potential division site.

(3) The third possibility is FtsZ. The two-hybrid assay showed that FtsK interacted directly with FtsZ *in vivo* (Di Lallo *et al.*, 2003). Methylation of FtsZ might be required for FtsK recruitment into the septum.

(4) The Fourth candidate is FtsQ. FtsQ is the late division protein immediately after FtsK in the linear dependency pathway. Its localization requires FtsK. The two-hybrid assay showed that FtsQ acted as a connection protein and interacted with FtsL and FtsN, but no evidence of FtsQ-FtsK interaction was described (Di Lallo *et al.*, 2003). Methylation might be required for

FtsQ migration into the septum and for stabilization of the FtsK rings through interaction with other known stabilizing factors like FtsN.

(5) Molecules other than division proteins might need to be methylated in order for late proteins to enter the division rings, and for the resulting cell cytokinesis to take place.

The process of cell division is complex and as yet, little understood. Little information is available concerning the role of division proteins. Even with my present conclusion that *metK84* filamentation is due to the absence of late division proteins, the question of how SAM affects cell division remains open. Further studies are needed to explore the role of SAM in cell division, such as using mass spectrometry to look for potential methylation targets or radiolabeling to monitor the pathway of SAM utilization *in vivo*.

In any case it is clear that the cells can make a stable partial ring that resists the fixation methods I used. Whatever proteins can enter the membrane do so, and they apparently can stay in this partially formed structure for long periods of time.

Summary

S-adenosylmethionine (SAM) is a central metabolite involved in a myriad of pathways with various biological roles in all cells, mainly as a universal methyl group donor. SAM synthesizes from methionine and ATP in a reaction catalyzed by SAM synthetase. In *E.coli*, *metK* is the only gene to encode SAM synthetase. The *metK84* mutant, which carries an A→G transition mutantion, shows a remarkable decrease in its level of SAM synthetase activity, and exhibits filamentation morphology under certain growth conditions, indicating that SAM might play a role in cell division. However, the point at which cell division is blocked is not yet determined.

Cell division is a complex and essential process for survival of all cells. In *E. coli*, a set of at least eleven proteins is required for septal recruitment at the potential division site during cell division, and their localization has a defined co-dependent order. In order to investigate a division step at which cell division is blocked, I mainly used merodiploid strains with the aid of fluorescence microscopy to examine localization of the cell division proteins in *metK84* filaments. Aztreonam, a specific division inhibitor of the late division protein FtsI, was also used for verification of the localization methods in this work.

I examined the localization of eight division proteins in the *metK84* filaments. The early division proteins, FtsZ, FtsA and ZipA, showed a regular distribution of division

rings, and their frequency of localization was similar in the filaments. The septal rings of all late division proteins, FtsQ, FtsW, FtsI and FtsN, were rarely observed in the filaments, and their localization frequencies decreased dramatically compared to the early division proteins. Furthermore, localization of the cell division proteins in aztreonam-treated filaments confirmed the absence of all late division proteins in *metK84* mutants. However, localization of FtsK, a mediate recruit during the cell division, was not yet clear because of the change in morphology as a result of the carbon source supplied.

Based on these results, I conclude that (1) *metK84* filamentation is caused by the failure in the assembly of FtsQ or FtsK during the cell division process; and (2) the techniques used in this study can detect the localization of all division proteins at mid cell. Thus, the fact that some division proteins were not seen in the *metK84* filaments is due to the fact that they really did not localize to the division site. Furthermore, based on my results, several potential SAM-dependent methylation targets that might be involved in the septum assembly may be identified

References

Addinall, S.G., Bi. E., and J. Lutkenhaus. 1996. FtsZ ring formation in *fts* mutants. J Bacteriol. **178**:3877-84.

Addinall, S.G., and B. Holland. 2002. The tubulin ancestor, FtsZ, draughtsman, designer and driving force for bacterial cytokinesis. J Mol Biol. **318**:219-36. Review.

Addinall, S.G., and J. Lutkenhaus. 1996. FtsA is localized to the septum in an FtsZ-dependent manner. J Bacteriol. **178**:7167-72.

Arends, S.J., and D.S. Weiss. 2004. Inhibiting cell division in *Escherichia coli* has little if any effect on gene expression. J Bacteriol. **186**:880-4

Arigoni, F., F. Talabot, M. Peitsch, M.D. Edgerton, E. Meldrum, E. Allet, R. Fish, T. Jamotte, M.L. Curchod, and H. Loferer. 1998. A genome-based approach for the identification of essential bacterial genes. Nat Biotechnol. **16**:851-6.

Aussel, L., F.X. Barre, M. Aroyo, A. Stasiak, A.Z. Stasiak, and D. Sherratt. 2002. FtsK Is a DNA motor protein that activates chromosome dimer resolution by switching the catalytic state of the XerC and XerD recombinases. Cell. **108**:195-205.

Bernhardt, T.G., and P.A. de Boer. 2003. The *Escherichia coli* amidase AmiC is a periplasmic septal ring component exported via the twin-arginine transport pathway. Mol Microbiol. **48**:1171-82.

Bi, E.F., and J. Lutkenhaus. 1991. FtsZ ring structure associated with division in *Escherichia coli*. Nature. **354**:161-4.

Boyd, D., D.S. Weiss, J.C. Chen, and J. Beckwith. 2000. Towards single-copy gene expression systems making gene cloning physiologically relevant: lambda InCh, a simple *Escherichia coli* plasmid-chromosome shuttle system. J Bacteriol. **182**:842-7.

Boye, E., and A. Lobner-Olesen. 1990. The role of dam methyltransferase in the control of DNA replication in *E. coli*. Cell. **62**:981-9.

Bramhill, D., and C.M. Thompson. 1994. GTP-dependent polymerization of *Escherichia coli* FtsZ protein to form tubules. Proc Natl Acad Sci U S A. **91**:5813-7.

Buddelmeijer, N., and J. Beckwith. 2002. Assembly of cell division proteins at the *E. coli* cell center. Curr Opin Microbiol. **5**:553-7.

Buddelmeijer, N., and J. Beckwith. 2004. A complex of the *Escherichia coli* cell division proteins FtsL, FtsB and FtsQ forms independently of its localization to the septal region. Mol Microbiol. **52**:1315-27.

Buddelmeijer, N., N. Judson, D. Boyd, J.J. Mekalanos, and J. Beckwith. 2002. YgbQ, a cell division protein in *Escherichia coli* and Vibrio cholerae, localizes in codependent fashion with FtsL to the division site. Proc Natl Acad Sci U S A. **99**:6316-21.

Carion, M., M.J. Gomez, R. Merchante-Schubert, S. Dongarra, and J.A. Ayala. 1999. *mraW*, an essential gene at the dcw cluster of *Escherichia coli* codes for a cytoplasmic protein with methyltransferase activity. Biochimie. **81**:879-88.

Carson, M.J., J. Barondess, and J. Beckwith. 1991. The FtsQ protein of *Escherichia coli:* membrane topology, abundance, and cell division phenotypes due to overproduction and insertion mutations. J Bacteriol. **173**:2187-95.

Chalfie, M., Y. Tu, G. Euskirchen, W.W. Ward, and D.C. Prasher. 1994. Green fluorescent protein as a marker for gene expression. Science. **263**:802-5.

Chen. J.C., and J.Beckwith. 2001. FtsQ, FtsL and FtsI require FtsK, but not FtsN, for co-localization with FtsZ during *Escherichia coli* cell division. Mol Microbiol. **42**:395-413

Chen, J.C., D.S. Weiss, J.M. Ghigo, and J. Beckwith. 1999. Septal localization of FtsQ, an essential cell division protein in *Escherichia coli.* J Bacteriol. **181**: 521-30.

Corbin, B.D., X.C. Yu, and W. Margolin. 2002. Exploring intracellular space: function of the Min system in round-shaped *Escherichia coli.* EMBO J. **21**:1998-2008.

Dai, K., and J. Lutkenhaus. 1992. The proper ratio of FtsZ to FtsA is required for cell division to occur in *Escherichia coli.* J Bacteriol. **174**:6145-51.

Dai, K., Y. Xu, and J. Lutkenhaus. 1996. Topological characterization of the essential *Escherichia coli* cell division protein FtsN. J Bacteriol. **178**:1328-34.

Dewar, S.J., and R. Dorazi. 2000. Control of division gene expression in *Escherichia coli.* FEMS Microbiol Lett. **187**:1-7.

Di Lallo, G., D. Fagioli m Barionovi, P. Ghelardini, and L.Paolozzi. 2003. Use of a two-hybrid assay to study the assembly of a complex multicomponent protein machinery: bacterial septosome differentiation. Microbiology. **149**:3353-9.

Eberhardt, C., L. Kuerschner, and D.S. Weiss. 2003. Probing the catalytic activity of a cell division-specific transpeptidase *in vivo* with beta-lactams. J Bacteriol. **185**:3726-34.

Errington, J., R.A. Daniel, and D.J. Scheffers. 2003. Cytokinesis in bacteria. Microbiol Mol Biol. **67**:52-65.

Gayda, R.C., M.C. Henk, and D.Leong. 1992. C-shaped cells caused by expression of an ftsA mutation in *Escherichia coli.* J Bacteriol. **174**:5362-70.

Ghigo, J.M., and J. Beckwith. 2000. Cell division in *Escherichia coli*: role of FtsL domains in septal localization, function, and oligomerization. J Bacteriol. **182**:116-29.

Goksor, M., A. Diez, J. Enger, D. Hanstorp, and T. Nystrom. 2003. Analysis of molecular diffusion in ftsK cell-division mutants using laser surgery. EMBO Rep. **4**:867-71.

Greene, R.C., R,D, Williams, H.F. Kung , C. Spears, and H. Weissbach. 1973. Properties of metK mutants of *Escherichia coli* K-12. J Bacteriol. **115**:57-67.

Guzman, L.M., J.J. Barondess, and J. Beckwith. 1992. FtsL, an essential cytoplasmic membrane protein involved in cell division in *Escherichia coli.* J Bacteriol. **174**:7716-28.

Guzman, L.M., D. S. Weiss, and J. Bechkwith. 1997. Domain-swapping analysis of FtsI, FtsL, and FtsQ, bitopic membrane proteins essential for cell division in *Escherichia coli.* J Bacteriol. **179**:5094-103.

Hale, C.A., and P.A. de Boer. 1999. Recruitment of ZipA to the septal ring of *Escherichia coli* is dependent on FtsZ and indeoendent of FtsA. J. Bacteriol. **181**:167-76

Hale, C.A., and P. A. de Boer. 2002. ZipA is required for recruitment of FtsK, FtsQ, FtsL, and FtsN to the septal ring in *Escherichia coli.* J Bacteriol. **184**: 2552-6.

Hughes, J.A., L.R. Brown, and A. J. Ferro. 1987. Expression of the cloned coliphage T3 S-adenosylmethionine hydrolase gene inhibits DNA methylation and polyamine biosynthesis in *Escherichia coli.* J Bacteriol. **169**:3625-32.

Kossykh, V.G., and R. S. Lioyd. 2004. A DNA adenine methyltransferase of *Escherichia coli* that is cell cycle regulated and essential for viability. J Bacteriol. **186**:2061-7.

Kouzarides, T. 2002. Histone methylation in transcriptional control. Curr Opin Genet Dev. **12**:198-209.

Liu, G., G.C. Draper, and W.D. Donachie. 1998. FtsK is a bifunctional protein involved in cell division and chromosome localization in *Escherichia coli.* Mol Microbiol. **29**: 893-903

Lobner-Olesen, A., M.G. Marinus, and F.G. Hansen. 2003 Role of SeqA and Dam in *Escherichia coli* gene expression: a global/microarray analysis.Proc Natl Acad Sci U S A. **100**:4672-7.

Markham, G.D., J. DeParasis, and J. Gatmitan. 1984. The sequence of *metK*, the structural gene for S-adenosylmethionine synthetase in *Escherichia coli.* J Biol Chem. **259**:14505-7.

Markham, G.D., Z.J. Lu, J.C. Taylor, and K. Geisser. 2002. Structure and function of enzymes. Fox Cancer Center. Scientific Report.

Mathews, Van Holde, Ahern. The *third edition* of biochemistry on the compansion web site. (http://www.aw-bc.com/mathews/)

Mengin-Lecreulx, D., J. Ayala, A. Bouhss, J. van Heijenoort, C. Parquet, and H. Hara. 1998. Contribution of the P_{mra} promoter to expression of genes in the *Escherichia coli* mra cluster of cell envelope biosynthesis and cell division genes. J Bacteriol. **180**:4406-12.

Mercer, K.L., and D.S. Weiss. 2002. The *Escherichia coli* cell division protein FtsW is required to recruit its cognate transpeptidase, FtsI (PBP3), to the division site. J Bacteriol. **184**:904-12.

Merlin, C., S. McAteer, and M. Masters. 2002. Tools for characterization of *Escherichia coli* genes of unknown function. J Bacteriol.**184**:4573-81.

Miller, D.J., N. Quellette, E. Evdokimova, A. Savchenko, and A. Edwards. 2003. Crystal complexes of a predicted S-adenosylmethionine-dependent methyltransferase reveal a typical AdoMet binding domain and a substrate recognition domain. Protein Sci. **12**:1432-42.

Mukherjee, A., C. Cao, and J. Lutkenhaus. 1998. Inhibition of FtsZ polymerization by SulA, an inhibitor of septation in *Escherichia coli*. Proc Natl Acad Sci U S A. **95**:2885-90.

Mulder, E., and C. L. Woldringh. 1989. Actively replicating nucleoids influence positioning of division sites in *Escherichia coli* filaments forming cells lacking DNA. J. Bacteriol. **171**:4303-14.

Newman, E.B., L.I. Budman, E.C. Chan, R.C. Greene, R.T. Lin, C.L. Woldringh, and R. D'Ari. 1998. Lack of S-adenosylmethionine results in a cell division defect in *Escherichia coli.* J Bacteriol. **180**:3614-9.

Norris, V., C. Woldringh, and E. Mileykovskaya. 2004. A hypothesis to explain division site selection in *Escherichia coli* by combining nucleoid occlusion and Min. FEBS Lett. **561**:3-10

Pichoff, S., and J. Lutkenhaus. 2002. Unique and overlapping roles for ZipA and FtsA in septal ring assembly in *Escherichia coli.* EMBO J. **21**:685-93.

Rothfield, L.I., and S.S. Justice. 1997. Bacterial cell division: the cycle of the ring. Cell. **88**:581-4.

Sun, Q., and W. Margolin. 2004. Effects of perturbing nucleoid structure on nucleoid occlusion-mediated toporegulation of FtsZ ring assembly. J Bacteriol. **186**:3951-9.

Taschner, P.E., P.G. Huls, E. Pas, and C.L. Woldringh. 1988. Division behavior and shape changes in isogenic ftsZ, ftsQ, ftsA, pbpB, and ftsE cell division mutants of *Escherichia coli* during temperature shift experiments. J Bacteriol. **170** :1533-40.

Tuan, L.R., R. D' Ari, and E.B. Newman. 1990. The leucine regulon of *Escherichia coli* K-12: a mutation in rblA alters expression of L-leucine-dependent metabolic operons. J Bacteriol. **172**:4529-35.

Wang, L., M.K. Khattar, W.D. Donachie, and J.Lutkenhaus. 1998. FtsI and FtsW are localized to the septum in *Escherichia coli.* J Bacteriol. **180**:2810-6.

Wang, L., and J. Lutkenhaus. 1998. FtsK is an essential cell division protein that is localized to the septum and induced as part of the SOS response. Mol Microbiol. **29**:731-40.

Wang, X., J.Huang, A. Mukherjee, C. Cao, and J. Lutkenhaus. 1997. Analysis of the interaction of FtsZ with itself, GTP, and FtsA. J Bacteriol.**179**:5551-9.

Ward, J.E. Jr., and J. Lutkenhaus. 1985. Overproduction of FtsZ induces minicell formation in *E. coli*. Cell. **42**:941-9.

Wei, Y. 2001. A study of the *metK84* gene encoding S-adenosylmethionine synthetase in *Escherichia coli* K-12. MSc.Thesis. Concordia University, Montreal. Quebec.

Wei, Y., and E.B. Newman. 2002. Studies on the role of the metK gene product of *Escherichia coli* K-12. Mol Microbiol. **43**:1651-6.

Weiss, D.S., K. Pogliano, M. Carson, L.M. Guzman, C. Fraipont, M. Nguyen-Disteche, R. Losick, and J. Beckwith. 1999. Localization of the *Escherichia coli* cell division protein Ftsl (PBP3) to the division site and cell pole. Mol Microbiol. **25**:671-81.

Wissel, M.C., and D.S. Weiss. 2004. Genetic analysis of the cell division protein FtsI (PBP3): amino acid substitutions that impair septal localization of FtsI and recruitment of J Bacteriol. **186**:490-502.

Yang, F., L.G. Moss, and G.N. Jr. Phillips. 1996. The molecular structure of green fluorescent protein. Nat Biotechnol. **14**:1246-51.

Yu, X.C., A.H. Tran, Q. Sun, and W. Margolin. 1998. Localization of cell division protein FtsK to the *Escherichia coli* septum and identification of a potential N-terminal targeting domain. J Bacteriol. **180**:1296-304.