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Loss of its Three L-Serine Deaminases Causes Major Changes in Metabolism of *Escherichia coli* K-12

Xiao Zhang

A Thesis

In

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ABSTRACT

Loss of its Three L-Serine Deaminases Causes Major Changes in Metabolism of

Escherichia coli K-12

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Concordia University, 2009

The *Escherichia coli* genome codes for three highly homologous enzymes which use a 4Fe-4S mediated catalysis to deaminate L-serine and generate pyruvate and ammonia. The three enzymes are encoded by the genes *sdaA, sdaB* and *tdcG*. Enzymes of this type are found in many prokaryotes, and are not found in any eukaryotes. Despite the fact that the first *E. coli* L-serine deaminase (L-SD) was identified in 1955, and the regulation of its expression has been extensively studied, the physiological function of this delicately regulated enzyme is still unknown.

In part one of this work, the impact of L-SD on the metabolism of *E. coli* K-12 was studied by creating a strain from which all three genes were deleted. This strain has serious growth problems. While the triple mutant grows well in glucose minimal medium even with L-serine, it forms long filaments on subculture into Luria Broth (LB). On subculture into minimal medium with glucose and casamino acids (CAA), it forms very large, abnormally shaped cells, many of which are osmotically sensitive and lyse. The processes of DNA replication and cell division are both abnormal in the triple mutant cell grown with CAA. Further study indicated that the triple mutant is unable to maintain sufficient production of one-carbon (C1) units. Provision of an exogenous supply of S-adenosylmethionine (SAM)

restores cell division in LB, and repairs much of the difficulty in growth in medium with CAA. Whereas cells grown with CAA in the absence of SAM show abnormal FtsI production and localization within filaments, the addition of SAM produces filaments and normal recruitment of FtsI into the division septum.

In part two, a mutant MEW128 which had been shown to be deficient in post-translational activation of L-SD was investigated. The mutation in that strain was located in *ygfZ,* a gene of unknown function. Neither MEW128 nor a strain known to carry a *ygfZ* deletion produces an active L-SD.

Very little is known about the function of YgfZ, though it has been crystallized and shown by Teplyakov *et al.* to bind folates. The possibility that it is involved in assembly of the iron sulfur cluster (Fe-S) is considered.

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List of Abbreviations

aTc	Anhydrotetracycline
C1	one-carbon
CAA	casamino acids
Crp	catabolite repressor protein
DAPI	4'-6-Diamidino-2-phenylindole
DMGO	dimethylglycine oxidase
DNPH	2,4-Dinitriphenylhydrazine
DTT	Dithiothreitol
EMP	Embden Meyerhof pathway
EPR	Electron paramagnetic resonance spectra
Fe-S	iron sulfur
GCV	glycine cleavage
GFP	green fluorescent protein
LB	Luria Broth
L-SD	L-serine deaminase
Lrp	leucine-responsive regulatory protein
NAcG1P	N-acetylglucosamine-1-phosphate
Nacmur	N-acetylmuramic acid
NO	nucleoid occlusion
ORF	open reading frame
PBP	penicillin-binding protein
PGDH	phosphoglyceric acid dehydrogenase
SAM	S-adenosylmethionine
SHMT	L-serine hydroxymethyltransferase

SMC	structural maintenance of chromosomes
TDH	threonine dehydrogenase
THF	tetrahydrofolic acid
UDP	uridine-diphosphate

General introduction

This thesis deals with the metabolic role of an unusual *Escherichia coli* enzyme, L-serine deaminase (L-SD). It is not easy to understand why *E. coli* deaminates L-serine to pyruvate. Like many other organisms, *E. coli* converts glucose to pyruvate by the Embden Meyerhof pathway, and makes L-serine by a 3-enzyme pathway from an Embden Meyerhof intermediate, 3-phosphoglyceric acid. The L-serine thus synthesized serves as the precursor for many other substances (reviewed below). It is not clear why the cell would have enzymes dedicated to degrading this serine to pyruvate and wasting the ATP used to synthesize it.

Nonetheless the *E. coli* genome codes for three highly homologous L-SDs, all using a 4Fe-4S mechanism (Cicchillo *et al.*, 2004). Enzymes like this are widespread in the bacterial world, but are totally absent from eukaryotes. All organisms have enzymes or that can deaminate L-serine, usually one that uses pyridoxal phosphate as cofactor, and is involved in energy metabolism (Bairoch and Boeckmann, 1993).

The fact that *E. coli* codes for three such enzymes, SdaA, SdaB and TdcG, suggests that they must play a special role in *E. coli*. *sdaB* and *tdcG* show 73 and 74% nucleotide identity respectively to *sdaA*. They clearly function in the same manner, but they are expressed under different circumstances. The regulation of expression of these genes assures that L-SD is made in all environmental conditions.

It is a puzzle that L-SD produces pyruvate, which is a good energy source, but *E. coli* cannot use L-serine as carbon and energy source (Newman *et al.*, 1981). L-alanine on the other hand is a good carbon and energy source, and is metabolized through pyruvate just

as serine would be. SdaA is expressed in glucose minimal medium (Su *et al.*, 1989). Why then can the cell not adapt to using L-serine via SdaA?

In this thesis I approach the study of the metabolic role of L-serine deamination by studying cells totally deficient in L-SD. I use two approaches for this. In the first, I study a mutant in which all three L-SD coding genes are deleted. This is known in this work as the triple mutant. A mutant deficient in only *sdaA* was studied earlier (Su and Newman, 1991). However it had no particular metabolic problems other than the inability to deaminate L-serine under particular conditions. In this study I show that the triple mutant has major metabolic problems in a medium containing amino acids, and has gross problems in growth and cell division.

L-SD as coded from any of the three genes is inactive when released from the ribosomes and must be activated by a system of enzymes not yet completely elucidated. Two mutants in which one or more of these enzymes is nonfunctional were described in the Newman laboratory. I have identified one of these, mutant MEW128, as occurring in an as yet uncharacterized gene, *ygfZ*. Because this activation system is used for all three gene products, mutant MEW128 should affect all three enzymes. If *ygfZ* were totally inactive, this mutant should be similar to the L-SD triple mutant. I present here the characterization of the MEW128 mutation.

In part one of this introduction, I review information related to the role of L-serine metabolism. This is intended to support an understanding of the experiments on the triple mutant presented in part one of the results. It includes a review of the pathways of biosynthesis of serine and related amino acids, glycine and threonine, and a description of the three L-SD coding genes and their gene products, as well as a short list of other

enzymes which deaminate L-serine as side-reactions. These pathways are closely integrated with the biosynthesis of one carbon (C1) units, so a review of C1 synthesis is also included, as is a review of S-adenosylmethionine (SAM) biosynthesis and its involvement in cell division. Since the major effect of loss of L-SD is on cell division, I also include a short review of what is known about the stages of *E. coli* cell division.

Since part two of the results deals with the activation of L-SD, which may correspond to the insertion of the 4Fe-4S cluster into an apoenzyme, in part two of the introduction I review what is known about the reaction mechanism of L-SD, and about the formation of Fe-S clusters. Whereas most of the information underlying part one has been available for some years, the biosynthesis of Fe-S clusters is poorly understood and information is accumulating.

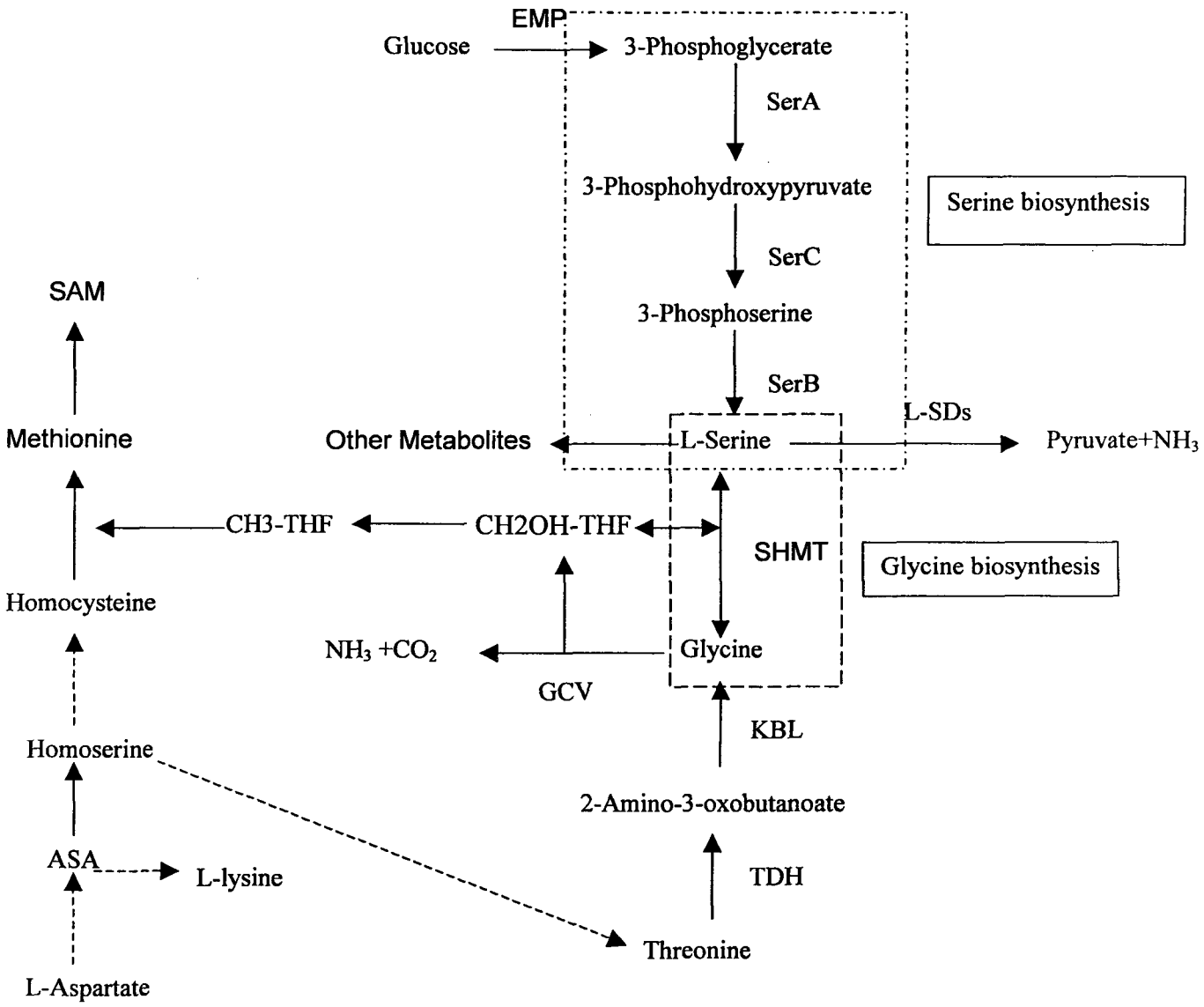
PART 1 STUDY OF THE METABOLIC FUNCTION OF L-SERINE DEAMINASE

Chapter 1 Introduction

L-serine is at the crossroads of several metabolic pathways (Figure 1). Much of its biosynthesis and degradation has been clarified in the past 50 years. Serine is used as the precursor for many different metabolites such as cysteine, tryptophan, glycine, phospholipids etc. (Keseler *et al.*, 2005). In cells grown on minimal media with glucose as carbon source, about 15% of the carbon is assimilated through the serine biosynthesis pathway and then flows to the derived cell constituents (Britten *et al.*, 1955). This high carbon flux through this pathway illustrates the important role of L-serine in the overall cell metabolism of the cell. Variation of the intracellular concentration levels of L-serine might act as a control point regulating many metabolic activities of the cell.

The enzyme L-SD which degrades L-serine to pyruvate and ammonia was first described in *E. coli* by Pardee and Prestidge (1955). Three genes are now known to encode homologous L-SDs in *E. coli*, *sdaA*, *sdaB* and *tdcG*. The expression of these enzymes has been shown to be highly regulated. A number of intracellular and environmental regulatory factors have been shown to be involved in L-SD regulation (Lin *et al.*, 1990; Newman *et al.*, 1981; Newman *et al.*, 1982; Newman, personal communication).

A


EMP
Glucose
3-Phosphoglycerate
SerA
3-Phosphohydroxypyruvate
Serine biosynthesis
SerC
3-Phosphoserine
SerB
SAM
L-SDs
Methionine
Other Metabolites
L-Serine
Pyruvate+NH3
CH3-THF
CH2OH-THF
SHMT
Glycine biosynthesis
Homocysteine
NH3 +CO2
Glycine
GCV
KBL
Homoserine
2-Amino-3-oxobutanoate
ASA
L-lysine
TDH
Threonine
L-Aspartate

B

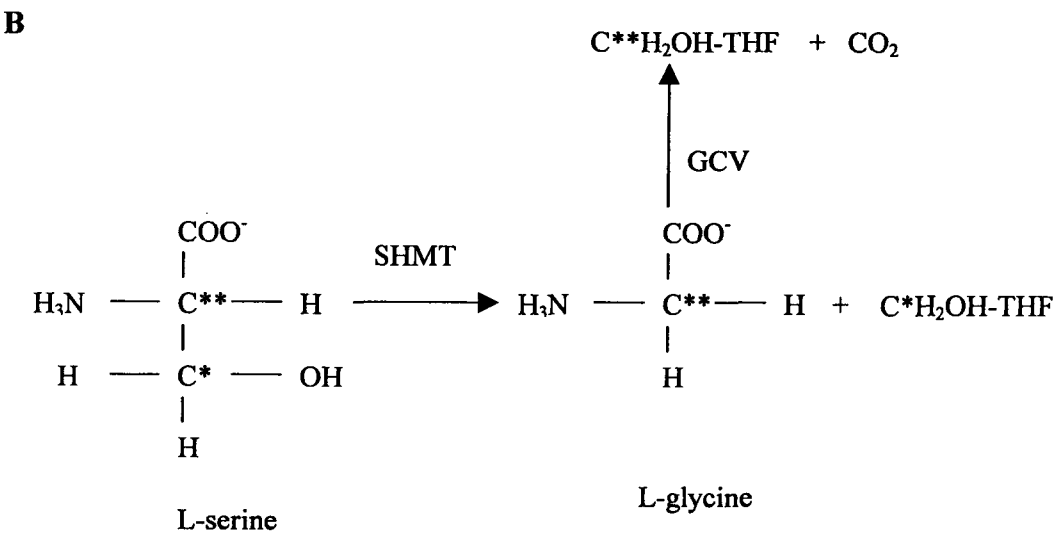


Figure 1 L-serine, glycine and one-carbon unit metabolism pathways

A) Glucose is converted to phosphoglycerate and pyruvate via the Embden Meyerhof pathway (EMP). Phosphoglycerate is converted to L-serine through a three-step pathway by 3-phosphoglycerate dehydrogenase (SerA), 3-phosphoserine aminotransferase (SerC) and phosphoserine phosphatase (SerB), respectively. L-serine may be converted to pyruvate by L-serine deaminases (L-SD) or alternatively to glycine and hydroxymethyl tetrahydrofolic acid (CH_2OH-THF) via reversible Serine hydroxymethyltransferase (SHMT). CH_2OH-THF may also be formed via the glycine cleavage enzymes (GCV). CH_2OH-THF can be reduced further to methylTHF (CH_3-THF), which is used by methionine synthase together with homocysteine to produce methionine, the immediate precursor of S-adenosylmethionine (SAM). Glycine can be generated through a two-step pathway from theronine by threonine dehydrogenase (TDH) and 2-amino-3-ketobutyrate CoA ligase (KBL). L-aspartate-semialdehyde (ASA) is the precursor of methionine, theronine and L-lysine. The dashed square frames indicate the biosynthesis pathways. The dashed arrows indicate the enzymatic reactions which need more than one enzyme.

B) Carbon atoms in L-serine flow through SHMT and GCV enzyme system generating two one-carbon units.

The Newman laboratory demonstrated the existence of two of the three *E. coli* L-SD genes, *sdaA* and *sdaB*. The *sdaA* gene is expressed in cells grown in both glucose minimal medium and Luria Broth (LB) (Su *et al.*, 1989). The *sdaB* gene, which is 73% identical to *sdaA* in primary structure, is only expressed in LB in the absence of glucose (Su and Newman, 1991). The regulation of the *sdaA* and *sdaB* genes has been studied in great detail, while the physiological roles of L-SD in the cells are still not understood completely. The synthesis of L-serine requires the expense of energy, but L-SD is still detectable even when the cells grow in glucose minimal medium where they have to synthesize L-serine. One will expect that the balance between synthesis and degradation is critical for maintaining a proper concentration of L-serine. This may be why the activity of L-SD is highly regulated in *E. coli*.

The goal of the first part of this thesis is to study the role of L-SD in overall cell metabolism. To introduce part one of the thesis, I will first provide the background of L-serine metabolism in *E. coli* and then review early studies on L-SD. Since L-serine metabolism relates to some other very important metabolic processes, such as the synthesis and use of C1 units and glycine metabolism, I will review these as well.

1.1 A survey of L-serine, glycine and C1 metabolism in *E. coli* K-12

1.1.1 The biosynthesis of L-serine and its conversion to glycine and C1 units

1.1.1.1 L-serine biosynthesis

The details of L-serine biosynthesis and its conversion to other metabolic intermediates are well understood. In *E. coli* growing in glucose minimal medium, there

is only one pathway of L-serine biosynthesis (Figure 2), as judged by the fact that mutants in *serA* require an exogenous supply of L-serine to grow. This requirement can also be supplied by glycine supplementation. However, the cell cannot grow without one or the other, indicating that this is the only pathway that functions under these conditions.

The three steps of L-serine synthesis initiated from 3-phosphoglycerate are coded by three genes, *serA serC* and *serB*. In the first highly regulated *serA*-encoded step, 3-phosphoglycerate is oxidized to 3-phosphohydroxypyruvate by 3-phosphoglycerate dehydrogenase. Then, 3-phosphoserine aminotransferase, the *serC* gene product, transaminates 3-phosphohydroxypyruvate to form 3-phosphoserine and SerB dephosphorylates 3-phosphoserine to generate L-serine.

This pathway is not exclusively involved in L-serine biosynthesis. The *serC*-encoded enzyme is also involved in pyridoxine biosynthesis and is properly named for both functions as a phosphoserine/phosphohydroxythreonine aminotransferase. This means that pyridoxal phosphate is needed for synthesis of L-serine as a cofactor of the transaminase, and similarly for synthesis of pyridoxal phosphate itself. Inhibition of *serA* would not impede pyridoxal synthesis, but sufficient repression of enzyme synthesis might cause problems.

This is a major pathway of carbon flow. Cells grown in minimal medium with glucose as carbon source assimilate about 15% of their carbon through the L-serine biosynthesis pathway (Britten *et al.*, 1955) but this may be a serious underestimate.

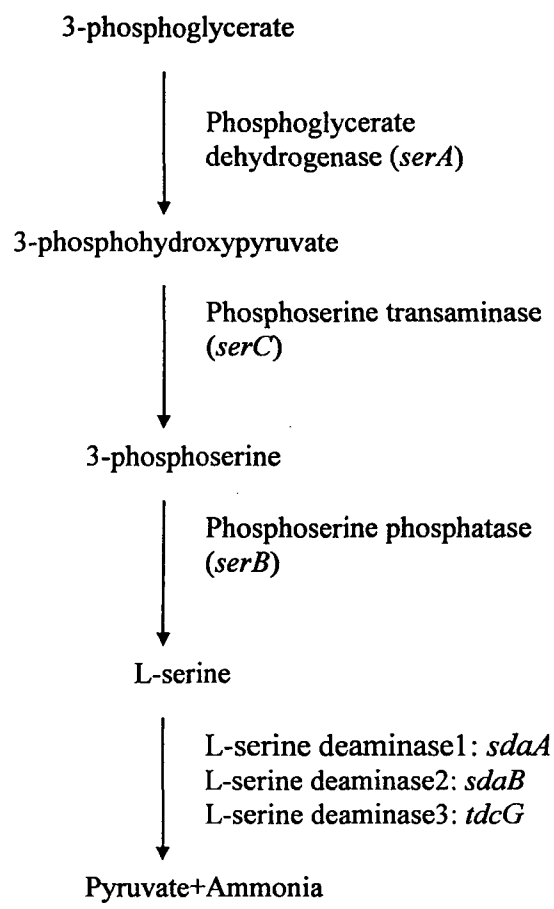


Figure 2 L-serine metabolism in *E. coli* K-12

The biosynthesis and degradation pathways of L-serine in *E. coli* K-12, adapted from Tang (2004).

1.1.1.2 Interconversion of L-serine and glycine and its relation to C1 biosynthesis

In cells growing with glucose in minimal medium, L-serine is produced by the pathway just described, and converted to glycine in a single step which also produces C1 units carried by tetrahydrofolic acid (THF). This is the only pathway of glycine biosynthesis (Figure 1) in cells growing in glucose minimal medium, as judged by the fact that a *glyA* mutant requires an exogenous supply of glycine (Pizer, 1965). Any aberration in L-serine biosynthesis will thus affect the supply of glycine and of C1-THF, and everything derived from them (Newman and Magasanik, 1963).

The interconverting enzyme is the highly reversible L-serine hydroxymethyltransferase (SHMT) (Figure 1), coded by the *glyA* gene. This enzyme also uses pyridoxal phosphate as cofactor so that biosynthesis of both L-serine and glycine depends on a supply of pyridoxal phosphate. Because this enzyme is reversible, glycine can serve as a source of L-serine (Miller and Newman, 1974; Stauffer and Brenchley, 1974) whenever a source of single carbon units is available.

C1 units are usually produced by SHMT or by GCV enzymes (Mudd and Cantoni, 1964). In general, L-serine is converted to glycine and equimolar C1-THF. However the cell frequently needs more C1-THF than glycine, and balances these requirements by cleaving some of the glycine to form CO_2 and C1-THF. A *serA* mutant cannot make L-serine *de novo* and depends on an exogenous supply of either L-serine or glycine. If the strain is also deficient in the GCV system, it cannot use glycine as a source of L-serine, indicating that this is the only pathway which converts glycine to C1 units. (Newman *et al.*, 1974a).

1.1.2 The glycine cleavage enzyme system

Glycine is cleaved by a loosely associated complex of four enzymes. Three of these, GcvP, GcvT and GcvH, are specific to the GCV system. Glycine is decarboxylated by the P protein generating an aminomethyl group and CO_2. The aminomethyl group is bound to a sulfur atom on the lipoyl group of the H protein. The T protein transfers a methylene group from the H protein to THF, reducing the H protein and generating ammonia. This leaves the dihydrolipoyl group of H protein to be regenerated by the one nonspecific enzyme in the complex, lipoamide dehydrogenase, well known as a component of pyruvate dehydrogenase (Stauffer, 1996).

Overall this reaction can be summarized as

$$NAD^+ + \text{glycine} + THF \rightleftharpoons N_5, N_{10}\ \text{methyleneTHF} + \text{ammonia} + CO_2 + NADH$$

1.1.3 Mutant studies on C1 production

From the preceding account a mutant deficient in *gcv* must produce C1 units from L-serine, and thus overproduce and excrete glycine. On the other hand, a *glyA* mutant must produce its C1 units from glycine by the GCV system. A *serA* mutant would be able to make C1 units from exogenous supplies of either L-serine (via SHMT) or from glycine (via GCV).

If this were true, it should be possible to isolate mutants of a *serA* strain with an additional mutation in *gcv*, and these should require L-serine but be unable to use glycine as a source of C1 units. The isolation of such mutants confirmed the role of *glyA* and *gcv* as the two sources of C1 in *E. coli* (Newman *et al.*, 1974a). This was demonstrated in much more detail, using the same mutant isolation, and directly demonstrating a

deficiency in glycine cleavage in the strains which could not use glycine (Plamann *et al.*, 1983).

Though this is perfectly logical, it may be only part of the picture. One would suppose that a mutant deficient in both *glyA* and *gcv* would be unable to make C1 units and would require a supply of all the end products of C1 units. However Newman *et al.* isolated a *glyA gcv* double mutant and showed that in that mutant, all C1 units arose from L-serine-3-^{14}C even though the strain was shown to be deficient in SHMT. This was taken to indicate that a third pathway of C1 synthesis exists, originating from L-serine, but not involving SHMT (Newman *et al.*, 1974a). No such pathway has ever been described, and this situation remains unresolved.

1.1.4 Alternative source of glycine

Whereas a third source of C1 units has not been identified, an alternative source of glycine has been described (Figure 1). *E. coli* K-12 makes its glycine from L-serine as described above. However it can establish an alternative pathway from threonine by a series of mutations (Fraser and Newman, 1975). Given exogenous threonine, a *glyA* mutant could convert threonine to glycine increasing the level of threonine dehydrogenase (TDH), which is usually expressed at a very low level. The strain can convert aspartate to threonine and threonine to glycine, thus suppressing the *glyA* mutation, and growing entirely without supplementation.

The mutant cells grew as well as the original strain. In this case it must derive its glycine from threonine and its C1 units entirely from glycine, unless it too uses the third unidentified pathway from L-serine. In any case it seems that a series of mutations will

allow the cell to dispense with *glyA* and still grow with no other carbon compound except glucose in its medium.

1.1.5 Uses of L-serine, glycine and C1 units as biosynthetic precursors

As the high flow of carbon through L-serine indicates, L-serine is the precursor of many metabolites, either directly or through glycine and C1 units. Its direct products include:

a) cysteine and tryptophan both made from an unaltered L-serine carbon skeleton

b) phosphatidylethanolamine, which is made from carbons two and three by decarboxylation of phosphatidylserine

c) glycine and N_5, N_{10} methyleneTHF

Thus L-serine is used as the precursor for four amino acids (itself included) and of all C1 units. The conversion to glycine accounts for a particularly large amount of L-serine, since glycine is incorporated as an intact molecule into purines. Synthesis of each purine base in DNA and RNA therefore represents the use of C1 and C2 of L-serine.

Many biochemical syntheses require insertion of a single carbon unit, formed initially as N_5, N_{10} methyleneTHF via *glyA* or *gcv*. The C1 units are required for many reactions in cells grown in minimal medium and for producing thymidylate and SAM in complex media like LB. the cell carries out a variety of methylations even in complex media, using SAM synthesized from methionine or L-serine found in the medium. Single carbon units are also involved in membrane processes, like the formation of cyclopropane derivatives, even in rich media (see below).

In minimal medium, in its biosynthetic role, N_5, N_{10} methyleneTHF is oxidized and reduced for various biosynthetic purposes. These reactions include:

a) synthesis of purine molecules which requires not only the glycine molecule just mentioned but two C1 units, which represents in total two L-serines if only *glyA* is used, and one if the *gcv* is also active

b) histidine is derived from a purine and thus involves the same requirement;

c) each thymidylate synthesized requires one N_5, N_{10} methyleneTHF;

d) in the form of formylmethionine, formylTHF is used to start every protein chain made by *E. coli*;

e) its reduced form, methylTHF, is used directly in a few methylation reactions including the biosynthesis of methionine;

and f) methionine is converted to SAM, the donor for most of the methylation in the cell.

E. coli has many methyltransferases, and produces many methylated compounds. Among these, reactions producing only a few molecules may be of critical functional importance. However among the quantitatively important ones, methylation of chromosomal and plasmid DNA account for a considerable amount of L-serine, as does methylation of tRNAs and rRNAs. However one reaction which is rarely considered, but quantitatively very important though of uncertain physiological importance, is the production of cyclopropane derivatives in *E. coli* membranes by cyclopropane fatty acyl phospholipid synthase (Cronan, 2002).

1.2 Degradation of L-serine to pyruvate by L-SDs

In view of the many synthetic reactions in which L-serine is involved, and the use of ATP in its synthesis from phosphoglycerate, it is surprising that *E. coli* codes for not one but three enzymes that degrade it in an apparently wasteful reaction to pyruvate- and not only one but three of them. This activity is known both as L-SD and L-serine dehydratase. It was first described in 1955 (Pardee and Prestidge, 1955), as generating pyruvate and ammonia, and further analyzed by Isenberg and Newman (1974).

There are in fact three homologous enzymes which deaminate L-serine, and these are coded by the *sdaA, sdaB* and *tdcG* genes (Hesslinger *et al.*, 1998; Su and Newman, 1991). All of these are dedicated L-SDs. Even TdcG, which is coded by a gene in an operon dedicated to anaerobic threonine metabolism and recognizes only L-serine as substrate and has no significant activity against threonine.

The nature of the regulation of expression of these three genes indicates their importance, in that it assures that *E. coli* contains L-SD under any circumstance the cell is likely to meet. SdaA is made under anaerobic and aerobic conditions, in glucose minimal medium and in LB glucose, and at 37 and 42 °C. This might seem to be enough. However, SdaB is made in complex media in the absence of glucose, and TdcG is made in anaerobic media with at least four particular amino acids, and in the absence of glucose. It is not easy to specify a natural environment in which *E. coli* would not make L-SD.

1.2.1 Description of the genes coding for L-SDs and the proteins they encode

The genes coding for L-SD are described in Table 1. They code for 454 or 455 amino acids containing an unusually high number of cysteines for an *E. coli* protein. *sdaB* shows 74% nucleotide identity to *sdaA,* and *tdcG* shows 70% nucleotide identity. *sdaB* and *tdc* show 70% identity.

The biochemical nature of the reaction was described in detail in 2004 (Cicchillo *et al.*, 2004). The three enzymes use a 4Fe-4S cluster for catalysis (Cicchillo *et al.*, 2004). With three of the sulfurs coming from the highly conserved residues at positions 339, 381 and 392 (Tang, 2004). The enzyme as synthesized from the ribosome does not have a 4Fe-4S cluster and is correspondingly inactive. It is activated *in vivo* by at least two gene products, one of which is identified in this work. This activation is likely to involve formation of the 4Fe-4S cluster and is mimicked *in vitro* by incubation in reducing conditions, e.g. Fe, dithiothreitol (DTT). (Newman *et al.*, 1990), but is not yet understood. The limited amount of information known about the formation of 4Fe-4S clusters will be reviewed in part two of the introduction.

1.2.2 Inhibition of growth by L-serine: the complexity of interlocking and reversible pathways

L-serine is one of the 20 amino acids, that *E. coli* needs to synthesize itself or find in its medium. As noted above, L-serine can be synthesized directly from glucose, or it can be derived from two molecules of glycine if glycine is provided in the medium, and it can be made from threonine by conversion to glycine using TDH. Which pathway is used will depend on growth conditions and nutrient availability, among other considerations.

Table 1 The properties of L-SDs in *E. coli*

Gene	Map position (min)	Protein length	Number of Cysteine residues	Nucleotide identity		
				sdaA	*sdaB*	*tdcG*
sdaA	40.85	454	9	100%	74%	70%
sdaB	63.11	455	10	74%	100%	70%
tdcG	70.18	454	8	70%	70%	100%

I have described above the reactions which make up biosynthetic pathways most relevant to this work. However the actual functioning of the cell requires not only the enzymes of the pathway but also a way of regulating their function so that carbon flow through the pathway is appropriate to the particular environmental and physiological conditions in which the cell finds itself. The most specific regulation of carbon flow through a particular pathway is carried out by one or more end-products inhibiting the first step of the pathway. This occurs when the end-product of a pathway inhibits the enzyme catalyzing the first step. The enzyme has separate binding sites for substrate and inhibitor(s), and binding of inhibitor decreases binding or metabolism of the substrate. The function of one such pathway, that for pyrimidine biosynthesis, was discovered by Arthur Pardee (1956a, 1956b), the discoverer also of L-SD.

Pathways may be short, or long, with different numbers of branches. The simplest regulation occurs with the compound prior to a branch inhibiting the first step- as in the case of pyrimidine. Since it is expensive to produce a regulated enzyme, bacteria have evolved to have the fewest possible regulated enzymes.

The biosynthesis of L-serine from phosphoglyceric acid is regulated in just this way. The end product L-serine inhibits the first enzyme phosphoglyceric acid dehydrogenase (PGDH) so that when the L-serine pool increases, its synthesis stops. When L-serine is degraded, and the cellular level decreases, more carbon would flow through the pathway unless some other control intervenes.

Feedback inhibition provides a simple and logical control for a linear irreversible pathway. Often coupled with transcriptional controls governing the amount of the enzymes made, it allows for efficient control of carbon flow. However cells have evolved

quite haphazardly and while metabolic controls are efficient, they are not necessarily the most efficient one could design. Moreover regulation is particularly difficult when pathways are reversible or interlock with other pathways.

Regulation of SHMT presents problems of this kind. When glycine is made from L-serine via SHMT and accumulates, SHMT is inhibited. However this arrangement would prevent the conversion of glycine to L-serine, and would thus be catastrophic when glycine is the sole source of L-serine. On the other hand, if glycine was converted to L-serine in an uncontrolled manner, L-serine would accumulate and L-serine is known to be toxic, as reviewed below. I suggest in this thesis that the reason why L-SD is so important is that it prevents L-serine overaccumulation from internal sources when L-serine is being made from compounds other than phosphoglyceric acid, and thus prevents the cell from being damaged by its own production of L-serine in circumstances when it can't avoid making it.

Feedback inhibition and repression of enzyme synthesis as described above are pathway specific. In addition, the cell has a variety of general activators and inhibitors, some governing a few reactions and some regulating many. Using specific binding sites in the promoter region, these govern the availability of mRNA for synthesis of particular proteins and often integrate the function of many pathways. The number of these which affect L-SD synthesis is surprisingly large including catabolite repressor protein (Crp), leucine-responsive regulatory protein (Lrp) and Cpx.

Moreover once mRNA is translated into a polypeptide, it may have problems assuming an active form, and this activation may also be regulated. L-SD is in fact made in an inactive form and must be activated by an as yet unknown mechanism. This allows

a very rapid interconversion of active and inactive forms, as in the 10 seconds or so needed to inactivate glutamine synthetase (Rhee *et al.*, 1989). A particularly relevant model 4Fe-4S enzyme, aconitase, has the additional feature that the form which is not active as an aconitase is active in catalyzing another reaction- binding on the iron-responsive elements- an RNA stem-loop structure of transferrin receptor and ferritin mRNAs to stabilize the transferrin receptor mRNA or prevent translation of the ferritin mRNA message (Beinert and Kennedy, 1993).

The actual environment of the cell will also have an effect on enzyme activity and synthesis. Less specific binding factors control the structure of the DNA with transcriptional effects of varying specificity. One of the most dramatic demonstrations of this action is the extraordinary change in form and function of the *E.coli* cell as the result of a mutation increasing the binding ability of the regulatory factor Hu (Kar *et al.*, 2005).

1.2.3 L-serine toxicity

The preceding account is based on the idea that internal accumulation of L-serine would be toxic. On the one hand, *E. coli* grows well with exogenous L-serine, as judged by the ability of a *serA* mutant to grow at the expense of an external supply of L-serine, and it needs a lot- up to 500 μg/ml for a *serA* mutant. Externally provided L-serine accumulates only as fast as the transport system allows. Nonetheless, externally provided L-serine is toxic, and L-serine-resistant mutants have been described. Accumulation from internal sources as a result of regulatory problems may present more difficult metabolic problems.

L-serine toxicity is powerful, but only partially understood. The best understood reason for L-serine inhibition of growth is its inhibition of homoserine dehydrogenase I leading to a deficiency in aspartic acid and its derivatives (Hama *et al.*, 1991). Cosloy and McFall showed that L-serine sensitivity could be counteracted by supplying isoleucine (1970). This inhibition is irrelevant for our experiments using strains derived from our lab isolate of *E. coli* because they all contain an *ilvA* deletion and are supplied with isoleucine.

This however is not the only reason for L-serine toxicity. L-serine also inhibits aromatic amino acid biosynthesis, probably by its action on prephenate dehydrogenase (Tazuya-Murayama *et al.*, 2006). Chlorate is toxic to *E. coli* by virtue of an effect in aromatic biosynthesis, and both L-serine and formate also modulate chlorate toxicity, though the mechanism is entirely unknown (Newman *et al.*, 1974b). However none of this accounts for the effects of L-serine on DNA synthesis, cell form and cell division as will be described here.

1.2.4 A comparison of effects of regulators of L-serine, glycine and C1 metabolism

1.2.4.1 *De novo* L-serine biosynthesis

The first enzyme of L-serine biosynthesis, phosphoglycerate dehydrogenase, PGDH, is inhibited by L-serine, and this is the way in which L-serine influences its synthesis since expression of the corresponding gene, *serA*, is not influenced by L-serine. Exogenously provided L-serine, in fact, inhibits incorporation of glucose carbon into L-serine. In accordance with this, PGDH activity is very sensitive to L-serine, with 50% inhibition at $4x10^{-5}$ M in *in vitro* enzyme assays (Pizer, 1963). Glycine is much less

inhibitory (Pizer, 1963) and does not inhibit incorporation of glucose into L-serine (Roberts *et al.*, 1955). That this inhibition occurs at a regulatory site and not at the substrate site was shown conclusively in 2004 (Grant *et al.*, 2004) and the mechanism of the inhibition is currently being examined in detail (Dey *et al.*, 2007).

The biosynthesis of L-serine is thus adequately controlled by feedback inhibition of the first enzyme. The expression of *serA*, which codes for PGDH, is not repressed by L-serine (McKitrick and Pizer, 1980), thus might not have major metabolic consequences, and indeed the expression of *sdaA* is not regulated by L-serine either (Isenberg and Newman, 1974; Pardee and Prestidge, 1955). However the fact that the level of both enzymes is nonetheless regulated by so many environmental factors and regulators other than L-serine suggests that control of their expression must also be important.

The first studies on L-SD showed that their expression was increased by glycine and leucine but not by L-serine (Isenberg and Newman, 1974; Pardee and Prestidge, 1955). That is, to the extent that enzyme levels matter in the face of L-serine inhibition of its own biosynthesis, the major controls on these enzymes have the apparent effect of increasing L-serine degradation and decreasing its *de novo* synthesis. The effect of leucine is very likely mediated by the Lrp which has similar effects as described in the next paragraph.

This counterintuitive reciprocity is a recurring theme, but is not understood. The transcriptional regulator Lrp represses *sdaA* expression and activates *serA* transcription. Its effect on *glyA* expression or SHMT function is not known. However it is absolutely essential for expression of the *gcv* operon. An *lrp* mutant is entirely deficient in *gcv* expression (Lin, 1992). From this one would expect that the *lrp* strain would make less L-

serine (decreased *serA* expression), convert no glycine to C1 units and thus make all its C1 from L-serine via SHMT, and degrade more serine than usual to pyruvate via L-SDs.

Another influence on PGDH expression is the carbon source which supports growth on glucose (McKitrick and Pizer, 1980). The major regulator differentiating glucose metabolism from that of other sugars is Crp. This has little effect on *sdaA* levels, but is required for *sdaB* and *tdcG* expression. Both of these genes have appropriate binding sites for Crp as expected (Sawers, 2001; Shao, 1993). Thus *sdaB* and *tdcG* are not highly expressed except in the absence of glucose. In both cases, the uninduced levels are so low that inhibitors of enzyme function would not matter for cells grown, as in this work, in glucose minimal medium or LB with glucose.

Crp and Lrp are not the only global regulators with strong effects on *sdaA* expression. Another major regulator is CpxA. The level of *sdaA* expression was particularly high in mutants in a gene known as *ssd* (Morris and Newman, 1980). Anne Monette (2006), in the Newman laboratory, showed recently that *ssd* mutants all carry a promoter mutation in *cpxP*, and usually also a compensating mutation in *cpxA*. These mutations, and also several isolated in *cpxA* in the Silhavy laboratory, all have very high L-SD levels. Therefore *sdaA* expression is induced in *cpxA* mutants. The effect of *cpxA* on *serA* expression is clearly of interest but has not been studied.

1.2.4.2 Regulation of *glyA* and *gcv* expression

The synthesis and functioning of SHMT and the GCV enzymes has turned out to be crucial to an understanding of the triple mutant. The repression of the *glyA* gene by its end products, glycine and the end products of C1 metabolism (histidine, methionine,

adenine, guanine and thymidine) is clear and almost understandable (Miller and Newman, 1974). Cells grown with glucose and glycine made approximately 45% as much enzyme activity as cells grown without. This was also true in *Salmonella* (Stauffer *et al.*, 1974). The inverse situation, starvation for endproducts, increased SHMT levels: starvation for purines in *Salmonella* and *E. coli*, and starvation for methionine in *E. coli* (Greene and Radovich, 1975). When the cells were starved for purines but given all other endproducts, SHMT levels increased.

The inhibitors of SHMT are much harder to study and much less well understood despite elegant work of Schirch and Szebenyi who reviewed this subject in 2005. For the physiological understanding which I am trying to achieve, regulators of the enzyme in both directions are important. If addition of glycine represses *glyA* expression, how does this affect growth when glycine is the sole source of C1 or of nitrogen or both?

In any case, it seems clear that the C1-THF derivatives are major regulators (inhibitors) of SHMT function. As written above, SHMT converts L-serine to glycine and CH_2OH-THF which may be oxidized to CHO-THF and reduced to CH_3-THF (Fig. 1). Of those, CH_2OH-THF and CH_3-THF bind strongly to the enzyme and inhibit it (Stover and Schirch, 1991). The reduced THF derivatives on the pathway to methionine and SAM are regulators of SHMT, and as seen below, in turn methionine and the regulators MetJ and MetR influence their concentration. The enzyme which converts CH_2OH-THF to CH_3-THF is inhibited allosterically by SAM (Sumner *et al.*, 1986) and this limits the accumulation of one of the inhibitors of SHMT.

The mechanism of SHMT is still a matter for investigation and discussion (Szebenyi *et al.*, 2004) as is the nature of possible inhibitors. If a combination of glycine and

CH_2OH-THF inhibits this enzyme, it is easy to see how this functions in the usual metabolic direction of the cell. However, it is not obvious how it works when glycine is the source of L-serine, a reaction which needs glycine and CH_2OH-THF, and should be inhibitory. A further complication in our understanding, alluded in the mechanism review just cited, is that inhibition has been studied seriously only in the direction from L-serine to glycine.

The regulation of the GCV enzyme system has been well studied and reviewed by Stauffer (1996). The GCV enzyme system is critical for cell metabolism to balance the cell's glycine and C1 requirements, so precise regulation by sensing and responding to the levels of different metabolic intermediates and end products would be expected. GcvA is a transcriptional regulator of the GCV operon, which acts in two ways: as both an activator in the presence of glycine and a repressor in the presence of inosine (Stauffer, 1996). As might be expected for a system producing a precursor for purine biosynthesis, the degradation of glycine is subjected to regulation by PurR protein (Stauffer and Stauffer, 1994). There is a PurR-binding site at about nucleotides -3 to +17 relative to the transcription initiation site in the *gcvT* promoter region (Wilson *et al.*, 1993).

Furthermore, another global regulator, Lrp, positively regulates the expression of the GCV enzymes. Mutations causing defects in Lrp function result in low and non inducible expression of the GCV enzymes (Lin *et al.*, 1992; Stauffer and Stauffer, 1994). Activation by Lrp likely functions by binding to the Lrp binding sequence found at position -69 to -244 upstream of the transcription initiation site of the *gcvT* promoter (Stauffer and Stauffer, 1994).

In addition to the regulatory factors reviewed above, there may be more regulators of the GCV system (Stauffer, 1996).

1.3 *E. coli* growth and cell division

1.3.1 General problems of a small cell with a rigid cell wall

E. coli is a rod-shaped organism about 0.5 by 0.5 by one micron when grown at 37 °C in rich medium. Because of the high concentration of molecules it contains, it is subject to tremendous osmotic pressure from its much more dilute surroundings, and it resists this by synthesizing a rigid cell wall of peptidoglycan. Indeed, *E. coli* is enclosed by a single huge molecule forming a sacculus around it.

The sacculus can be isolated as a single molecule which maintains its shape in vitro (Holtje, 1998). If this molecule is interrupted anywhere, the cell lyses. This poses a major problem for growth and cell division. How can the cell grow without at least a transient gap in the wall? Yet we know from the action of penicillin that a gap leads rapidly to lysis.

This problem occurs with any cell protected by a rigid cell wall, as is the case with plant cells. These cells are surrounded by cellulose rather than the peptidoglycan used by *E. coli* and other bacteria. However growth and cell division present the same problem. The strategy used is to increase in size before creating a gap in the existing wall. The cell gets larger by synthesizing a loop of cell wall material outside the cell from point A to point B, and then hydrolyzing the old inner wall between A and B allowing for an extension of the wall equal to the length of the loop. Growth is then a coordinated series of extensions and hydrolyses, and hydrolases are as important as synthetases in growth.

A second problem the cell faces is the maintenance of its shape as it grows longer. *E. coli* is a rod-shaped organism, and maintains this shape by adding wall along its long axis. This implies that the machinery for peptidoglycan synthesis has a corresponding spatial orientation resulting in elongation and not an increase in diameter. Because of this, when cell division is prevented but growth is still possible, the cells become longer and longer, maintaining their original diameter. This process, known as filamentation, is seen in many instances, e.g. when SulA is produced (Huisman *et al.*, 1980; Huisman *et al.*, 1984) or when the *metK84* mutant is starved of leucine (Newman *et al.*, 1998).

During normal growth the cell elongates until it doubles in size, and then makes a septum at right angles to the long axis, cutting the cytoplasm into two cells, each close to the mother cell size, with DNA partitioned into each. This septation involves chemical reactions similar to those used in elongation, but they are carried out by a different set of enzymes. *E. coli* thus alternates elongation and septation. At the onset of septation, elongation is thought to be inhibited. How this is organized, both in terms of the time at which the septation occurs and its location in space, is currently a matter of intense investigation.

E. coli maintains a constant size in whatever medium allows it the opportunity for exponential growth. However this size is not the same in all media. *E. coli* grows much faster in rich medium like LB and it forms cells which are larger. This is necessary because it takes a great deal more machinery (ribosomes, DNA polymerase, RNA polymerase) to make cells grow faster, and there has to be room in the cell for these. The mechanism by which *E. coli* manages this growth rate control over cell size is still largely

unknown. One suggestion, however is that as more machinery is accumulated the turgor pressure in the cytoplasm also increases and causes the cell wall to expand (Koch, 2000).

In this work, I describe cells filamenting, and ascribe this to difficulties in septation. I also describe increases in cell size. This implies a disruption in the integrity of the cell. Filaments can easily reach 50 cell lengths of the original cross-section dimensions with properly segregated DNA (Newman *et al.*, 1998). Cells seem to increase in cross-section only when there is some defect in the regulation of wall synthesis. They become huge, but they are correspondingly subject to lysis.

1.3.2 The nature of peptidoglycan

E. coli cytoplasm is surrounded by a membrane and a cell wall, with a space between them known as the periplasmic space. The cell wall consists of a single molecule of peptidoglycan, also known as murein, which is responsible for the shape of the cell. Peptidoglycan is a long polymer made up of long chains of two alternating sugar molecules, N-acetylmuramic acid and N-acetylglucosamine, cross-linked with a short peptide linker of three to five amino acids. This forms a single molecule surrounding the cytoplasmic membrane and conferrs rigidity upon the cell, the degree of rigidity depending upon the extent of cross linking by the peptide molecule.

The structure of peptidoglycan was reviewed recently as part of a general peptidoglycan review by Vollmer and Bertsche (2008). The N-acetylmuramic acid of the alternating sugar chain is linked to a pentapeptide, L-alanyl, D-glutamyl, mesodiaminopimelyl-D-alanyl-D-alanine. The last D-alanine is lost when this peptide is cross-linked to a second strand. It thus requires a pentapeptide to synthesize the

peptidoglycan, but the final structure is cross-linked by a tetrapeptide. With its muramic acid, two D-amino acids, and diaminopimelic acid, this macromolecule is very different from the other cell components of *E. coli*. Moreover, all of the components are synthesized internally within the cytoplasm and have to find their way to the exterior of the cell to be incorporated into peptidoglycan.

Peptidoglycan varies somewhat in different species of bacteria. However it follows the same general pattern. This molecule is of intense commercial interest because it is found only in prokaryotes and thus is a major target of antibiotics.

1.3.3 MreB and the determination of *E. coli* cell shape

The role of Mre proteins in cell shape determination has been of great interest in recent years and has been recently reviewed by Osborn and Rothfield (2007), as follows. The addition of peptidoglycan depends on a group of at least 12 transpeptidases and transglycosylases known as penicillin binding proteins (PBPs) because they form covalent bonds with penicillin. Among these, PBP2 catalyzes elongation and PBP3 catalyzes septation. Mutants deficient in PBP2 are correspondingly round, and those deficient in PBP3 form filaments. Growth of *E. coli* requires a periodic activation and inactivation at least of PBP3.

A question of current interest is the nature of the track along which the cell elongates. There is a great deal of enthusiasm for the protein MreB as some sort of scaffold, supported by the finding that mutants in *mreB* are spherical (Wachi *et al.*, 1987) as are mutants in two contiguous genes *mreC* and *mreD*. In *Bacillus subtilis* MreB forms long helical filaments under the membrane. This led to the current model that an array of

interacting proteins MreB, MreC, MreD and PBP2 interact to insert peptidoglycan helically. This model is appealing but a great deal of inconsistent evidence has accumulated (Osborn and Rothfield, 2007).

This is complicated by the fact that mutants in *rodA* are also spherical, and seem to make only septal (polar) peptidoglycan, and display no elongation-which is what causes the spherical shape. However though *rodA* mutants have been known for quite some time, nothing more about the function of this protein is known (Osborn and Rothfield, 2007).

1.3.4 Cytoplasmic synthesis of components of an exterior structure

The cell wall is external to the cytoplasm and the periplasmic space, causing enormous logistic problems, similar to those incurred in organizing the assembly of extracellular structures e.g. flagella. As reviewed by Barreteau *et al.* (2008), the small molecule precursors are synthesized in the cytoplasm. They are transferred to a large lipid molecule on the inner side of the cytoplasmic membrane, and then must be transferred to the exterior, where polymerization occurs. This is a considerable biosynthetic feat, but also means that the information in a cytoplasmic helix like that described for *mreB* would have to be transferred somehow across a considerable distance to influence the localization of new peptidoglycan synthesis.

In the cytoplasm, the cell must carry out the following reactions:

a) it converts fructose-6-phosphate to N-acetyl-glucosamine-1-phosphate (NAcG1P) and condenses that with uridine-diphosphate (UDP) to form UDP-N-acetyl-D-glucosamine (NacG).

b) it converts this to UDP-N-acetylmuramic acid (Nacmur) in two steps condensing with phosphoenolpyruvate and reducing with NADPH, using MurA and MurB.

c) it then uses MurC, MurD, MurE and MurF to add L-alanine, D-glutamate, diaminopimelic acid and the dipeptide D-alanyl-D-alanine forming UDP-NacMurpentapeptide.

The preceding reaction requires the prior synthesis of two specific precursors that are not found in other cell structures, D-alanine synthesized from L-alanine by DadX, and D-alanyl-D-alanine synthesized from D-alanine by Ddl.

One can understand this as the *de novo* synthesis of two sugar nucleotides, and the sequential addition of five amino acids to one of these by the Mur ligases. However another ligase is involved in recycling material after hydrolysis of peptidoglycan which as noted is essential for growth. This ligase, UDP-Nacmur: L-alanyl-γ-D-glutamyl-*meso*-diaminopimelate ligase (Mpl), adds the tripeptide L-ala-D-glu-DAP directly to Nacmur and thus by passing MurC, MurD and MurE. Mpl is not essential but presumably aids in the economy of the cell.

All these reactions produce the two constituents of peptidoglycan, N-acetylmuramic pentapeptide, and N-acetylglucosamine, each carried on a UDP handle. Of course these two nucleotides are located inside the cytoplasm where they are synthesized. On the other hand, the tripeptide used by Mpl or its direct precursor must be outside.

Many of the genes involved in cell wall synthesis are located under a single promoter in a single cluster known as *dcw* or *mra* at two minutes (reviewed in cell division section). These include *murC*, *mur D*, *murE* and *murF* which add the pentapeptide, but not *murA* (69 min) nor *murB* (90 min) which convert N-acetylglutamic enolpyruvate to

Nacmur, while *ddlB* is part of the *mra* cluster; *ddlA* (8.5 min) which can substitute for it is not. Neither of the two genes which convert L-alanine to D-alanine, *dadX* 27 min and *alr* (92 min) is part of the cluster. It is odd that on the one hand *E. coli* organizes 12 genes under a common promoter at two minutes and scatters the rest at 96, 92, 90.27, 8.5 among other locations. This may imply that there is a general regulator which operates at the *mra* promoter at two minutes, and also at some or all of the other promoters.

1.3.5 Getting things where they belong: the transport molecule Undecapryl phosphate

The N-acetylglucosamine and the N-acetylmuramic pentapeptide are not transferred as such to the exterior. Instead they are transferred to a huge lipid molecule bound to the inside membrane and externalized with its peptidoglycan precursors by an unknown mechanism.

This externalized molecule acts as a scaffold on which peptidoglycan can be extended. This lipid is both a transport molecule and a structural scaffold, and also prevents the precursors from diffusing away before they can be used. If there were some way in which the position of the lipid in the internal membrane was correlated with the extension of the *mreB* helix, this could provide an organizational mechanism for cell wall synthesis.

Undecaprenyl phosphate is a 55 carbon lipid. Its biosynthesis is known but not reviewed here. As described by Bouhss *et al.* (2008), the transferase MraY transfers phospho-N-acetylmuramic pentapeptide to undecaprenyl phosphate forming lipid I. MurG then transfers N-acetylglucosamine from its UDP handle forming lipid II. This is still inside the cell though membrane bound. The next step by which it is exteriorized and

forms the substrate for cell wall elongation is not known. The crucial role of this lipid has been known for many years. When it is sequestered by the antibiotic bacitracin, cell wall synthesis is blocked and the cell lyses. It is thus clearly needed to maintain the integrity of the cell. However the next steps of getting the derivatized lipid outside and incorporating the peptidoglycan precursors is not currently understood.

1.3.6 Peptidoglycan hydrolysis

As noted earlier, extension of the cell wall occurs by hydrolysis as well as by synthesis. Lysis of the cell can thus occur by a failure in the regulation of either synthetases or hydrolases. The cell wall hydrolases of bacteria have been recently reviewed by Vollmer *et al.* (2008).

These authors point out that these enzymes are difficult to study first because there are so many of them with overlapping specificities, and because any one of them may have several substrates. Thus *E. coli* has "five N-acetylmuramic acid-L-alanine amidases, six membrane bound lytic transglycosylases, and three peptidoglycan endopeptidases all involved in cleaving the septum when the daughter cells separate" (Vollmer *et al.*, 2008).

The biochemistry of the hydrolases is given in detail in the Vollmer review. For the purposes of this work the physiological roles are of much more interest. If the increase in cell length depends on a hydrolase, it should be possible to find conditional mutants that cause growth arrest. The supply of hydrolases in *E. coli* is, however, too large to permit this.

Nonetheless, their function is clearly indicated since peptidoglycan fragments are released from the wall during growth. Vollmer *et al.* estimate that 40% of the existing peptidoglycan is lost per generation.

Clearly hydrolysis has to be closely correlated with synthesis. This could be done by incorporating both activities into a multienzyme complex. During septation in *E. coli*, hydrolysis follows quickly after cell wall synthesis, and defects in the process can be seen with mutants in any of the six amidases genes, including Slt70, MltA-D and EmtA (Holtje, 1996).

1.3.7 Scavenging of hydrolyzed peptidoglycan fragments

It would not be very efficient to cleave 40% of the peptidoglycan every generation and throw it away. Indeed, *E. coli* has permeases devoted to bringing these fragments back into the cell and recycling them. Vollmer *et al.* (2008) list AmpG permease for disaccharide peptide complexes. AmpD cleaves off the tri-, tetra- and pentapeptides which can be reincorporated by Mur enzymes, as mentioned above. Otherwise diaminopimelic acid is released by MpaA, and the L-Ala-γ-D-glu dipeptide can be racemized to L-Ala-L-Glu by YcjG, and then hydrolyzed by PepD. This is only the start of the list. One would not expect any of these enzymes to be essential but they must add greatly to the efficiency of the operation.

1.3.8 Physiological consequences of the *ldc* mutation

The *ldcA* gene of *E. coli* codes for a L, D-carboxypeptidase which acts on the pentapeptide releasing on recycling the peptidoglycan, cleaving the terminal D-alanines and releasing the tripeptide which is reincorporated as indicated above (Templin *et al.*,

1999). This gene is essential for *E. coli* survival if one uses the correct conditions. *E. coli* cells devoid of *ldcA* lyse in stationary phase. The cells accumulate the tetrapeptide, which becomes incorporated into new peptidoglycan but forms a much weaker structure and so the cells lyse.

LdcA is inhibited by dithiazolidines and this causes lysis of 90% of the population in stationary phase. This is consistent with the preceding mutant study. It is also relevant to my work in that it would not be noticed at all if one just grew populations and used them without looking at the late stationary phase culture. Similarly malformed cells were noted in the present work only by the examination of individual cells, this time early in development of the culture.

1.4 *E. coli* cell cycle

The triple mutant deficient in L-SD shows big differences in cell division from the parent strain. Therefore, a large amount of my work has focused on the study of cell division. For this reason, the following review about the cell cycle and cell division is provided.

Bacterial cytology and caryology have been popular topics for microbiologists for more than a hundred years. By the 1990s, progress in fluorescence microscopy, the development of new methods of selectively staining specific DNA fragments and aggregates of specific proteins brought a breakthrough of this area. New discoveries in this field have been reviewed by Prozorov (2005).

The bacterial cell cycle integrates two major processes, a DNA cycle that includes chromosome replication and segregation; and a division cycle that includes elongation,

coordinated such that the cell septum is completed only after the DNA has segregated (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.

Conditional mutants which cannot perform proper cell division can have a quite different phenotypes: long threadlike cells (filamentous phenotype), deprived of a septa (sep phenotype), disorderly distributed nucleoids (par phenotype), small cells deprived of nucleoids (min phenotype), and very often decelerated growth and decreased viability (Prozorov, 2005).

1.4.1 Chromosomal DNA replication and segregation

Initiation of chromosomal DNA replication is precisely modulated during the cell cycle. DnaA triggers DNA replication at a certain critical level (Moriya *et al.*, 1990; Zyskind and Smith, 1992). In *E. coli*, regulation of replication initiation is achieved through the assembly and disassembly of DnaA-*oriC* complexes (Fuller *et al.*, 1984; Leonard and Grimwade, 2005; Margulies and Kaguni, 1996; Weigel *et al.*, 1997). DnaA binds to at least eight specific sequences in *oriC* (Nievera *et al.*, 2006). DNA helicase binds to DnaA-*oriC* complexes, untwisting the double-stranded DNA and thus enabling replication. Fluorescence microscopy revealed that the replisome does not move during replication and thus is probably attached to the cytoplasmic membrane (Lemon and Grossman, 1998, , 2000).

To prevent re-initiation of DNA replication before a cell needs it, *E. coli* discriminates between the newly synthesized DNA and old template DNA by methylation. Only the old

template DNA remains methylated, and nonmethylated newly synthesized DNA cannot serve as a template in the next cycle (Russell and Zinder, 1987). In *E. coli*, right after initiation, the Seq protein binds to hemimethylated *oriC*, quarantining *oriC* while levels of active DnaA are reduced, preventing re-initiation. Sequestration requires hemimethylated GATCs located at 11 positions within *oriC* interacting with the Seq protein (Bach and Skarstad, 2004; Brendler T, 1995; Lu *et al.*, 1994; Slater S, 1995). In *seqA* mutants, the *oriC* region has a multifork structure because of the repeated duplication (Lu *et al.*, 1994).

During the replication of the DNA, the nucleoid turns so as to be positioned across the cell, while the labeled *terC* still remains in the center of the cell and simultaneously, the *oriC* duplicates and both of the *oriC*s move towards the poles and stop in a short distance from them, then duplication of *terC* occurs. The duplicated nucleoids move to the cell poles without changing their orientation, and the intercellular septum is formed (Jacob and Brenner, 1963; Prozorov, 2005).

Bacterial proteins analogous to centromeres and kinetochores were suggested by some microbiologists (Hiraga *et al.*, 1989; Holmes and Cozzarelli, 2000; Niki *et al.*, 1991). *E. coli* MukB protein, which belongs to the ubiquitous family of structural maintenance of chromosomes (SMC) proteins, plays an important role in chromosome organization and processing. It possibly has functions similar to those of the eukaryotic chromosome pulling spindle. In *mukBEF* mutants, viability is restricted to low temperature and 15% of cells are produced as anucleate (Britton *et al.*, 1998; Niki *et al.*, 1991). It was shown that in *mukB* mutant cells, the two chromosome arms do not separate into distinct cell halves,

but extend from pole to pole with the *oriC* region located at the old pole (Danilova *et al.*, 2007).

It is difficult to distinguish between the end of chromosome segregation and separation with the beginning of cell division. They are closely related to and occur almost at the same time.

1.4.2 *E. coli* cell division

The study of bacterial cell division has been greatly promoted by new techniques in the past decade. The new achievements in cell division were reviewed by Weiss (2004) and Errington *et al.* (2003).

E. coli cells assemble a septal protein complex at the midcell during binary fission. This process involves the recruitment of at least 15 proteins to the division site (Buddelmeijer and Beckwith, 2002; Weiss, 2004). These cytoplasmic, membrane, and periplasmic proteins localize into a ring across the cell width at midcell. FtsZ, the first protein to localize, forms a ring (Z ring) between the two segregated nucleoids right after the termination of replication (Den Blaauwen *et al.*, 1999). The rest of the septal proteins then are recruited to the Z ring one after another in a particular order.

The selection of the correct division site at midcell is modulated by two global systems, which have important topological roles in identifying this site (Margolin, 2001; Yu and Margolin, 1999). One is negative spatial regulation called nucleoid occlusion (NO), which involves the nucleoid itself (Woldringh *et al.*, 1990; Woldringh *et al.*, 1991). By an unknown mechanism, Z ring assembly on the membrane is inhibited by the presence of the nucleoid at the same site. Indeed cell division was largely inhibited in

cells in which DNA replication and nucleoid segregation failed (Mulder and Woldringh, 1989). Therefore, it is proposed that bacteria may sense the sites from which DNA is absent and other sites are occluded. There are three possible sites in the cell where DNA is absent or reduced in concentration: one is between the replicated and segregated nucleoids at mid cell and the other two lie at the cell poles. As a result, three potential division sites are available in the cell: one correct site at mid cell and two incorrect sites at the poles.

The molecular mechanism of nucleoid occlusion is as yet unknown. However, optimum nucleoid occlusion may require specific organization and structure of the nucleoid, for example assuring the correct local density of the nucleoid and/or nucleoid-associated proteins. Drugs which block transcription, translation or protein secretion influence nucleoid structure, and lead to defective nucleoid occlusion (Sun and Margolin, 2004). Disruption of MukB, a homolog of SMC involved in condensation of the nucleoid (Sawitzke and Austin, 2000), results in thermosensitive growth and unexpected Z ring formation above the unsegregated nucleoid (Dasgupta *et al.*, 2000; Niki *et al.*, 1991; Sun *et al.*, 1998).

The other mechanism for division site selection is the Min system, which prevents unwanted division events at nucleoid free cell poles, MinC, an inhibitor of Z ring formation, continuously oscillates between the two cell poles. The membrane-associated ATPase (MinD) and the enhancer of the MinD ATPase (MinE) keep MinC away from the midcell site (de Boer *et al.*, 1989; Hale *et al.*, 2001; Hu *et al.*, 1999; Hu and Lutkenhaus, 2001; Raskin and de Boer, 1999a, 1999b). The Min system may be important for the accurate placement of midcell Z ring (Huang *et al.*, 2003), or it may

simply function to prevent polar divisions as it does in *Bacillus subtilis* (Migocki *et al.*, 2002).

The nucleoid and Min system appear to function independently in *E. coli*. In a mutant which frequently makes cells without nucleoids, Z rings localize anyway near midcell, probably because the Min system remains functional in such anucleate cells (Sun *et al.*, 1998). On the contrary, without the Min system, nucleoids can still inhibit septum formation in their surrounding (Sun and Margolin, 2001; Yu and Margolin, 1999). Arresting chromosomal DNA replication by therminactivation of *dnaA* does not affect nucleoid occlusion activity, indicating that chromosome replication is not required to maintain nucleoid occlusion (Sun and Margolin, 2001).

In *E. coli*, the assembly of septa involves the recruitment of at least 15 currently known proteins at mid cell (Buddelmeijer and Beckwith, 2002; Weiss, 2004). Many of these are encoded by division genes which were originally recognized in conditional mutants forming long aseptate filaments under non-permissive conditions, and so they were named filamenting temperature sensitive mutants (*fts*) (Eberhardt *et al.*, 2003). These proteins are recruited into the ring one by one in a sequential and almost linear order. The order is as follow: FtsZ>FtsA/ZapA/ZipA> (FtsE, FtsX)> FtsK> FtsQ> (FtsB, FtsL)> FtsW >FtsI >FtsN> AmiC> EnvC (Errington *et al.*, 2003; Vicente and Lowe, 2003; Weiss, 2004). The proteins FtsA, ZapA and ZipA are independent of each other but dependent on FtsZ. Those within parentheses assemble simultaneously (Vicente *et al.*, 2006). The failure in the recruiting of a protein in the sequence may prevent the assembly of those that incorporate later to localize into the ring. However, it is not known if the

incorporation of a particular protein into the ring requires the presence of all those proteins in ahead of it (Vicente *et al.*, 2006).

FtsI (PBP3) is the only division protein with an identified enzymatic function. It is involved in the synthesis of peptidoglycan at the septal site (Spratt, 1977). Pogliano *et al.*, (1997) found inactivation of FtsI not only delayed the assembly of the Z ring but also decreased the total number of rings per unit cell mass.

FtsI protein has three domains: a cytoplasmic domain, a transmembrane helix and a periplasmic domain (Bowler and Spratt, 1989). Wissel and Weiss (2004) showed the mutant FtsI which had lesions in one of three amino acids-R23, L39 or Q46, which are in or near the septal localization region of FtsI, could not be localized to the septal ring.

1.4.3 *mra* cluster located at two min on the *E. coli* genetic map

On the *E. coli* genetic map two min location, there is a large operon which is comprised of cell division and cell envelope biosynthesis genes. The majority of known division genes are located within this large *mra* cluster, except *ftsK* at 20.1 min, *ftsB* at 1.2 min and *amiC* at 63.5 min. All of the sixteen genes in the two min *mra* cluster are transcribed in the same direction, with many of the ORFs overlapping or separated by only very short gaps (Vicente *et al.*, 1998). Regulation of expression of these genes is extremely complex.

The *mra* promoter, the first promoter of the *mra* cluster, is required for full expression of at least the first nine genes, including the division genes *ftsL*, *ftsI* and *ftsW* (Hara *et al.*, 1997). The transcription of the genes located downstream from these nine genes, even the last gene in the cluster, is also mainly dependent on the Pmra promoter, although they are

also under the regulation of other promoters such as the ones present in the *ftsQ-A-Z* operon, regulate gene expression too (Dewar and Dorazi, 2000). Production of the FtsZ protein is significantly reduced when Pmra is repressed (Mengin-Lecreulx *et al.*, 1998).

The correct intracellular concentration of the division proteins is very important for cell growth. Unexpected changes in the levels of some essential division proteins can disrupt cell division. For instance, the proper ratio of FtsZ to FtsA is critical for cell division (Dai and Lutkenhaus, 1992); overproduction of ZipA abolishes cell division (Hale and de Boer, 1999) .

The gene order and content of the *E. coli* cluster are conserved among bacteria, most closely in species with similar cell shape. For rod-shaped bacteria even from very distant groups there is a strong trend toward conservation. On the contrary, the genes tend to be dispersed in other morphological groups (Mingorance *et al.*, 2004; Tamames *et al.*, 2001).

There is no doubt that deletion or inactivation of division genes leads to blockage of cell division. Among interesting findings is the fact that some mutants exhibit abnormal cell morphology other than filaments. A *ftsL*-null mutant displayed Y-shaped filaments or contained bulges along the filaments (Guzman *et al.*, 1992). C-shaped cells were formed by a specific mutation in the *ftsA* gene (Gayda *et al.*, 1992).

1.5 SAM and cell division

A variety of strategies have been used to study the roles of SAM in cellular metabolism. Hughes and colleagues introduced a plasmid containing a SAM hydrolase encoding gene from the coliphage T3 into a *E. coli* cells and tried to deplete SAM in the

cells (Hughes *et al.*, 1987). High levels of SAM hydrolase did not affect the growth of the markedly cells obviously. However, there was one noticeable phenotypic trait conferred by the SAM hydrolase gene, namely the cells were shown to elongate and occasionally filaments during early log-phase growth (Hughes *et al.*, 1987). When the SAM hydrolase gene was under the control of a even stronger promoter trc, more extensive filamentation was observed in minimal medium (Posnick and Samson, 1999).

Previous work from the Newman laboratory showed that SAM synthetase-deficient *metK84* mutants led to a decrease in SAM synthetase activity and to filamentation under certain condition (Newman *et al.*, 1998). When starved of SAM, these cells can metabolize for some generations, but cannot divide normally, and therefore, form long filaments with segregated DNA (Newman *et al.*, 1998; Wei and Newman, 2002). Later work on this mutant demonstrated that the early division proteins, FtsZ, FtsA and ZipA showed a regular distribution of division rings but the division proteins, FtsQ, FtsW, FtsI and FtsN, were rarely recruited to the filaments (Wang *et al.*, 2005).

The study above suggests that SAM plays a role in the regulation of cell division. However the mechanism in which SAM is involved is as yet unknown.

Chapter 2 Materials and methods

2.1 Strains, bacteriophages, and plasmids

The strains, bacteriophages, and plasmids used in this study are listed in Table 2.

2.2 Media, reagents, antibiotics and growth conditions

2.2.1 Luria-Broth (LB)

LB contained 1.0% Bacto-tryptone, 0.5% Yeast extract and 0.5% NaCl in distilled water. The pH of the medium was adjusted to 7.0 by either HCl or NaOH. Corresponding solid media were made by adding 2% Bacto-agar. The media were sterilized by autoclaving at 121°C for 45 min.

2.2.2 Minimal Medium (NIV)

NIV used contained 0.2% $(NH_4)_2SO_4$, 1.5% K_2HPO_4, 0.525% KH_2PO_4, 0.005% L-isoleucine and 0.005% L-valine in distilled water. The pH of the medium was made as 7.0. Corresponding solid media were made by adding 2% Bacto-agar. The media were sterilized by autoclaving at 121°C for 45 min. Sterile 1% $CaCl_2$, 10% $MgSO_4$ and 20% glucose were added to the autoclaved medium before using to final concentrations of 0.004%, 0.08% and 0.2% respectively.

2.2.3 SGL Medium

SGL medium contained 0.2% $(NH_4)_2SO_4$, 1.5% K_2HPO_4, 0.525% KH_2PO_4, 0.005% L-isoleucine and 0.005% L-valine in distilled water. The pH of the medium was made as

Table 2 *E. coli* strains and plasmids used in part 1

Strains and plasmids	Genotype and/or relevant characteristics	Source or reference
Strains		
Cu1008	*E. coli* K-12 *ilvA*	L. S. Williams
MEW1	Cu1008*Δlac*	Newman *et al.*, 1985b
MEW990	MEW1*ΔsdaA*	This study
MEW991	MEW1*ΔsdaB*	This study
MEW992	MEW1*ΔtdcG*	This study
MEW993	MEW1*ΔsdaAΔsdaB*	This study
MEW994	MEW1*ΔsdaAΔtdcG*	This study
MEW995	MEW1*ΔsdaBΔtdcG*	This study
MEW999	MEW1*ΔsdaAΔsdaBΔtdcG*	This study
MEW191	SGL-derivative by Mu::dX insertion from CAG5050 into MEW1	Newman *et al.* 1985b
JW1803	Keio collection *E. coli* K-12 BW25113*ΔsdaA* Km^R	Baba *et al.*, 2006
JW2768	Keio collection *E. coli* K-12 BW25113*ΔsdaB* Km^R	Baba *et al.*, 2006
JW5520	Keio collection *E. coli* K-12 BW25113*ΔtdcG* Km^R	Baba *et al.*, 2006
EC436	MC4100 *Δ (λattL-lom)::bla lacI^q P$_{207}$-gfp-ftsI*	Weiss *et al.*, 1999
EC448	MC4100 *Δ (λattL-lom)::bla lacI^q P$_{208}$-ftsZ-gfp*	Weiss *et al.*, 1999
EC791	MC4100 *Δ (λattL-lom)::bla lacI^q P$_{209}$-gfp-ftsW*	Mercer *et al.*, 2002
MetK84/ftsZ-gfp	MEW402 *Δ (λattL-lom)::bla lacI^q P$_{208}$-ftsZ-gfp*	Wang *et al.*, 2005
MEW999/*ftsZ-gfp*	MEW999 *Δ (λattL-lom)::bla lacI^q P$_{208}$-ftsZ-gfp*	This study

Cont.

MEW999/*gfp-ftsI*	MEW999 *Δ (λattL-lom)::bla lacI^q P$_{207}$-gfp-ftsI*	This study
MEW999/*gfp-ftsW*	MEW999 *Δ (λattL-lom)::bla lacI^q P$_{209}$-gfp- ftsW*	This study
MEW1/ *ftsZ-gfp*	MEW1 *Δ (λattL-lom)::bla lacI^q P$_{208}$- ftsZ-gfp*	This study
MEW1/*gfp-ftsI*	MEW1 *Δ (λattL-lom)::bla lacI^q P$_{207}$-gfp-ftsI*	This study
MEW1/*gfp-ftsW*	MEW1 *Δ (λattL-lom)::bla lacI^q P$_{209}$-gfp- ftsW*	This study
JE7968[I]	W3110 P_{mra}::P_{lac}	Hara *et al.*, 1997
MEW999h[I]	MEW999 P_{mra}::P_{lac}	This study
Plasmids		
p*sdaA*	pBR322 carrying *sdaA* gene and part of its upstream and downstream region Amp^R	Su *et al.*, 1991
psamT	plTET plasmid carrying the Rickettsial SAM transporter Cm^r	Wood *et al.*
pCP20	Amp^R and Cm^R plasmid with temperature-sensitive replication and thermal induction of FLP recombinase	Cherepanov and Wackernagel 1995
PGS146	A expression plasmid carries the *E. coli* gcv system	Stauffer *et al.*, 1993
Phages		
P1 *vir*		E. McFall

I: A IPTG dependent strain whose *mra* promoter was disrupted by inserting the *lac* promoter

7.0. Corresponding solid media were made by adding 2% Bacto-agar. The media were sterilized by autoclaving at 121°C for 45 min. Sterile 1% $CaCl_2$, 10% $MgSO_4$ were added to the autoclaved medium before using to final concentrations of 0.004%, 0.08% respectively. Different from NIV, instead of adding glucose, L-serine, glycine and L-leucine are added into the medium to final concentrations of 600 μg/mL, 300 μg/mL and 300 μg/mL respectively as the only carbon sources unless otherwise noted.

2.2.4 R-top agar

R-top agar contained 1% Bacto-tryptone, 0.1% Yeast extract, 0.8% NaCl and 0.8% Bacto-agar in distilled water. The pH of the medium was adjusted according to the requirement of the experiment by either HCl or NaOH. The media were sterilized by autoclaving at 121°C for 45 min. Sterile 1M $CaCl_2$ and 20% glucose were added to the autoclaved medium to final concentrations of 2 mM and 0.1% respectively.

2.2.5 SOC medium for electro-transformation

SOC medium contained 2% Bacto-tryptone, 0.5% Yeast extract, 0.058% NaCl, 0.0185% KCl and 0.203% $MgCl_2$ in distilled water. The pH of the medium was adjusted to 7.0 by either HCl or NaOH. The media were sterilized by autoclaving at 121°C for 45 min. Sterile 20% glucose was added to the autoclaved medium to a final concentration of 0.38%.

2.2.6 Preparation of S-Adenosyl Methionine (SAM) Stock

SAM-e, the nutritional supplement of SAM was obtained from Foodscience Of Vermont as 200 mg tablets. The tablets were ground and dissolved in 1 mM HCl to a

final concentration of approximately 100 mM. The mixture was centrifuged at 4000 rpm for five min to pellet the insoluble material and the supernatant was filtered through a 0.4 μm pore size filter and stored as aliquots at -20 °C. The real concentration of SAM was obtained by measuring the absorbance at 260 nm (ε= 15, 400 M-1cm-1).

2.2.7 Antibiotics and Supplements

Antibiotics were used at the following concentrations, in μg/mL: Ampicillin (Amp) 100, tetracycline (Te) 25, kanamycin (Kan) 40, chloramphenicol (Cm) 25 and streptomycin (Sp) 100.

Additions were made to the following concentrations unless otherwise noted: glucose (0.2%), amino acids (100 μg/mL), casamino acids (0.5%). Overnight cultures were diluted 10^4 fold into fresh medium to start the experiments unless otherwise noted.

2.3 Buffers and solutions

2.3.1 SM buffer

SM buffers contained 0.58% NaCl, 0.2% $MgSO_4 \cdot 7H_2O$, 0.05M Tris·Cl (pH 7.5) and 0.01% gelatin in distilled water. The buffers were sterilized by autoclaving at 121°C for 45 min.

2.3.2 2,4-Dinitriphenylhydrazine (DNPH) solution

DNPH stock solutions contained 0.02% DNPH and 1% HCl the distilled water and were stored at 4 °C in the dark.

2.3.3 Fix solution for microscopy

Fix solutions were prepared by adding paraformaldehyde and glutaraldehyde into distilled water to final concentrations of 16% and 0.1% respectively.

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

DAPI (Sigma Co.) was dissolved in PBS buffer to a final concentration of 20 mg/mL and stored at -20 °C in the dark.

2.3.5 Enzymes, oligonucleotides, DNA ladder and DNA kits

Restriction enzymes, *Taq* DNA polymerase, *Pfu* DNA polymerase, GeneRuler^{TM} 1 kb DNA ladder, T4 DNA polymerase, T4 DNA ligase, CIAP etc. were purchased from MBI Fermentas (Montreal, Canada). QIAprep Spin Miniprep Kit and GenElute Plasmid Miniprep Kit were purchased from QIAGEN (Montreal, Canada) or SIGMA (USA) respectively. DNA mini Kit, QIAEX II Gel Extraction Kit and QIAquick PCR Purification Kit were purchased from QIAGEN (Montreal, Canada). Oligonucleotides used in this study were purchased from Biocorp Montreal.

2.3.6 Chemicals

Amino acids: L-serine was purchased from CALBIOCHEM (Germany). The other amino acids were purchased form SIGMA (USA). Casàmino acids was purchased from DIFCO (USA).

2.4 Experiment methods

2.4.1 P1 phage transduction

P1 phage-mediated transduction is performed as described by Miller (1972) with some modifications.

2.4.1.1 P1 phage lysate preparation

A single colony of donor strain was inoculated into LB+Ca^{2+} and incubated at 37 °C overnight without shaking. Subsequently 0.1 mL P1 phage (10^5-10^6 phage/mL) was added to 1 mL of overnight culture and incubated at 37 °C for 15-30 min to let the phage infect the donor strain. Then 3 mL melted R-top agar is added, and the whole mixture is poured onto a fresh LB+Ca^{2+} agar plate. After incubating the plate without inversion for eight hours, the phage lysate was collected and stored at 4 °C with two to three drops of chloroform.

2.4.1.2 P1 phage-mediated transduction

The recipient strain was incubated in LB+Ca^{2+} and then harvested and resuspended in 1/10 volume of LB+Ca^{2+} when it reached late log phase (OD_{600}=0.7-1.0). Then 0.1 mL of the resuspended culture was mixed with a properly diluted P1 phage lysate containing the desired gene. The mixture was incubated at 37 °C for 15 min, 1 mL of 1 M sodium citrate was added and the cells pelleted by centrifudge. Then the cells were resuspended in 1 mL of LB+glucose. After incubation at 37 °C for one hour, a 0.1 mL aliquot was plated on the appropriate selection plates.

2.4.2 Fixing cells

A 500 µl aliquot of the culture was added directly to 100 µl of the fix solution and 20 µl sodium phosphate buffer (pH7.4). The mixture was incubated at room temperature for 15 minutes, and then incubate on ice for 15 minutes. The cells were centrifudged at low speed of 4,000-5,000 rpm for five minutes. The pellet was washed 2-3 times in 1 mL of PBS buffer, and resuspended in PBS buffer at a concentration of 50-100 µl of PBS per 0.1 OD_{600} unit. At this point or later, the cells were stable and can be kept at 4 °C for even more than one month until needed.

2.4.3 Staining of DNA with DAPI

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

2.4.4 Fluorescence microscopy

Fluorescence micrographs were recorded on a Leica microscope equipped with a 100× 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 two – five seconds for GFP signal, 0.5-1 seconds for DAPI signal and 0.1-0.5 seconds for phase image.

The experiments of fluorescence microscopy were performed partially in BRI (Biotechnology Research Institute) and mostly in Dr. Boushel's lab (Exercise Science, Concordia) with Leica DMIRE2.

2.4.5 Slide cultures

To prepare slides, between 3 and 5 mL of melted medium with 2% agar were poured on a slide, the excess was poured off, and the rest allowed solidifying. Overnight cultures grown in liquid minimal medium with glucose 0.2% were diluted 1/1000 in the same fresh medium and 4 µl were pipetted onto slides of the appropriate composition prepared as above, and spread by covering with a cover glass. The slides were incubated in a covered Petri dish with wet kimwipes to maintain sufficient humidity.

2.4.6 Microscopy

The microscopes used in this study were Leica DMIRE2, Zeiss Axioplan and National DC3-163.

2.4.7 L-SD enzyme assays

L-SD was assayed as described previously in toluene-treated whole cells (Newman *et al.*, 1985a), using 30 min incubation. 1 unit of L-SD is defined as the amount of enzyme which catalyzed the formation of one µmol of pyruvate in 30 min.

2.4.8 Plasmid isolation and restriction enzyme digestion

2.4.8.1 Plasmid isolation

Plasmid was isolated by Sigma plasmid miniprep kit following the manufacturer's instructions.

2.4.8.2 Restriction enzyme digestion

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

2.4.8.3 DNA gel electrophoresis analysis

1% DNA agarose gels (1%) were used for electrophoresis analysis.

2.4.8.4 Electro-transformation

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

2.5 Construction of strains

2.5.1 Construction of MEW1 derivatives carrying single, double or triple L-SD deletion

The single, double and triple L-SD deletion strains MEW990, MEW991, MEW992, MEW993, MEW994, MEW995 and MEW999 were constructed by deleting one, two or three of the genes *sdaA*, *sdaB* and *tdcG* from the parent strain MEW1. Donor strains were from the systematic, single-gene knockout mutant Keio collection (Baba *et al.*, 2006) of all the nonessential genes of BW25113. These strains carry a kanamycin resistance gene replacing the targeted gene, flanked by a 50 bp FRT homologous sequence. This inserted

Table 3 Primers used to verify the gene deletions in MEW999

Gene	Front (F) and Rear (R) primers		Base pairs
sdaA	Internal F	5'-CGTTTTCATAACGGCAACCT-3'	20
	Internal R	5'-GGGCTGACCGATTCAATAAA-3'	20
	Outside F	5'-AGATATCTACCGCCGTGGTG-3'	20
	Outside R	5'-CTGTAAGCGTATGGCGACAA-3'	20
sdaB	Internal F	5'-ACGATCTGATTGCCCGTAAC-3'	20
	Internal R	5'-CGGGTCAGTGGTGGTTTTAT-3'	20
	Outside F	5'-CGTATTCGTTGTCGTGATGG-3'	20
	Outside R	5'-TCTGGTAAACGCTGATGACG-3'	20
tdcG	Internal F	5'-GAAGAACACTTCGGCCTGTC-3'	20
	Internal R	5'-ATTTGTACCTGTCCGGCAAC-3'	20
	Outside F	5'-TCTGAATGATTTTGCCACCA-3'	20
	Outside R	5'-ATGACGCCTTTATTCGATGC-3'	20

gene was transferred into the host strain by P1 transduction. Then plasmid pCP20 (Cherepanov and Wackernagel, 1995) was introduced and the resultant homologous recombination deleted the kanamycin resistance gene allowing the process to be repeated for the second and third genes. The deletions were confirmed by PCR using the following primers (Table 3), and Taq polymerase with 25 cycles and 50 °C annealing.

2.5.2 *gfp* Merodiploid cells

The merodiploid strains used in this study are in an MEW1 or MEW999 backgroud. Each of them contains two copies of a specific septal protein gene. The first one is the wild type copy, which is located at the original site. The other one, fused with green fluorescent protein (GFP) encoding gene, is located at the λ phage attachment site.

All the original merodiploid strains are the gifts from Dr. Weiss. The constructions of merodiploids were made using the λInCh insertion system (Weiss *et al.*, 1999). P1 phage lysates were made on Dr.Weiss's strains: EC436, EC448 and EC791, and used to infect MEW1 and MEW999, selecting transductants on LB plates with ampicillin 25 μg/mL. The nature of the transductants was confirmed by examining GFP fluorescence. The following genes were transferred to MEW1 and MEW999 respectively:

ftsZ-gfp	from strain EC448
gfp-ftsW	from strain EC791
gfp-ftsI	from strain EC436

2.5.3 Construction of MEW999h

The *mra* promoter in MEW999 was replaced with a *lac* promoter by P1 transduction to generate MEW999h. P1 phage lysate was made from Dr. Hara's strain JE7968.

Transductants were selected on LB plates with chloramphenicol (25 μg/mL) and IPTG (500 μM). Transductants were confirmed by examining the IPTG dependence of the cell.

2.6 Preparation the cell samples for GFP localization

2.6.1 Preparation of MEW1 merodiploid cell samples

A single colony was grown in glucose minimal medium with ampicillin 25 (μg/mL) at 37 °C over night. The overnight culture was diluted into the same medium with CAA (0.5%) and IPTG and without ampicillin for 10^4 fold and grown at 37°C to the sample time. A 500 μl sample was fixed for protein localization and DAPI staining was done for DNA localization.

2.6.2 MEW999 merodiploid cell preparation

Different from the parent strain, the triple mutant MEW999 strain showed misshapen cells tended to lyse in the presence of CAA, so when theese cells were prepared for the fluorescent microscopy, all the steps should be very gentle. No vortexing and violent pipetting should be used for MEW999 samples.

A single colony was grown in glucose minimal medium with ampicillin (25 μg/mL) at 37 °C for overnight. The overnight culture was diluted 10^4 fold into fresh glucose minimal medium with CAA (0.5%) and IPTG and grown at 37°C to the sample time. IPTG concentrations used to induce the expression of *ftsZ-gfp*, *gfp-ftsW* and *gfp-ftsI* were 25 μM, 1 mM and 25 μM respectively. If the samples were taken before six hours after the subculture, 10 mL culture was taken to collect all the cells for GFP localization. The 10 mL culture was centrifuged at 10,000 rpm for 10 min, and then 500 μl minimal

medium was added to resuspend the cells for fixing and DAPI staining. If the samples were taken six hours after the subculture, a 1 mL sample was taken for fixing and DAPI staining. The centrifuge speed should not be higher than 5000 rpm for the samples which had swollen cells.

2.6.3 *metK84/ftsZ-gfp*

The leucine-dependent strain *metK84/ftsZ-gfp* produces long filamants in glucose minimal medium when 5 μg/mL of L-leucine was added (Newman *et al.*, 1998).

A single colony was grown in glucose minimal medium with amplicillin (25 μg/mL) at 37 °C for over night. Cells were washed three times with minimal medium plus 5 μg/mL L-leucine and inoculated 1:1000 into fresh glucose minimal medium with leucine 5 (μg/mL) and IPTG (25 μM). The culture was incubated for 16 hours. Then samples were fixed for protein localization and DAPI stain.

Chapter 3 Results

As described in the introduction, although enzyme-catalyzed L-SD in *E. coli* has been known for more than 50 years, the metabolic function of L-serine deamination is still unclear. To try to understand what this metabolic function might be, I created an *E. coli* strain deficient in all three dedicated L-SDs, SdaA, SdaB and TdcG and compared its metabolism with that of a strain carrying all three genes.

To do this, I constructed various L-SD deletion strains, carrying single, double or triple L-SD deletions, and compared their physiological characteristics. This led to the surprising finding that when incubated in complex media, the triple mutant, MEW999 behaved differently from the parental strain MEW1.

Strain MEW999 growing in either LB or in glucose minimal medium with casamino acids could not conduct normal DNA replication or segregation, nor could it divide normally, nor could it maintain a normal shape. Correlated with this, is a deficiency in the production of C1 units and SAM. I conclude that the *E. coli* strain deficient in L-SD activity cannot supply C1 units in sufficient quantity, and this deficiency leads to failure of the cell cycle with respect to both DNA replication and cell division.

3.1 Construction of the L-SD deletion mutants

Earlier studies have shown that any of the three genes coding for L-SD can be lost without interfering growth in glucose minimal medium or LB: *sdaA* (Su *et al.*, 1989); *sdaB* (Shao and Newman, 1993; Su and Newman, 1991); *tdcG* (Burman *et al.*, 2004). However, there was no study using a mutant strain deficient in all three L-SDs. I therefore constructed such an L-SD triple deletion mutant and then investigated the

impact of total loss of dedicated L-SD on *E. coli* cell metabolism. The parent strain used was *E. coli* K-12 strain MEW1, a derivative of strain CU4, one of the earliest K-12 strains used for metabolic studies and the reference strain of *E. coli* common used in the Newman laboratory.

3.1.1 Construction of L-SD deletion mutants.

As described in the materials and methods section, the strain construction was done by introducing single deletions of *tdcG*, *sdaB* and *sdaA* one by one into the Keio collection of deletion-carrying strains (Baba *et al.*, 2006). I constructed three single deletion mutants: MEW1*ΔtdcG* (MEW992), MEW1*ΔsdaB* (MEW991) and MEW1*ΔsdaA* (MEW990), three double deletion strains: MEW1*ΔtdcG ΔsdaB* (MEW995), MEW1*ΔtdcG ΔsdaA* (MEW994) and MEW1*ΔsdaB ΔsdaA* (MEW993) and one triple deletion mutant MEW1*ΔtdcG ΔsdaB ΔsdaA* (MEW999).

I confirmed by PCR that the deletions were correctly made, using the oligonucleotides listed in Table 3. The results of this PCR verification are shown in Figure 3. Using primers located outside the coding sequence, we found fragments of the expected size: 1838, 1840 and 1935 bp for amplicons of parental *sdaA*, *sdaB* and *tdcG*, respectively and much smaller sizes of 575, 574 and 673 bp for the amplicons of the corresponding deletion strains. This can be seen on the agarose gels, where the

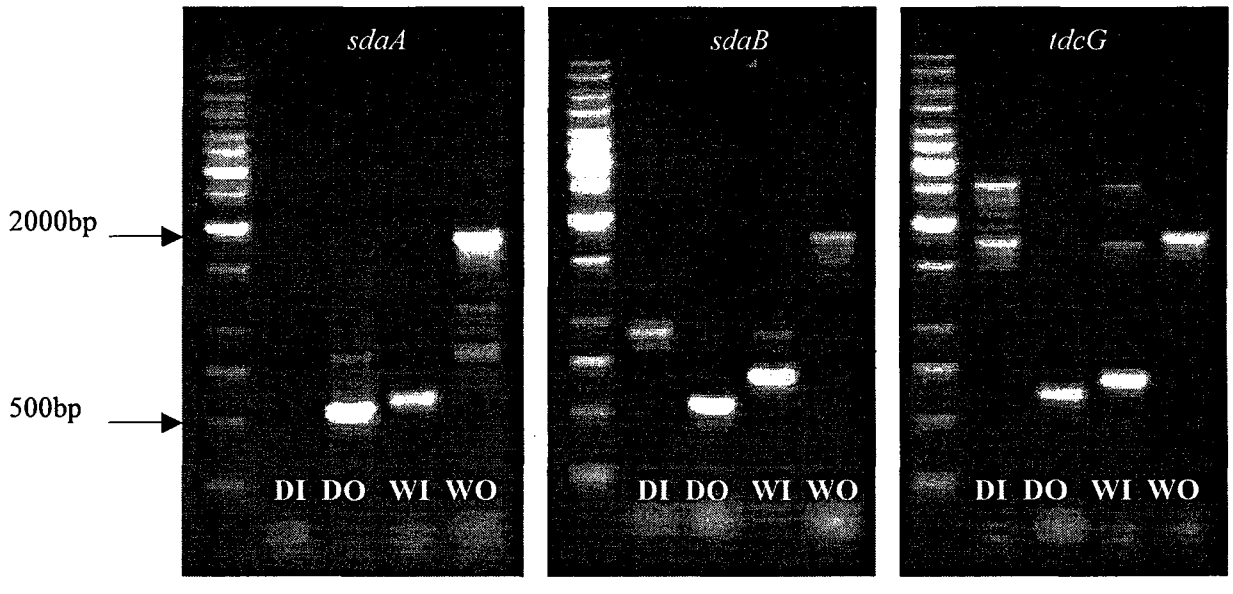


Figure 3 Verification of deletions in the triple mutant, strain MEW999.

Results of PCR amplification with gene-specific primers analyzed by agarose gel electrophoresis using primers inside (I) and outside (O) the gene indicated for the deletion strains (D) and the parent strain (W). Markers are displayed in the left hand lane for each gel. For sdaA,sdaB and tdcG respectively, we expected bands of 606,702,733 for WI, 1838,1840,1935 for WO, 575,574 and 673 for DO, and no corresponding band for DI. Primer sequences are listed in Methods.

parental strain amplicons with outside primers all show a size around 2000 bp (Figure 3), lanes marked WO) and the corresponding DNA fragments from the triple mutant migrated with a size corresponding to the marker of 500 to 600 bp. Furthermore, the primers inside the coding region were expected to generate 606, 702 and 733 bp amplicons for *sdaA*, *sdaB* and *tdcG* genes, respectively, and did in fact migrate in the range of 500 to 800 bp (WI lanes for *sdaA*, *sdaB* and *tdcG*). Since this is precisely the region which is deleted no DNA bands were expected from the amplicons generated from the same pairs of the primers (DI lanes in *sdaA*, *sdaB* and *tdcG*) and no corresponding band were seen. This data confirmed that the triple mutant, MEW999 does not have any coding sequence corresponding to *sdaA*, *sdaB* or *tdcG*. Similar experiments were done for the single and double mutant strains. It was confirmed that these mutant strains carried the expected deletions (data are not shown).

3.1.2 L-SD activity assay of the deletion mutants

It is known that L-SD coded by the *sdaA* gene can be expressed when cells are grown in both rich medium and minimal medium with and without the inducers, glycine and leucine (Isenberg and Newman, 1974). L-SD from the *sdaB* gene was only expressed in rich medium, such as LB, in the absence of glucose. The *tdcG* gene can only be expressed in anaerobic growth conditions, also in the absence of glucose. I therefore grew the various mutants in the appropriate media and assayed L-SD, to determine whether the enzyme levels followed the expected L-SD expression profiles.

L-SD was assayed in strains grown aerobically in glucose minimal medium with glycine and leucine the inducers of *sdaA* or in LB. As can be seen in Table 4 line 8, the triple mutant showed no detectable L-SD in either medium tested. I conclude therefore

Table 4 L-SD activity of the deletion mutants

Expt.	Strains	Remaining genes	Deleted genes	L-SD activity in*	
				LB	Glucose minimal medium with glycine and leucine
1	MEW1	*sdaA*,*sdaB* and *tdcG*	None	20	13
2	MEW992	*sdaB* and *sdaA*	*tdcG*	20	11
3	MEW991	*sdaA* and *tdcG*	*sdaB*	24	11
4	MEW990	*sdaB* and *tdcG*	*sdaA*	2	0
5	MEW995	*sdaA*	*tdcG* and *sdaB*	27	10
6	MEW994	*sdaB*	*tdcG* and *sdaA*	2	1
7	MEW993	*tdcG*	*sdaA* and *sdaB*	1	0
8	MEW999	none	*sdaA*, *sdaB* and *tdcG*	0	0

* L-SD activity was assayed in whole cells and is expressed as μmoles pyruvate synthesized by 0.1ml of a 0.6 OD 600nm suspension of cells in 30 minutes.

that the mutant has the intended phenotypic properties. No other enzyme in the cell deaminates L-serine in the conditions of this assay, though certain other enzymes can deaminate L-serine when overproduced (Brown *et al.*, 1990). These results also suggest that SdaA is the major enzyme expressed in either medium (expt. 4 vs expt. 1) since loss of SdaA decreased the L-SD level much more than loss of SdaB or TdcG (expts 2-4 vs 1). In strains grown in minimal medium, loss of SdaA left almost no activity- neither of the other enzymes were overexpressed to compensate. However, as expected, strains without *sdaA* did show activity in LB as *sdaB* was still present (line 4 vs 6), 2 units for both of them, compared with 20 from the parent strain MEW1.

The presence of a functional *tdcG* gene made very little difference to these results, no doubt because all assays were done in aerobic conditions. The strain which carried functional SdaB and TdcG (line 4) made 2 units in LB and only 0 unit in minimal medium. The corresponding values for the strain carrying only *sdaB* were only slightly different (2 and 1 (line 6)). Thus there is generally much more expression in aerobic medium from *sdaA* than from either of the other two genes, and *tdcG* is not expressed to any significant extent aerobically.

In short, these assays confirm that the triple mutant has no dedicated L-SD, and that the other strains derive most of their L-SD from *sdaA* and none from *tdcG*. Considering this together with the preceding amplicon analysis, I conclude that strain MEW999 with *sdaA*, *sdaB* and *tdcG* gene deletion has lost all L-SD.

3.2 Differences in the growth of the triple mutant and its parent

It is known that L-serine is toxic to *E. coli*. If L-SD decreases the L-serine concentration, it might protect against L-serine and thus allow the cell to grow at higher

concentrations of L-serine than a cell without L-SD activity. It is therefore possible that the strain MEW999 with three L-SD gene deletions would show different growth patterns and L-serine sensitivity than its parent in various media.

3.2.1 The triple mutant and the parent strain grew at the same rate in glucose minimal medium

When strains MEW999 and MEW1 were grown in glucose minimal medium at 37 °C, they showed similar growth rates with an apparent doubling time of 67 minutes. This data suggests that a loss of L-SD activity has no impact on *E. coli* growing in glucose minimal medium.

3.2.2 Nitrogen source and carbon source testing

The lack of L-SD in *E. coli* might affect growth on various carbon and nitrogen sources. To study that, I tested the ability of the triple mutant and its parent to grow on minimal agar plates provided with different carbon and nitrogen sources. The tests were done with either 0.2% $(NH_4)_2SO_4$ supplemented with various carbon sources or with 0.2% glucose supplemented with the various nitrogen sources. No difference was seen between the strains grown on any carbon source tested. The carbon sources tested included: Succinic acid, D-galactose, D-fructose, L-arabinose, β-D-lactose, α-L-fucose, D-gluconic acid, D-xylose, D-mannose, L-glutamic acid, α-D-melibiose, maltose, L-alanine, glycerol and D-glucose. However, while the parent strain could grow on SGL plates (i.e. with a mixture of L-serine, glycine and leucine as carbon source), the triple mutant could not.

The triple mutant was also unable to use L-serine as a nitrogen source which the parent strain could. Otherwise the strains used the same amino acids as nitrogen source.

3.2.3 Cell division problems of the *ΔsdaAΔsdaBΔtdcG* triple mutant growing in LB

The preceding studies indicate that there is not much difference in the metabolism of the triple mutant except in so far as L-serine deamination is directly concerned. However, when I tested the growth of the triple mutant in LB, I found that loss of the three L-SDs resulted in a remarkable growth difference from the parent strain.

It is understandable that triple deletion mutant MEW999 does not have any significant impact on cell growth in the minimal medium with various carbon and nitrogen sources because no massive concentration of L-serine is expected in any of those media, and therefore there should be no great need for L-serine deamination. However, in LB, the most commonly used medium, which supports the growth of our reference strain very well, the growth of the triple mutant was markedly delayed. An overnight LB glucose culture of the parent strain diluted 10000 fold into fresh medium reached a visible density 3.5 hours later and then grew steadily with an apparent mass doubling time of about 25 min in contrast to the triple mutant handled in the same way which was barely visible after four hours and grew thereafter with an apparent mass doubling time of 84 min. This was also true of the strain grown in LB without glucose. After five hours subculture in LB glucose, the parent strain was almost fully grown and in the same conditions, the triple mutant culture was only visibly turbid.

Cells were observed through the microscope and what surprised me was that MEW999 could not divide normally in LB. After the subculture, MEW999 formed long

filaments during the first few hours. Thereafter, the filaments became shorter and shorter, and finally all of the filaments subdivided into single cells. As shown in Figure 4, at two hours after subculture, the cells look short and small, though, already a little bit longer than the parent strain. In the following three hours, the length of the cells continued to increase and so did the turbidity of the culture, and at five hours many long filaments were formed. More and more filaments formed from five to six hours. However, the length of the filaments did not increase very much during this period. From six to 10 hours, the filaments begin to subdivide into short filaments or single cells and finally in the overnight culture (24 hours), only single cells were seen. The length of the filaments was measured using software ImageJ. At five hours, 87% of the triple mutant cells were longer than 10 μm , and the longest filament was about 101 μm (Table 5), over 25x the length of the parent strain. As the turbidity increased further, the number and proportion of cells of normal size in the triple mutant culture also increased, and the length of the remaining filaments shortened. By 11 hours, 69% of the cells were less than 10 μm long and by stationary phase, cells of normal size cells made up the vast majority of the units in the culture.

To be sure that the accumulation of cells of normal size was not due to selection of a non-filamenting subpopulation or alleviated mutants, I streaked out a culture that had gone through the filamentation and retested 10 randomly chosen colonies. All of these gave cultures which filamented and returned to normal size just as the original triple mutant. I conclude that a deficiency in L-SD interferes with replication and/or cell division in a rich medium, so that the cells form filaments starting shortly after subculture into this medium, and revert to normal size only later on in their growth cycle.

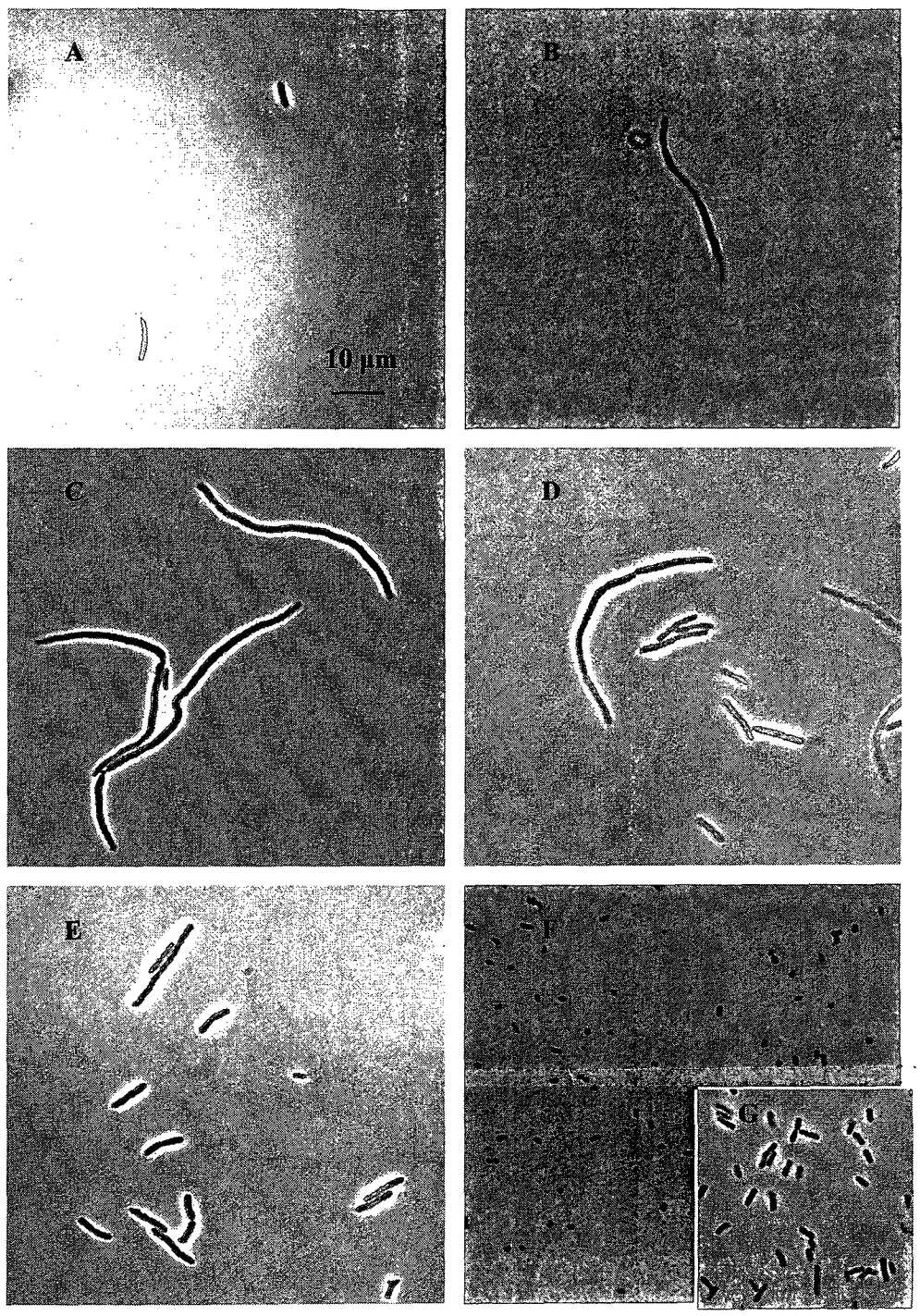


Figure 4 MEW999 grown in LB.

Strain MEW999 was grown in LB overnight and then diluted 10^4 fold into the same medium and examined at 2 hours (A), 3 hours (B), 5 hours (C), 6 hours (D), 10 hours (E) later and after over night (F). The parent strain treated in the same way was photographed at 5 hours (G) but was examined at various times with identical results.

Table 5 Length of triple mutant filaments in LB glucose

Strain	MEW1	Triple Mutant			
Time of measurement(hours)	5	5	8	10	11
# of cells measured	10	104	156	128	111
Mean length(μm)	4.7	39.9	28.7	23.9	13.6
Min length(μm)	1	2.8	3.6	3.1	2.7
Max length(μm)	5.9	101.5	78	88.8	85
%cells > 50μm	N/A	39	21	14	3
%cells 10-50μm	N/A	48	61	45	28
%cells < 10μm	100	13	18	41	69

Moreover addition of L-serine to LB glucose allowed the filaments to persist for longer than in LB alone. As shown in Table 6, when 600 μg/mL L-serine was added to LB glucose, by 11 hours, 11% of cells were filaments longer than 50 μm compared to 4% in the parent strain.

2.3 Gradual 'detoxification' of LB

During the period in which the triple mutant filaments, it accumulates considerable biomass and depletes some of the components of LB. If this depletion allows the cells to divide, one would expect recovery to be more rapid when there are more cells inoculated. I, therefore, compared cultures resulting from different degrees of dilution on subculture into LB (Figure 5). Filaments formed as described when cultures were diluted 10000 times. When the cultures were diluted less, (i.e. 10, 100 and 1000-fold), turbidity was obvious immediately, the lower the dilution, the fewer the filaments and the shorter the time taken to reach the final growth rate. That is, dilution into LB has a greater effect on dilute cultures, perhaps because they cannot detoxify something as rapidly, or because more heavy subcultures carry over some essential ingredient.

Table 6 MEW999 grown in LB with glucose in presence of 600 μg /mL L-serine

	5 hours	8 hours	10 hours	11 hours
Total cell number measured	101	147	123	112
Mean length(μm)	33.6	33.3	31.6	21.3
Min length(μm)	7.8	2.6	1.8	2.4
Max length(μm)	72.7	93.3	82.2	82.3
Cells longer than 50μm	17%	23%	27%	13%
Cells between 10 and 50μm	78%	69%	47%	35%
Cells shorter than 10μm	5%	8%	26%	53%

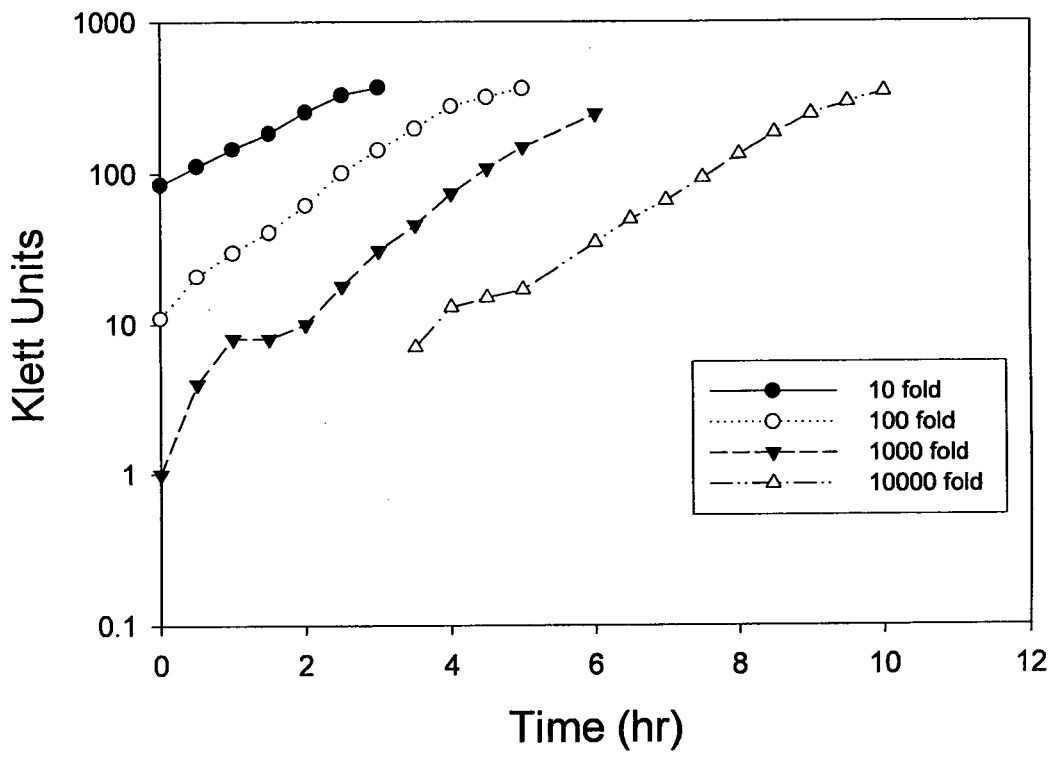


Figure 5 MEW999 growth in LB at different dilutions

MEW999 cells were diluted to the different extents in LB and the turbidity measured thereafter with a Klett-summerson photoelectric colorimeter with a 540 filter.

3.2.4 The triple mutant makes deformed cells on exposure to casamino acids.

The preceding experiments showed that the triple mutant forms filaments on subculture into rich medium LB but does not have growth problems in glucose minimal medium. To determine what LB components might be important, I tested the growth of the triple mutant in minimal medium with CAA, a commercial hydrolyzate of casein.

I subcultured cells from an overnight culture in glucose minimal medium with a 10^5 fold dilution into glucose minimal medium with 0.5% CAA. The parent strain increased in turbidity faster than in minimal medium, but the triple mutant was markedly delayed and produced a remarkable array of misshapen cells by 13.5 hr after subculture. These cells made up the majority of the population until after 17.5 hours when most cells were still deformed, but many had lysed (Figure 6). By 19 hours, almost all cells appeared normal.

This extensive lysis might result in the selection of a more resistant subpopulation. To test this, I isolated 10 survivors and grew and subcultured each of them as above, and each produced the same deformed cells after 14 hours as the original mutant strain, and again each population recovered later.

To be sure that the growth defect was due to the loss of L-SD, I constructed a derivative of the triple mutant carrying *sdaA* on a plasmid. This strain, MEW999 p*sdaA,* was resistant to CAA. Since the parent strain grew normally in glucose minimal medium with or without CAA, I conclude that a deficiency in L-SD results in the transient formation of deformed cells in the presence of CAA 0.5%.

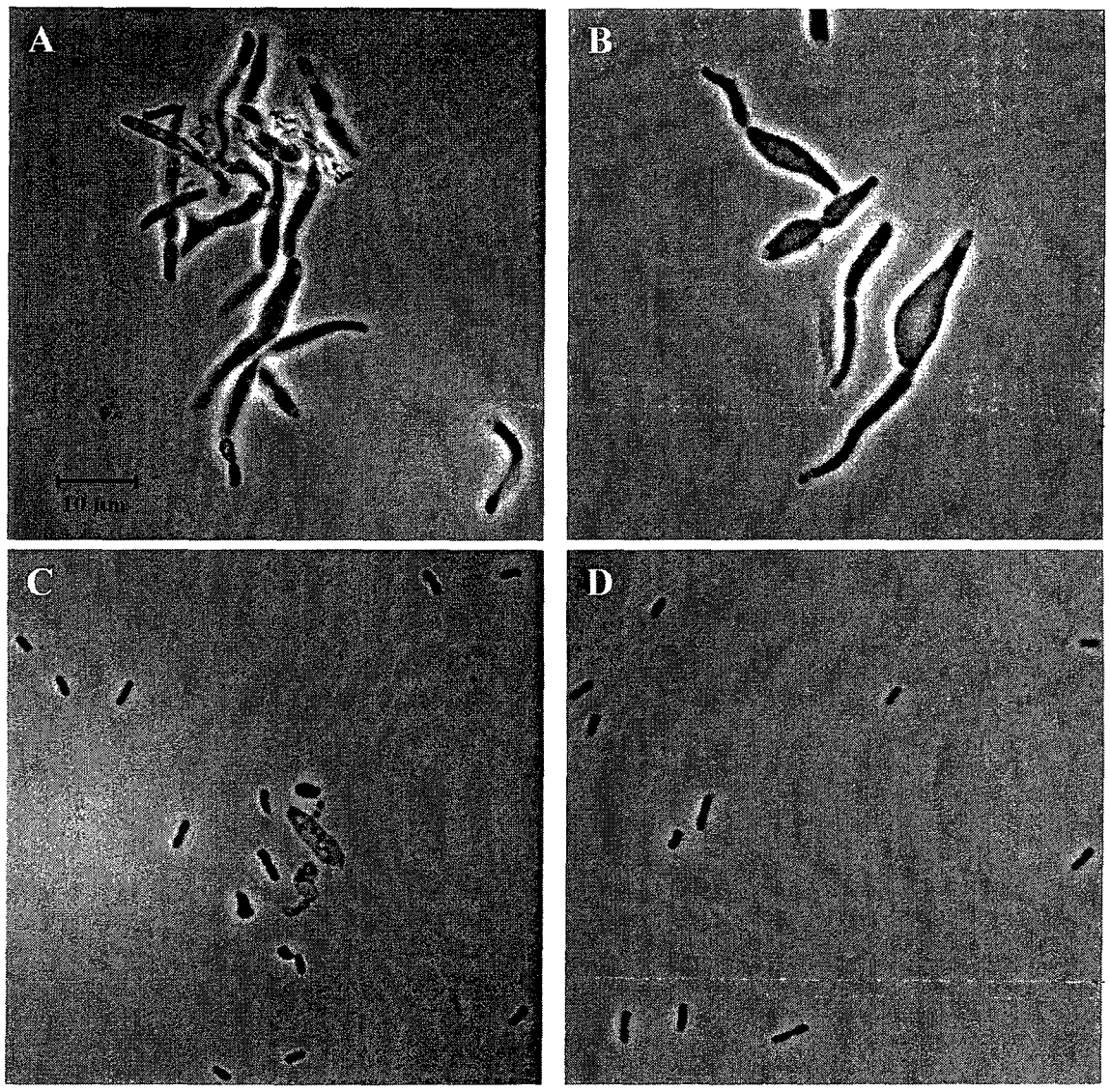


Figure 6 Effect of growth in liquid medium with casamino acids.

Strain MEW999 grown in glucose minimal medium was diluted 10^5 fold into the same medium with 0.5% CAA and examined at 13.5 (A) 15.5 (B) and 19 hours (C). The parent strain treated in the same way was photographed at 10.5 hours (D) but was examined at various times with identical results throughout its growth curve.

3.2.5 Cells increase in size at each cell division on solid medium with CAA

To follow changes within a microcolony derived from a single cell, I followed the growth of the triple mutant on slide cultures on glucose minimal medium with CAA. While growth in liquid media allows the study of large populations of cells, growth on solid media allows one to follow the fate of smaller numbers of individual cells over time. Moreover, solid media are likely to be osmotically protective, as in the case of *E. coli* L-forms which are generally grown in soft agar though they can also be formed in liquid medium (Joseleau-Petit *et al.*, 2007). Triple mutant cells clearly become osmotically sensitive during growth in liquid medium with CAA, since they can be seen to lyse, but this must be a borderline phenomenon since most cells survive. If it is borderline in liquid, a solid medium might show less lysis.

The triple mutant was grown on the slide with a layer of glucose minimal medium agar with 0.5% CAA. While there was less lysis on slide cultures, growth was very different from that seen with the parent strain. *E. coli* normally divides when it doubles its size. However, after a short lag, the triple mutant cells divided regularly once an hour for the next four to six hours, i.e. more slowly than the parent strain, but became larger at each division (Figure 7). That is, whereas the parent cell divided regularly when it reached about twice its original length, the mutant divided only when it was appreciably longer, thus increasing in both length and width, at every division. By five hours, the cells were much bigger and irregularly shaped.

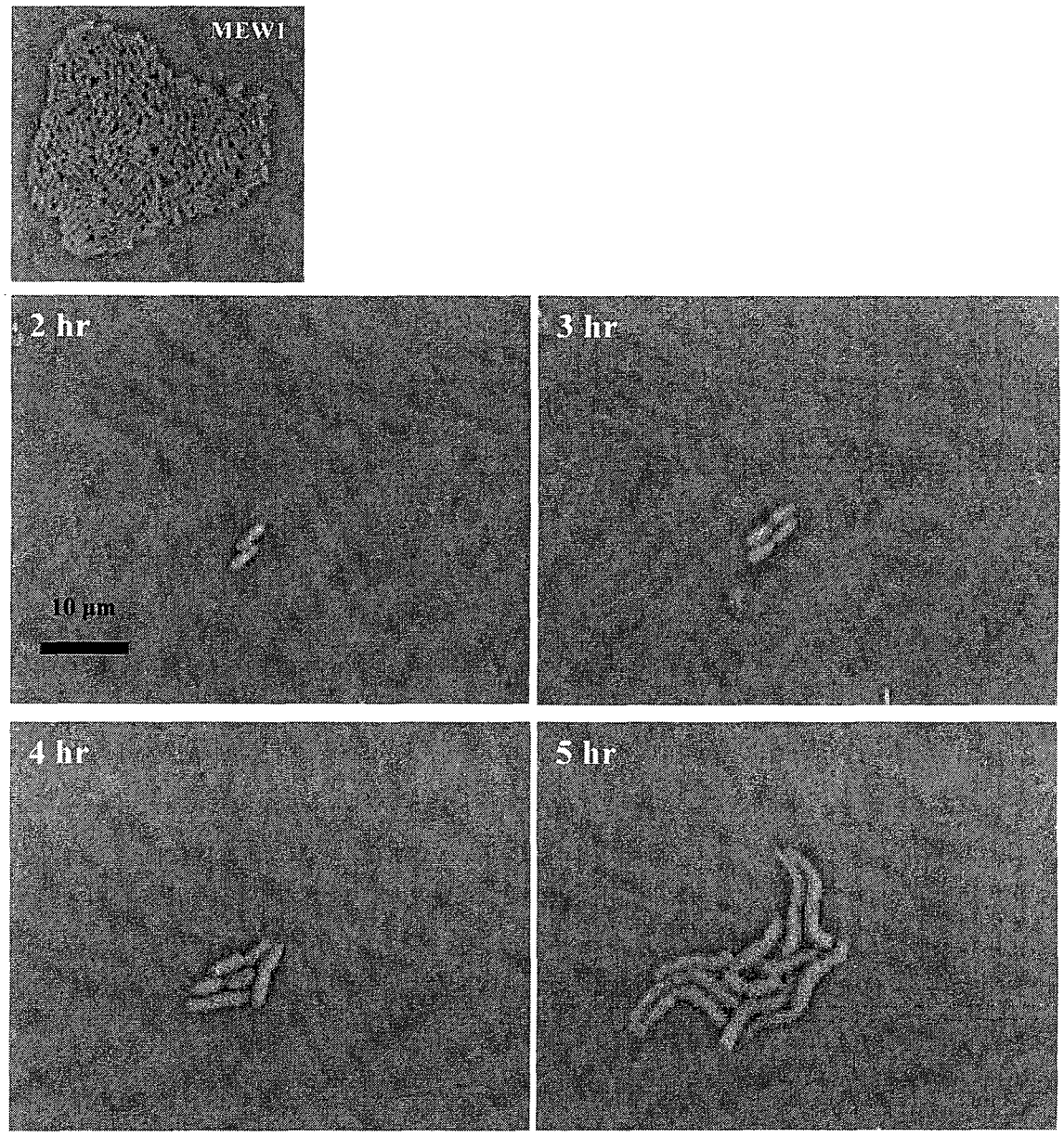


Figure 7 Early divisions in a triple mutant microcolony.

A culture of the triple mutant grown in glucose minimal medium was plated on a slide culture of the same medium with 0.5% CAA. A pair of cells was selected after 2 hours, and the same cells were photographed 3, 4, and 5 hours after plating. A microcolony of the parent strain MEW1 was photographed at 5 hours on a slide culture.

After the phase of elongation shown in Figure 7, much smaller cells accumulated rapidly, with occasional lysis of large ones, (Figure 8). A typical microcolony photographed approximately every 20 minutes between five and eight hours (i.e. 20 min at 37°C; two minutes handling time at room temperature) is shown. Five hours after subculture, all the cells were large and swollen with very irregular shapes. Between five and seven hours, some cells divided to make smaller, normal-looking cells. These markedly increased in number thereafter.

The cells shown in the microcolony on solid medium are much more regular in appearance than those grown in liquid cultures. However, even microcolonies within a population on slide cultures were quite variable in appearance. Those in wetter areas spread out and took on the astonishing diversity of forms regularly seen in cells grown in liquid (Figure 9A). This suggests that the triple mutant is somewhat osmotically challenged and its shape and growth characteristics are more like the parent strain when it is aided by external support. On the other hand, microcolonies in dryer areas may also show extremely deformed cells, as in Figure 9B where a combination of lysis and subdivision produced patchy microcolonies, with many swollen cells, and patches of much smaller cells particularly at the periphery of the colony.

2.6 Osmotic sensitivity of the triple mutant

In a population of triple mutant cells growing with CAA, a considerable number of cells lyse, and more in liquid glucose minimal medium with CAA than on agar slides with the same medium. The cells therefore must be osmotically sensitive, but not extremely so since so many cells do not lyse.

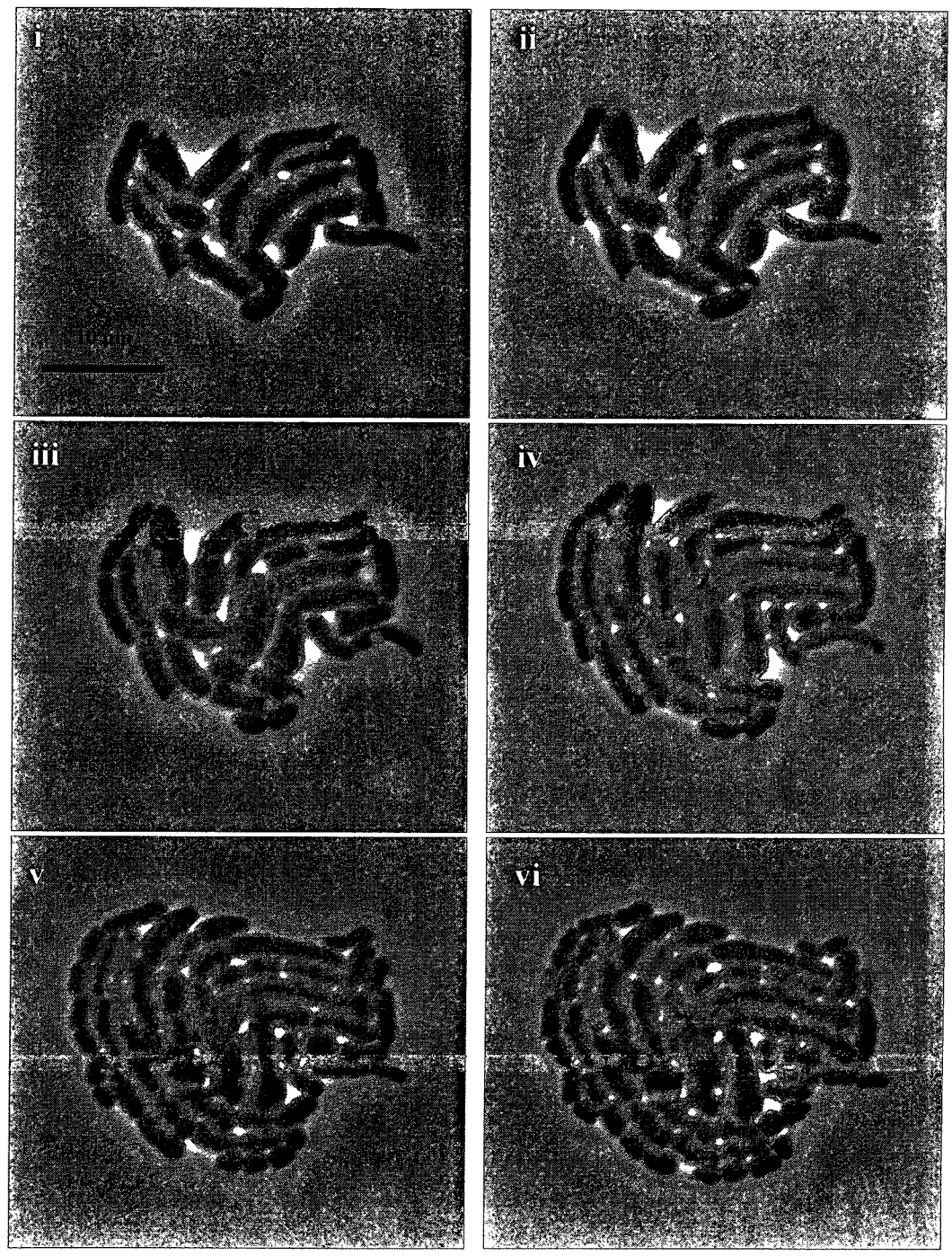


Figure 8 Later divisions in a triple mutant microcolony

As in Fig. 7, a different microcolony was photographed at 20 min intervals from 4 hours 50 minutes (i) to 6 hours 28 minutes (vi).

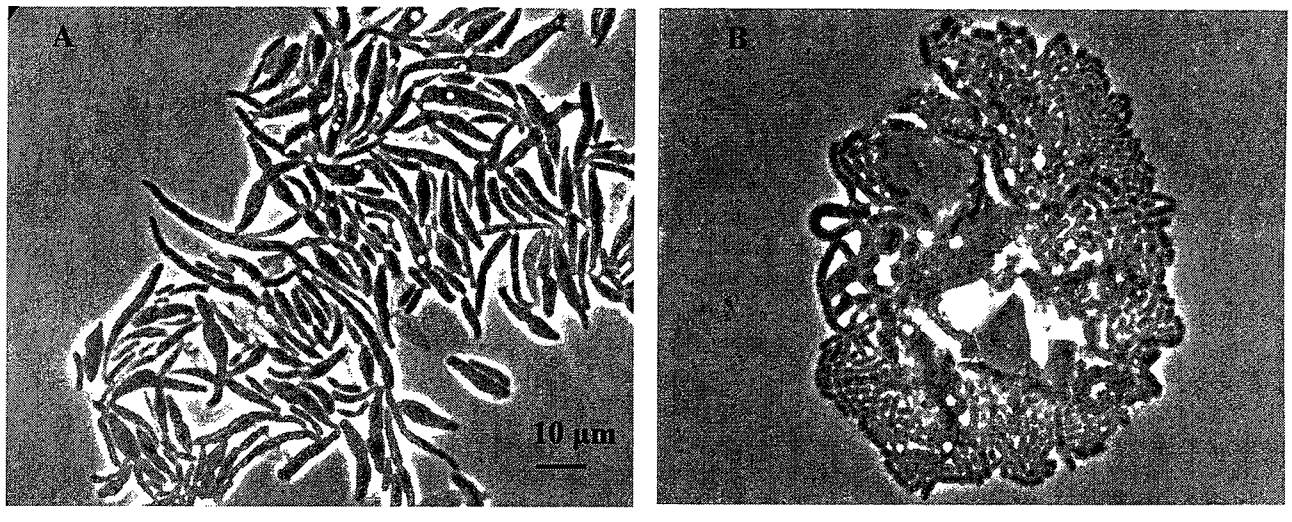


Figure 9 Extreme deformations of the triple mutant grown with casamino acids.

As in Fig. 7, appearance of (A) a microcolony in a wet area of a slide culture 5 hours after plating and (B) a different microcolony in a normal dry area 3 hours later.

This lysis might be due to a slight or intermittent deficiency in cell wall synthesis. In one of the most famous *E. coli* experiments, Lederberg demonstrated that penicillin caused a normal *E. coli* K-12 strain to become osmotically sensitive and to form protoplasts in liquid medium with concentrations of sucrose from 5 to 20% (Lederberg, 1956). I examined whether triple mutant cells grown with CAA might also form protoplasts in protective medium e.g. in the presence of 20% sucrose or on an agar slide. The presence of protoplasts would indicate that the cell membrane was strong enough to maintain the protoplasm but the cell wall of the misshapen cells was too weak to protect the cell from lysing without osmotic protection.

To test this, an overnight culture of MEW999 grown in glucose minimal medium was subcultured with a 10^4-fold dilution into glucose minimal medium with 0.5% CAA. After seven hours, sucrose was added directly into the culture to 20%. In one to two hours many cells formed spherical protoplasts as shown in Figure 10A. This seems to indicate that the cells formed at that time were sensitive to lysis but the membranes were intact and allowed protoplasts to form.

This was confirmed by plating the culture at seven hours on a layer of glucose minimal medium with CAA and 2% agar and incubating at 37 °C for one hour (Figure 10C). Many cells formed protoplasts on the slide culture. These results suggest that the misshapen triple mutant cells in glucose minimal medium with CAA had weak cell walls and tended to lyse in the liquid medium. The high osmotic pressure due to either a high concentration of sucrose or 2% agar may protect the exposed cell membrane and allow protoplasts to persist. This suggests that a deficiency in L-SD activity affects peptidoglycan synthesis or wall assembly in some way, leaving the

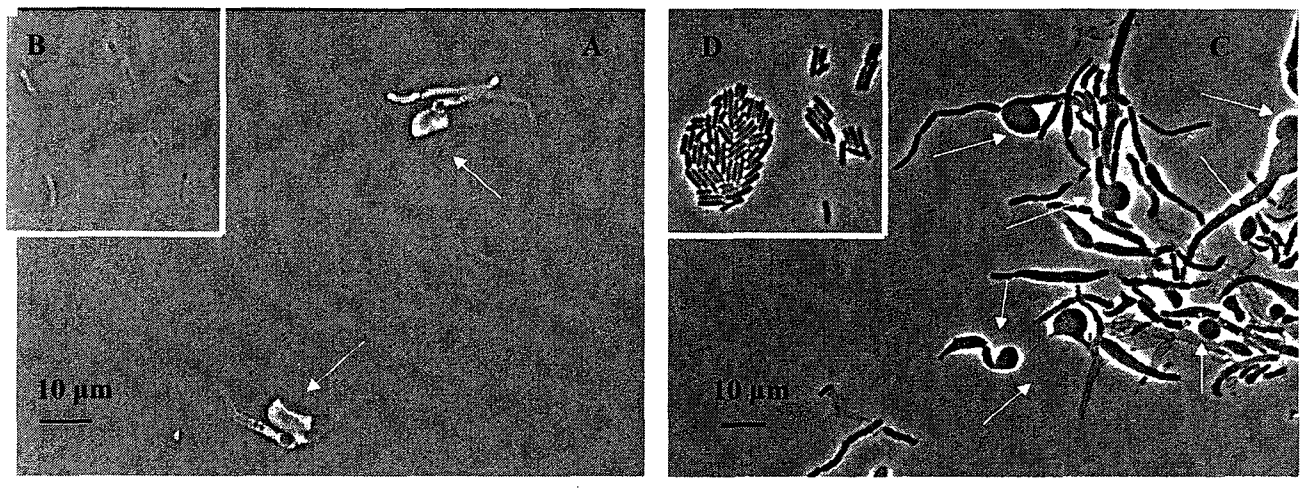


Figure 10 Triple mutant forming protoplast

The spherical protoplast was formed by growing the triple mutant cells in glucose minimal medium with CAA for 7 hours. A. Sucrose was then added to a final concentration of 20%, and cells placed on slides and photogrpaphed. B. The parent cells treated in the same way as in A. C. Cells of the 7 hours culture were plated on agar slides with glucose minimal medium and CAA and photographed an hour later. D. The parent cells treated in the same way as in C. White arrows indicate protoplasts forming.

cells at risk of lysis. However these problems are relatively mild or intermittent since much of the population does not lyse but recovers quickly.

3.3 The effect of particular amino acids on growth of the triple mutant

In the previous sections, I have described the effects of rich medium and minimal medium supplemented with CAA on the cell division of the triple mutant strain, MEW999. In this section, I present experiments designed to discover which amino acids have a strong impact on cell division.

3.3.1 Effect of L-serine on the growth of the triple mutant

It is clear that the triple mutant cannot divide properly in glucose minimal medium supplemented with 0.5% CAA. Though I used vitamin-free CAA, it is possible that contaminants of the CAA, such as vitamins or minerals could be involved. To confirm that the growth defects were due to amino acids, I made solutions of each of the 20 amino acids separately and then tested the growth of the triple mutant in the presence of a mixture of the 20 amino acids. The triple mutant grown in the glucose minimal medium and subcultured into glucose minimal medium supplemented with 20 amino acid mixture at concentrations of 100 μg /mL showed the same deformations as it did with 0.5% CAA (Figure 11A), whereas the parent strain grew normally (data not shown).

Since the major difference between the triple mutant and its parent is an alteration in L-serine metabolism, one might expect the formation of abnormal cells to be caused by L-serine. Support this hypothesis the triple mutant is more sensitive to L-serine than the parent strain on glucose-minimal medium with an L-serine gradient from 0-600 μg/mL (Figure 12). The parent strain grew well over the entire range while the triple mutant was

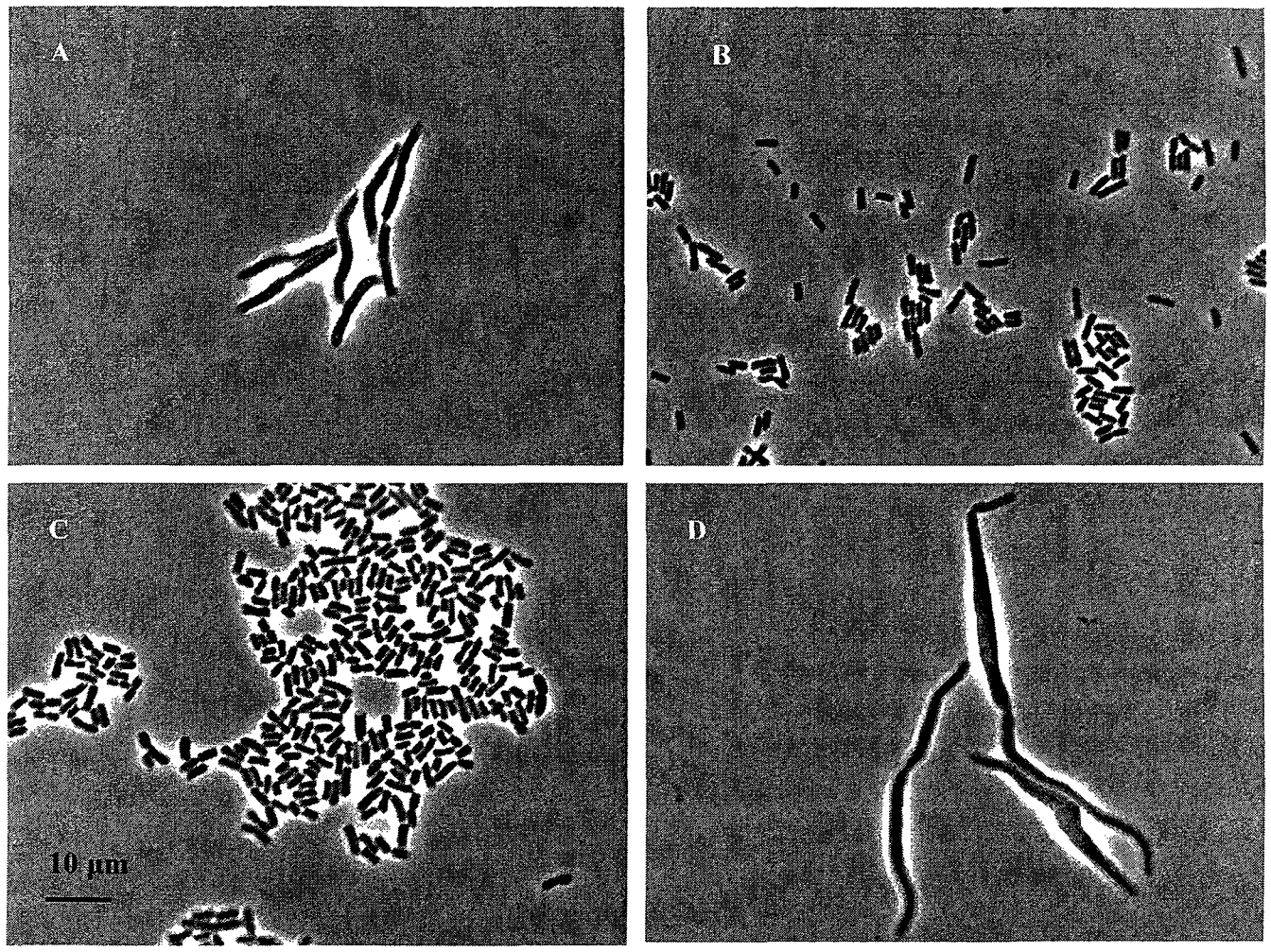


Figure 11 Effect of omission of amino acids from a 20-amino acid mix on growth of the triple mutant.

Cells were grown overnight in glucose minimal medium and subcultured into the same medium with 20 amino acids (A) or with mixes of 19 from which L-serine (B), glycine (C) or phenylalanine (C) were omitted. Cultures were photographed after 6 hours.

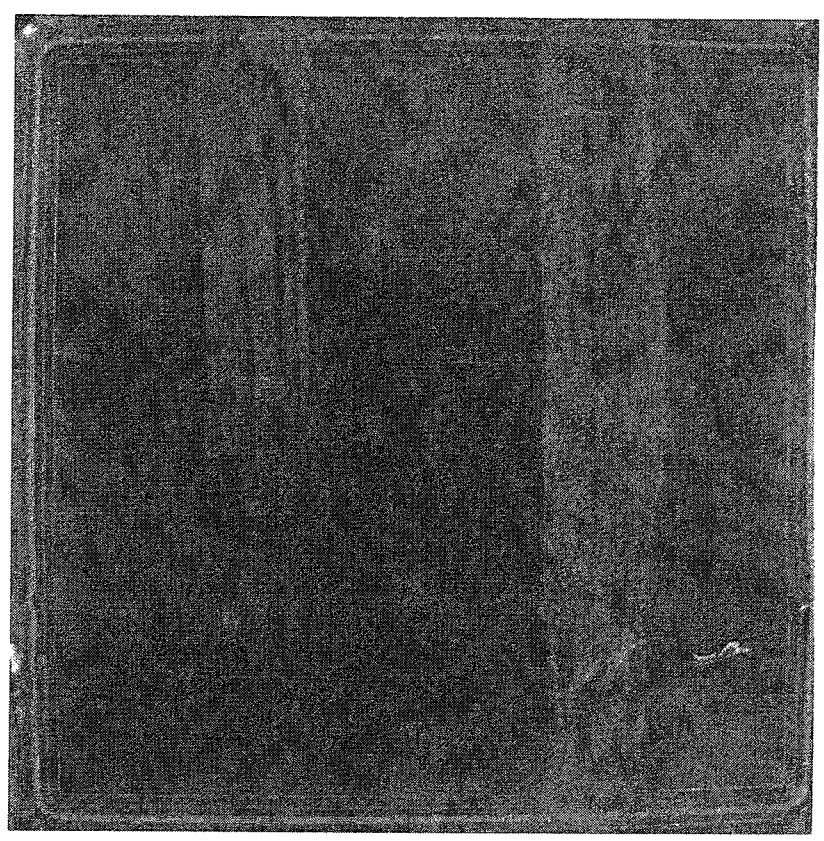


Figure 12 Effect of L-serine on growth of the triple mutant.

Strain MEW999 (left) and its parent MEW1 (right) were grown overnight in glucose minimal medium and then streaked on the same medium with a gradient from 0 (top) to 600 (bottom) μg/mL L-serine, and incubated for 48 hours.

unable to grow at much lower concentrations. This indicates that one of the physiological roles of L-SD is to defend against high L-serine concentrations by decreasing the intracellular L-serine pool.

However, when I subcultured cells from glucose minimal medium into glucose minimal medium with 100 μg/mL of L-serine, the triple mutant did not show the deformed cells seen in CAA.

To investigate which amino acid(s) in the 20 amino acid mixture cause the distortion of the triple mutant cells, I examined cells grown in glucose minimal medium with various 19 amino acids mixtures by removing each of the amino acids in turn from the 20 amino acids mixture. I found that L-serine must be included in the medium in order for abnormal cells to form, although it does not produce deformed cells on its own. Growth with 19 amino acids, but not L-serine, produced MEW999 cells of normal appearance (Figure 11B). Interestingly glycine was also important. The mixture of 19 amino acids including L-serine but not glycine, also did not produce deformed cells (Figure 11C). Without glycine, the triple mutant cells grew as well as the wild type cells even in the presence of L-serine. Removal of any of four other amino acids, threonine, methionine, lysine and leucine, also improved growth, though much less dramatically than the removal of glycine or L-serine. When any one of the remaining common amino acids (alanine, arginine, asparagine, aspartic acid, cystine, glutamine, glutamic acid, histidine, phenylalanine, proline, tyrosine and tryptophan) was removed, growth was not improved. (e.g. Figure 11D).

3.3.2 Effect of six amino acids on MEW999

I tested the triple mutant in glucose minimal medium with only L-serine and glycine added and with all six implicated amino acids. With only L-serine and glycine, the cells grew normally. When the cells were given all six amino acids, they formed long filaments, and occasional swollen cells (Figure 13). However, they did not form the grotesque cells seen in the presence of CAA.

3.4 Increased efficiency of C1 metabolism restores the growth of MEW999

As described in previous sections, I have demonstrated that L-serine and L-glycine are two critical amino acids that affect cell division in the triple mutant, and four others also affect division. These six amino acids are involved in C1 metabolism directly as L-serine, glycine and methionine, or in the synthesis of methionine and/or its regulation. This suggests that the effect on cell division maybe due to an effect on C1 metabolism, the more so since C1 deficiency has already been shown to cause filamentation in the *metK84* mutant (Newman *et al.*, 1998).

3.4.1 SAM aids growth in the presence of CAA

Wei and Newman previously reported that *E. coli* requires SAM, and by extension, a methylation reaction, to complete its intercellular septum (Wei and Newman, 2002). The availability of SAM (and other methyl donors) depends on the availability of L-serine, the ultimate donor of C1 units (Newman and Magasanik, 1963). If, as it seems, L-SD regulates the concentration of L-serine in the cell, one might

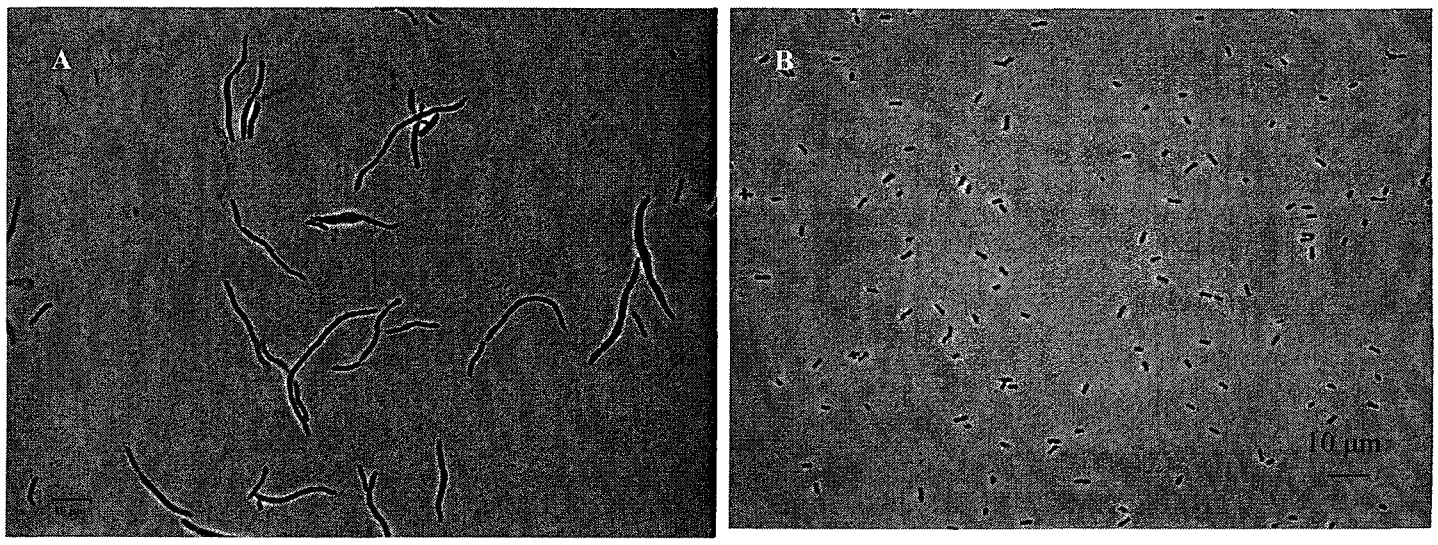


Figure 13 Growth of the triple mutant with six amino acids.

A). The triple mutant was subcultured from glucose minimal medium into the same medium with six amino acids: L-serine, glycine, leucine, methionine, lysine and threonine at 100 µg/mL and photographed after 12.5 hours. B) The parent strain treated in the same way was photographed at 10.5 hours.

expect that the triple mutant functions with a very different internal L-serine concentration than its parent, which might in turn change the availability of methyl donors.

I, therefore, made a derivative of the triple mutant which could transport SAM, strain MEW999 psamT by transforming a plasmid carrying the Rickettsial SAM transporter, kindly provided by Dr. D. Wood (Tucker *et al.*, 2003), into the triple mutant.

SAM profoundly altered the response of the triple mutant to CAA, largely restoring normal function. This suggests that availability of SAM in the triple mutant may be decreased. Addition of 2 mM SAM altered the morphology of the developing cells from swollen misshapen cells to filaments of even width much like those made transiently in LB (Figure 14). These filaments have not completely regained normal function, since they have a high probability of lysing whereas LB filaments do not lyse. Nonetheless, exogenous SAM restores many of the defects of cells grown in CAA, allowing them to elongate into filaments and increase in biomass, but it does not restore their ability to divide.

3.4.2 Division problems in LB are reversed by supplying SAM

The triple mutant does not divide normally in LB or in minimal medium with CAA, but its growth is much less severely affected in LB where it forms long filaments and usually does not lyse. In LB, normal growth was entirely restored by the addition of SAM. While the SAM-transporting triple mutant, strain MEW999 psamT, made filaments in liquid LB glucose when grown without SAM, it grew faster when SAM was provided and formed cells of normal size (Figure 15).

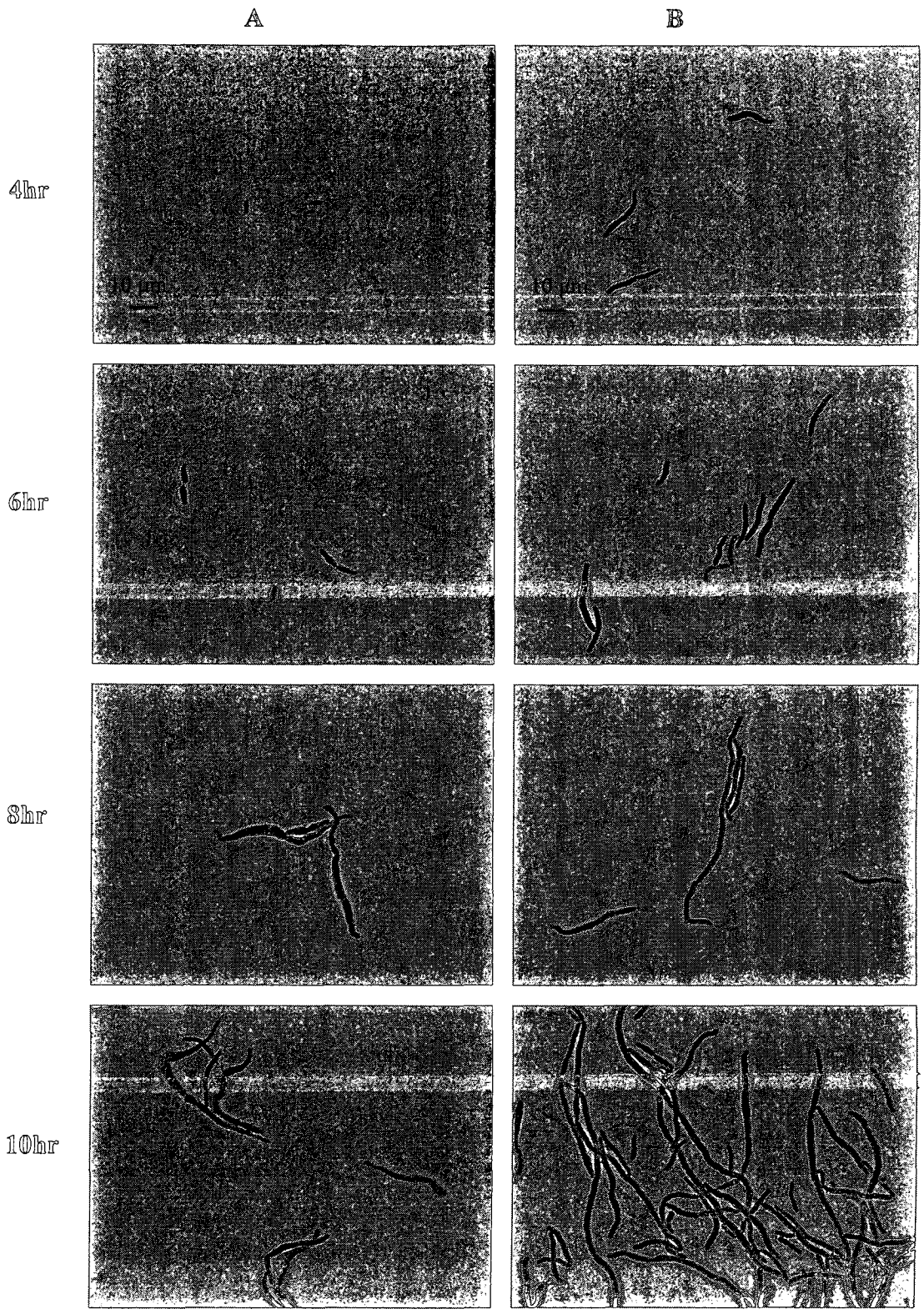


Figure 14 The effect of SAM on the growth of MEW999 psamT with casamino acids

Ttriple mutant cells carrying the SAM transporter were grown in glucose minimal medium with chloramphenicol 25 μg/mL and subcultured into the same medium with CAA (A), or with both CAA and SAM (2 mM) (B). Images were taken at 4, 6, 8 and 10 hours after subculture.

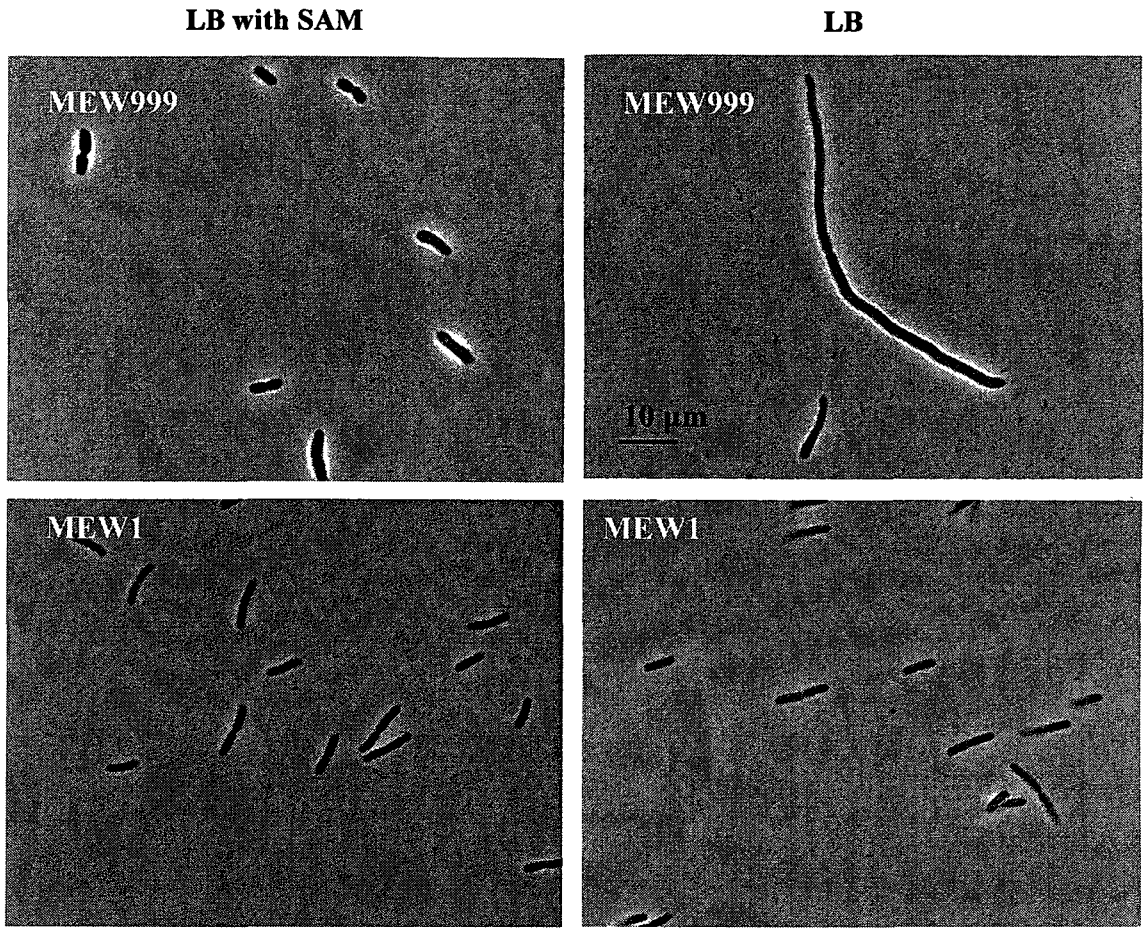


Figure 15 Effect of SAM on growth of the triple mutant in LB.

The triple mutant MEW999 with a plasmid-encoded SAM transporter was grown in LB glucose with chloramphenicol 25 μg/mL and subcultured in the same medium with and without SAM 2 mM then photographed at 5 hours. No filaments were seen at any time in the presence of SAM. The parent strain MEW1 was treated with the same way and photographed at 5 hours.

On slide cultures with LB glucose, the triple mutant does not filament at all and so an effect of SAM could not be demonstrated. It is clear that the relatively mild effects on growth in LB can be totally reversed by supplying SAM, suggesting again that the problems of the triple mutant are related to one-carbon metabolism and methylation.

3.4.3 A *gcv* system expression plasmid can restore the growth of MEW999

If C1 metabolism imbalance was the real reason for the growth difficulty of the triple mutant, one would expect that an increase in the availability of C1 units inside the cell would restore the growth capability of MEW999 in rich medium or minimal medium supplemented with amino acids. In vivo, C1 units can be generated from both L-serine and glycine via SHMT or GCV respectively. The availability of C1 units in the triple mutant cell maybe limited because of the L-SD gene deletion. One might not expect an inhibitory relation between L-SD deficiency and production of C1 units. If the problem was caused by C1 deficiency, over expression of SHMT or the GCV enzyme might be able to overcome this problem.

To investigate this, a plasmid pGS146, which carries an expressible *gcv* enzyme system kindly provided by Dr. Stauffer (Stauffer *et al.*, 1986), was transformed into the triple mutant MEW999. Both in LB medium and minimal medium supplemented with 0.5% CAA, normal growth of the triple mutant MEW999 was restored by the presence of plasmid pGS146 (Figure 16). This suggested that improving the supply of C1 units in the cell overcame the cell growth deficiency caused by deletion of the L-SD coding genes. This supported my previous hypothesis that the triple mutant had problems with one-carbon metabolism and methylation.

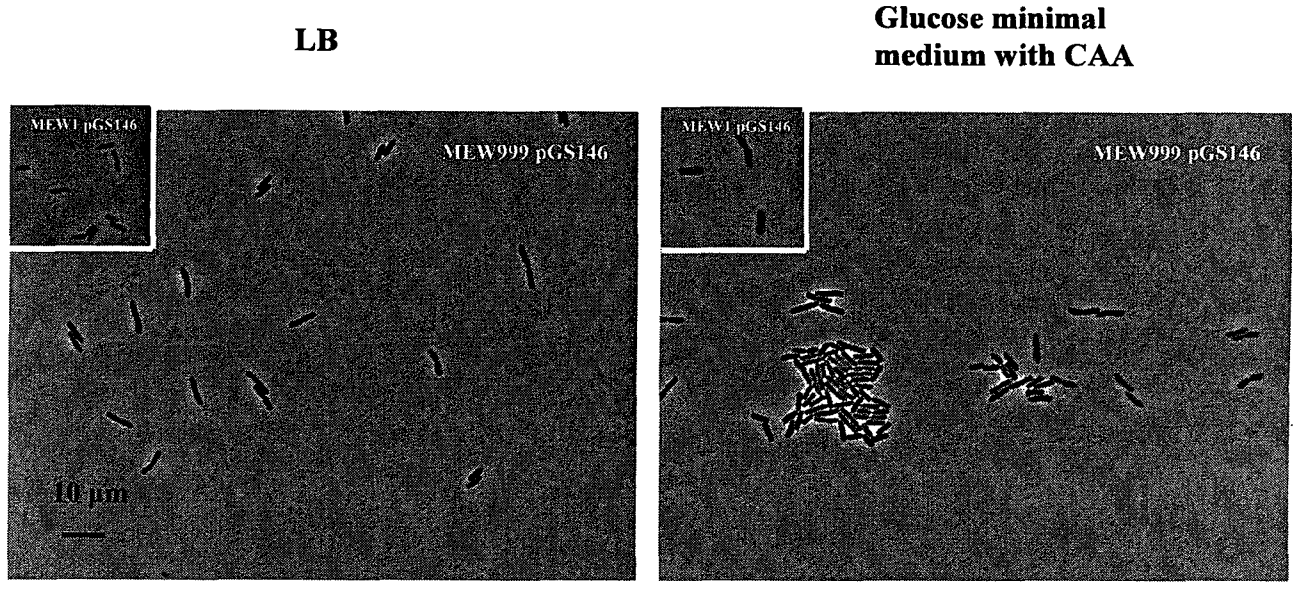


Figure 16 Effect of GCV expression plasmid pGS146 on growth of the triple mutant

The triple mutant MEW999 with a GCV expression plasmid pGS146 was grown in LB or in glucose minimal medium with chloramphenicol 25 µg/mL and subcultured for 5 hours in LB or in glucose minimal medium with CAA. No filaments were seen in LB nor misshapen cells in glucose minimal medium with CAA. The parent strain MEW1 with same plasmid treated in the same way was photographed at 5 hours.

3.4.4 Effect of the *gcv* expression inhibitor hypoxanthine on growth of the triple mutant

The GCV enzyme system is critical for cell metabolism to balance the cells' glycine and C1 requirements. It is regulated by sensing and responding to the levels of different metabolic intermediates and end products. One aspect of its regulation is that the expression of the GCV enzyme is under the negative regulation of the PurR protein (Stauffer and Stauffer, 1994) which can repress transcription by binding to a PurR-binding site between nucleotides -3 to +17 in the transcription initiation site in the *gcvT* promoter region (Wilson *et al.*, 1993). Therefore, adding hypoxanthine to the medium can repress the expression of the *gcv* operon.

I tested the effect of hypoxanthine on growth of the triple mutant. Hypoxanthine had a strong effect when L-serine was also present in the medium. On glucose-minimal medium plates with L-serine (300 μg/mL) and a hypoxanthine gradient from 0-40 μg/mL, the triple mutant was much more sensitive to hypoxanthine than the parent strain (Figure 17). The parent strain, MEW1, grew well over the entire range. The triple mutant could not grow at even a very low concentrations of hypoxanthine (with L-serine present). On plates with either L-serine or hypoxanthine alone, the triple mutant grew normally. The fact that increased expression of the GCV enzymes restored normal growth of the triple mutant MEW999, and hypoxanthine with L-serine prevented growth, strongly supports the hypothesis that the growth difficulties of the triple mutant are due to a decreased availability of C1 units.

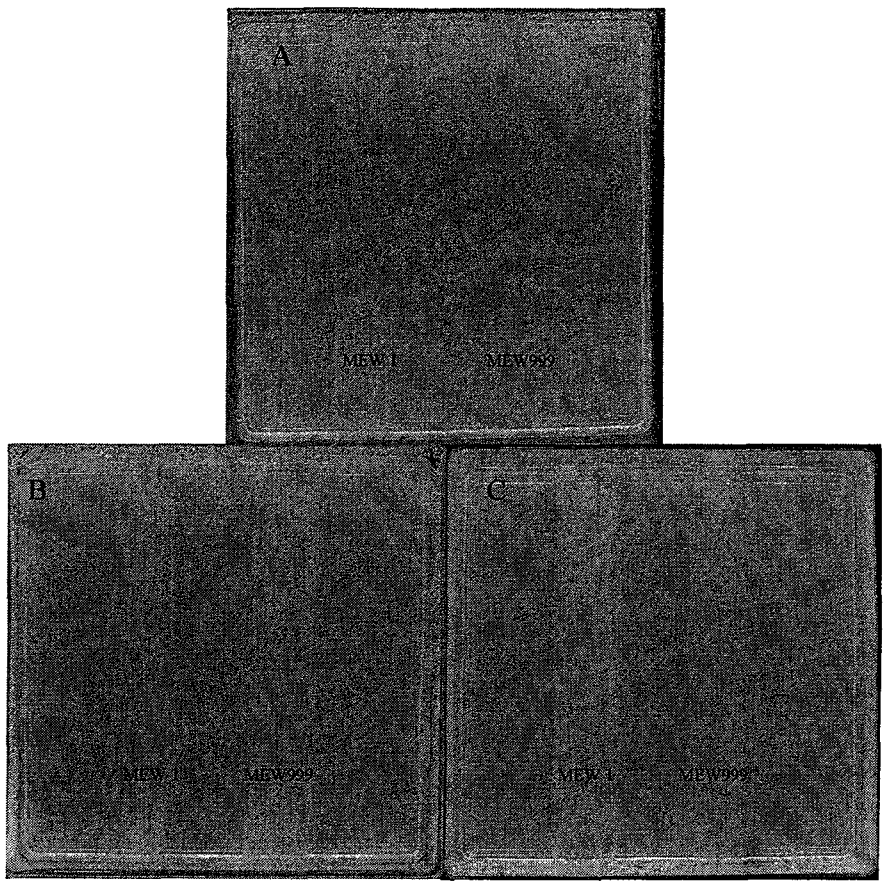


Figure 17 The growth of MEW999 on hypoxanthine gradient plates

Strain MEW999 and its parent strain MEW1 were grown in glucose minimal medium. A loopfull of a 1/100 dilution was streaked on to glucose minimal medium plates with a) L-serine 300 μg/mL throughout and a hypoxanthine gradient from 0-40 μg/mL, b) L-serine 300 μg/mL throughout, and c) a hypoxanthine gradient from 0-40 μg/mL.

3.5 DNA replication in the triple mutant

The fact that the triple mutant's growth problems can be ascribed to C1 deficiency is consistent with earlier work from this lab which showed that SAM synthetase-deficient *metK84* mutants formed long filaments during a gradual SAM starvation (Newman *et al.*, 1998). These cells could increase in mass and length for several hours, but could not divide normally, and therefore, formed long filaments (Newman *et al.*, 1998; Wei and Newman, 2002). Later work on this mutant demonstrated that chromosomal DNA from this strain could be replicated and segregated normally, but the division septum could not be assembled. FtsZ, FtsA and ZipA were recruited to the septum but the late division proteins, FtsQ, FtsW, FtsI and FtsN were not (Wang *et al.*, 2005).

DAPI is known to form fluorescent complexes with natural double-stranded DNA and was used in this study to examine the distribution of DNA inside the cells. I obtained similar images for strain *metK84* and its parent with the microscope and methods currently used in the lab. As shown in Figure 18, a 16-hour *metK84* filaments image gave the same results as seen earlier with both phase contrast and fluorescent microscopy (Wang *et al.*, 2005). Filaments of *metK84* did not show signs of septation (Figure 18, phase contrast image) but DNA can segregate at regular intervals. The parent strain, MEW1, grew normally, segregating its DNA, making septa and dividing. Since the methods in use were reliable, it was possible next to evaluate nucleoid segregation and septation in the triple mutant, MEW999.

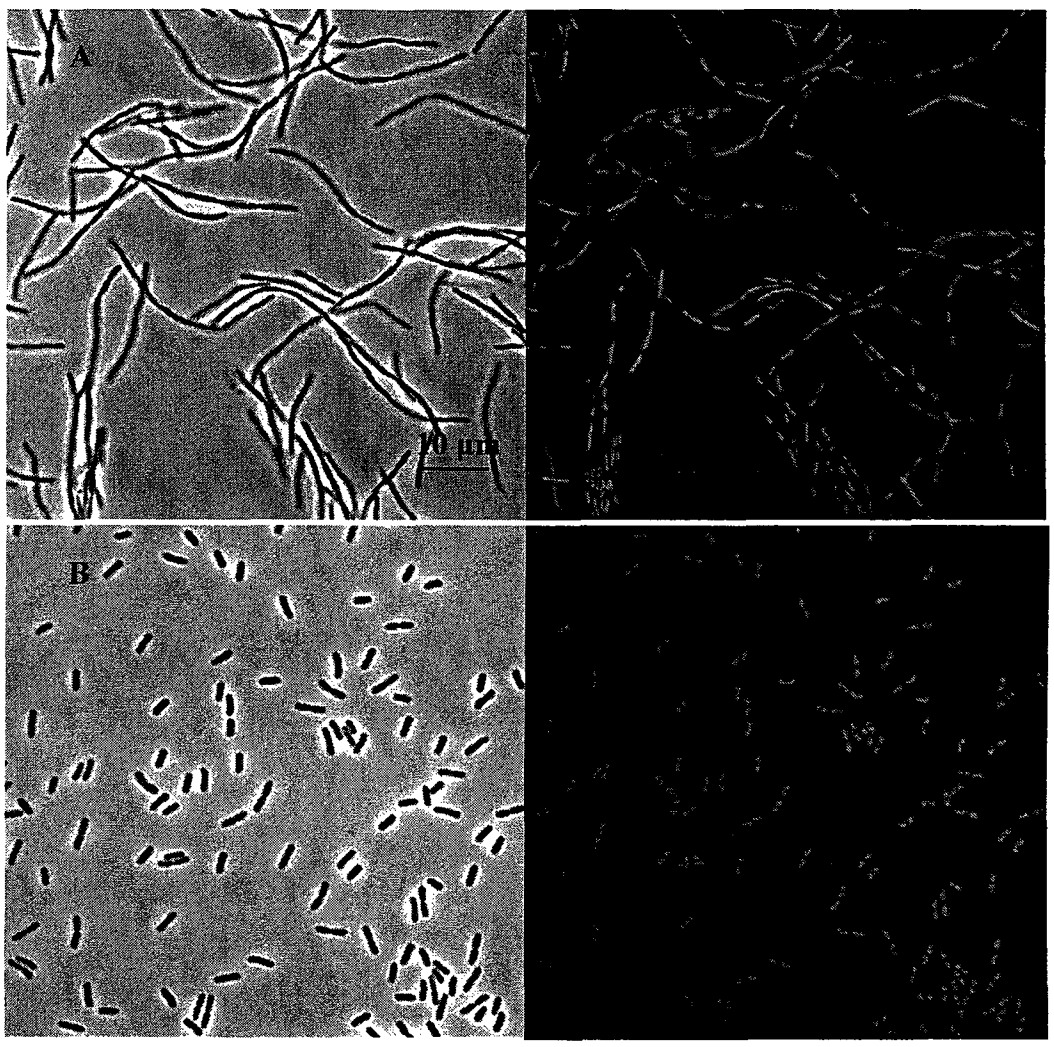


Figure 18 *metK*84 and the parent strain MEW1 can condense and segregate DNA

*metK*84 cells formed long filaments in glucose minimal medium with leucine 5 μg /mL. However, in most of the filaments the DNA can segregate normally (A). The parent strain MEW1 can divide normally in glucose minimal medium with CAA (B).

3.5.1 Triple mutant MEW999 DNA segregation in LB and minimal medium with CAA

I first used DAPI staining to investigate nucleoid segregation in the triple mutant filaments in LB. An overnight culture in LB with glucose was subcultured into fresh LB with glucose medium with a 10^4-fold dilution. In filaments collected at five hours, fixed, and stained with DAPI, the DNA was spread throughout most of the filaments (Figure 19). One of the few filaments showing segregated DNA is indicated by a white arrow, and even that filament contained unsegregated DNA for most of its length. The same experiment was also performed in LB without glucose and gave the same results. From this I concluded, that the triple mutant does not segregate its DNA at least during the early stage of filamentation in LB glucose.

Similar experiments were also done with cells grown in glucose minimal medium with CAA, recording images between four and 11 hours.

At four hours, most of the cells appeared larger than wild-type *E. coli* with little cell lysis (Figure 20A). Chromosomal DNA was not condensed but spread throughout the cell. At six hours, many of the cells had very swollen areas (bulges) (Figure 20B). The appearance of DNA varies in different cells. In some cells, chromosomal DNA was at least partially condensed. In cells with bulges, DNA seemed to accumulate at either end of the bulge with a gap between areas of DNA accumulation. This may indicate that septa were forming. The filament on either side of the bulge often had no DNA at the poles, suggesting that the expansion of the cytoplasm was pulling the cell membrane with its contents away from the wall. Segregation of DNA

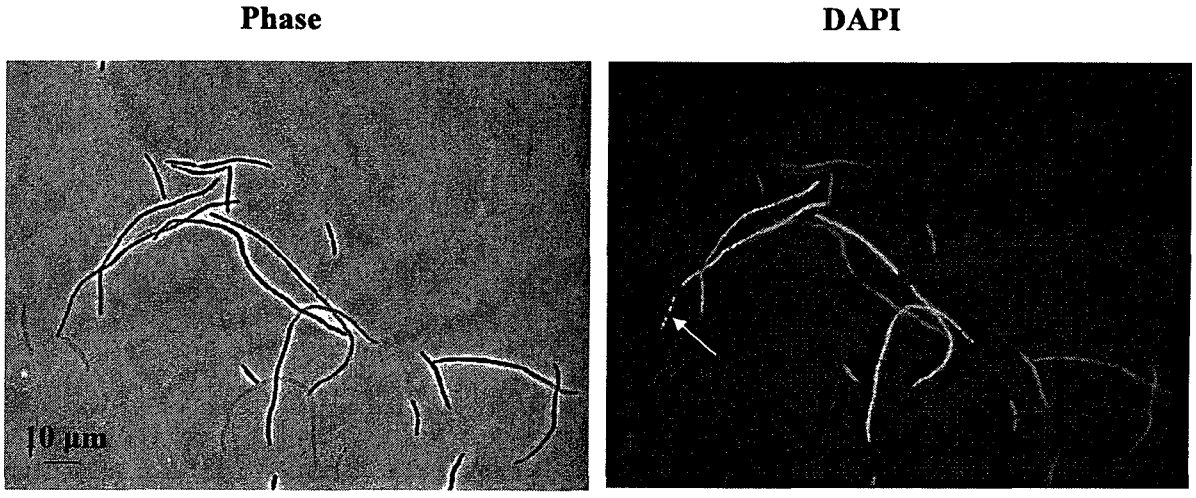


Figure 19 The triple mutant filaments in LB glucose show unsegregated DNA

Triple mutant cells grown in LB with glucose were subcultured in LB with glucose. The 5 hours filament sample was fixed and then strained with DAPI. As shown in the DAPI image, most of the filaments had unsegregated DNA. The white arrow showed the segregated DNA in part of a long filament.

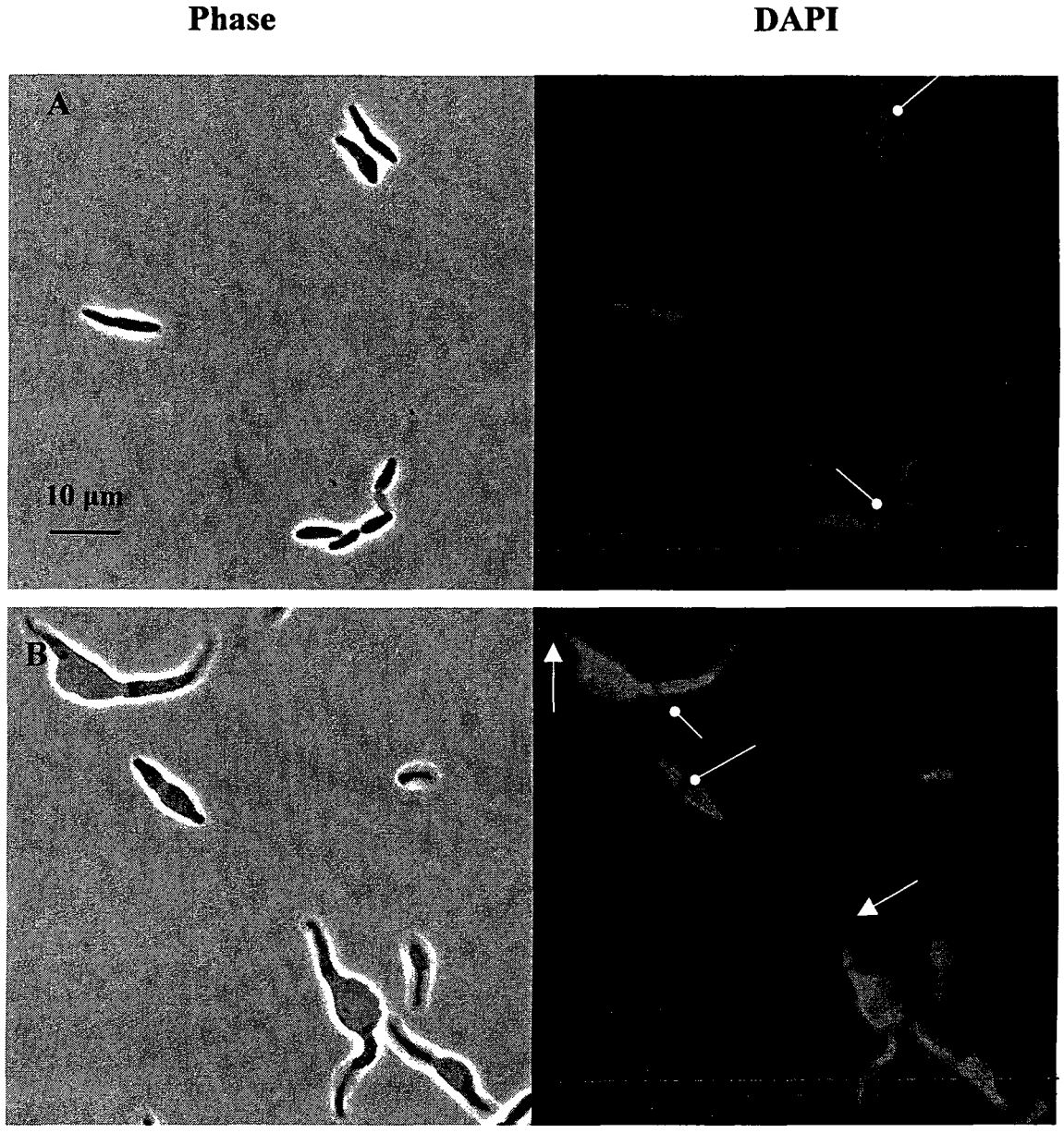


Figure 20 MEW999 grown in glucose minimal medium with casamino acids-the early stages

Cells of the triple mutant grown in glucose minimal medium were subcultured into the same medium with 0.5% CAA and after the subculture, (A) at 4 hours all the cells have uncondensed DNA filling the whole cell. (B) At 6 hours, some cells began to segregate the DNA. The white arrows in B DAPI point to the areas that lack nucleoids. The arrows with ball end indicate gaps between the uncondensed DNA.

was clearly achieved even when the DNA was not condensed (white ball-ended arrows, Figure 20).

By eight hours of incubation, many cells had lysed, but many others had segregated DNA (Figure 21A), although some cells still showed uncondensed DNA. In most of the cells, the DNA looked like small grains, spread unevenly inside the cell. Several cells seemed to be leaking DNA into the surrounding medium as judged by a cloud of DAPI-staining material surrounding them. This is probably an artifact of handling, but does indicate considerable osmotic sensitivity. These data suggest that although at eight hours most of cells could divide, DNA was still not properly condensed and segregated. However by 11 hours (Figure 21B), most of the cells had subdivided into single cells. There were still long filaments, but the DNA within them was obviously segregated so it seems likely that they would have subdivided with well-segregated DNA a short time later.

It is clear that DNA segregation is abnormal in the triple mutant while growing in LB or in minimal medium with CAA. It is not clear if the problem is due to problems in DNA replication or DNA condensation. Since in *E. coli* DNA hemimethylation is a critical step for the regulation of DNA replication initiation (Russell and Zinder, 1987), the phenotype might be that C1 deficiency inhibit DNA methylation in MEW999 and the absence of hemimethylated DNA results in filament formation.

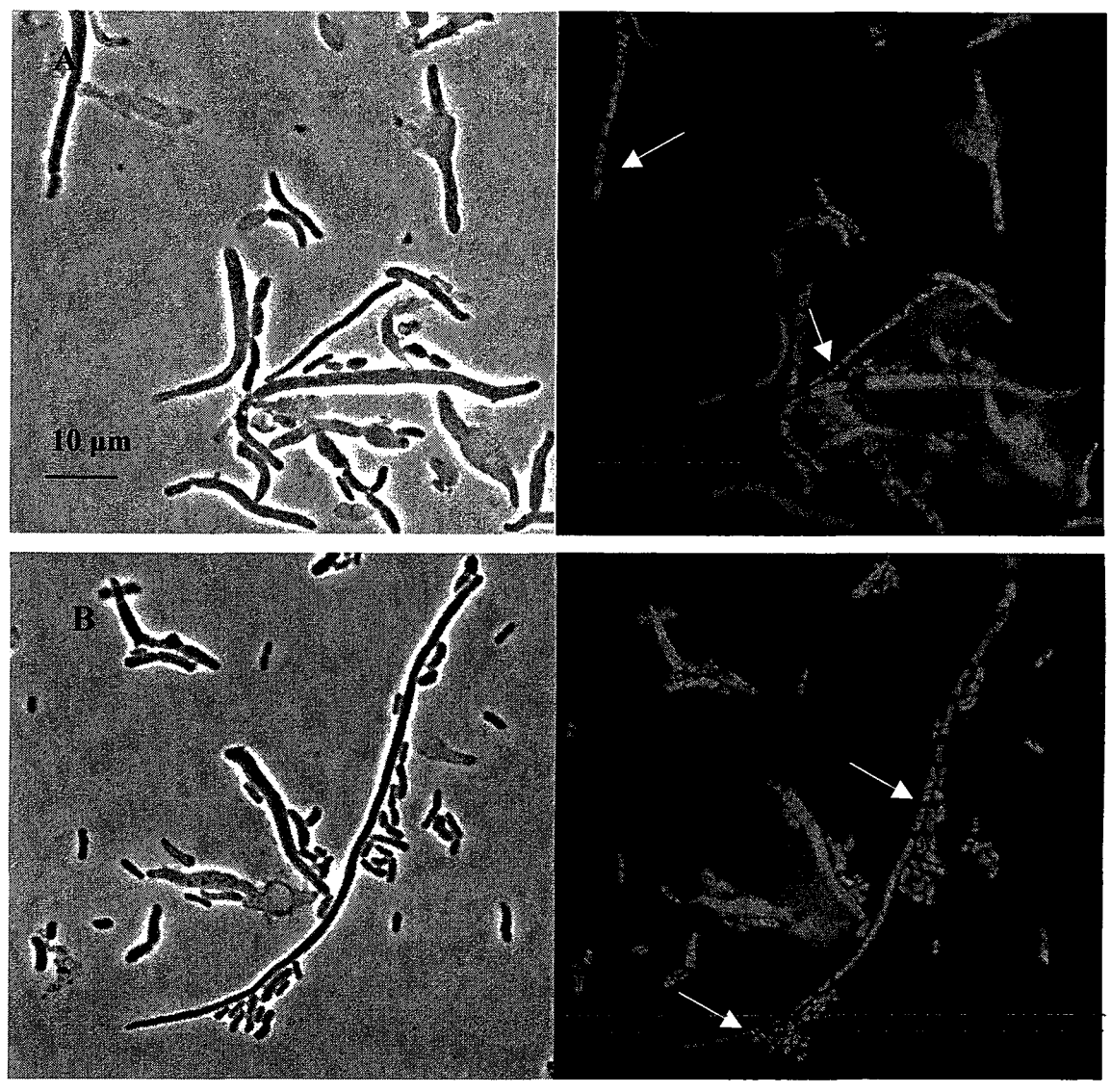


Figure 21 MEW999 grew in glucose minimal medium with casamino acids-the later stages

As in Fig 18, (A) after 8 hours subculture, many cells lysed, and the released DNA is seen as a diffuse fog around the swollen area. There are still many long cells left (B). At 11 hours, most of the cells are small single cells. A few long cells persist. The white arrows show the segregated DNA.

3.5.2 DNA methylation

To investigate the extent of DNA methylation, I used three enzymes-*Bsp*1431, which can digest both methylated or unmethylated Gm^6ATC sites, *Dpn*I which cuts the same sequence only when it is methylated Gm^6ATC and *Mbo*I which recognizes only unmethylated GATC sites. I isolated chromosomal DNA from the most dramatically affected cells, those grown in glucose minimal medium with CAA. The triple mutant, MEW999, and the parent strain MEW1 were subcultured into glucose minimal medium with 0.5% CAA and chromosomal DNA was isolated and purified from both cultures, at four and 24 hours. At four hours most of the cells were intact and showed noncondensed DNA and at 24 hours most of the cells had recovered the normal rod shape. As seen in Figure 22, there was no difference observed for the samples taken at four and 24 hours from MEW999 and MEW1. The chromosomal DNA purified from both strains at different time points was methylated as they could be digested by *Dpn*I, which recognizes only methylated DNA, and by *Bsp*1431, which recognizes both methylated and unmethylated DNA. No digested DNA bands were observed for *Mbo*I. Similar data were obtained with the samples taken at seven hours of subculture time (data not shown). The data demonstrated that even if the cell's main problem is in C1 unit availability, DNA methylation proceeded normally overall. Thus decreased average DNA methylation was not the reason that MEW999 cells could not divide properly.

All of the above results suggests that in the absence of L-SD, the cell cannot replicate and/or segregate DNA normally. However DNA methylation is not affected in this case, so there must be something else which affects or signals DNA replication and is sensitive

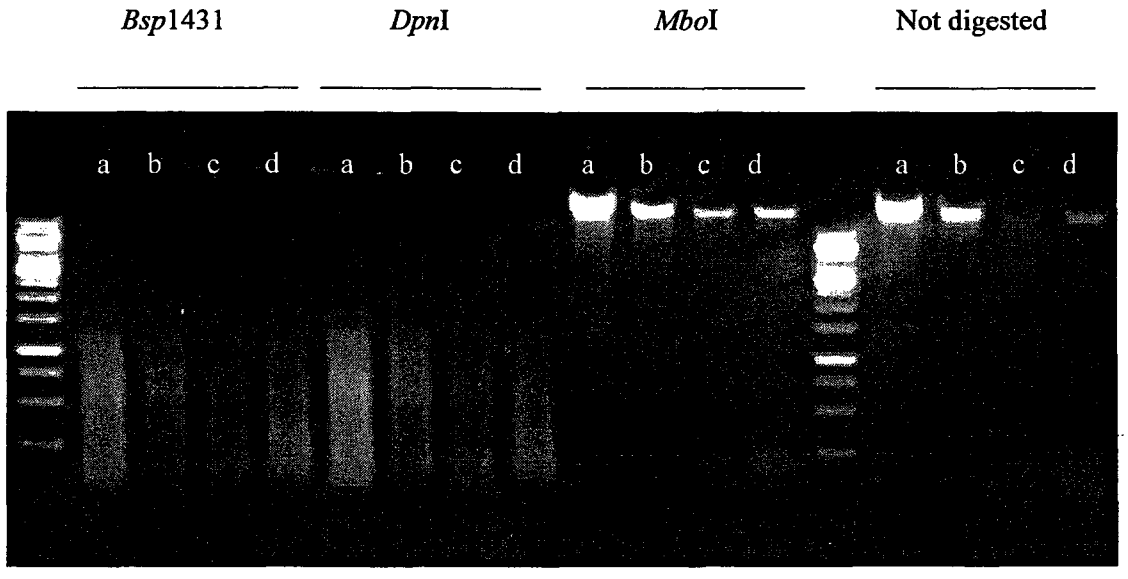


Figure 22 Chromosomal DNA methylation of MEW999

Triple mutant cells (b, d) grown in glucose minimal medium were subcultured in glucose minimal medium with CAA and DNA extracted 4 hours (sample b) or 24 hours (sample d) later. DNA was also extracted from the parent strain MEW1 (a, c) treated the same way after 4 hours (sample a) and 24 hours (sample c). Samples were loaded into a 1% agarose gel as follows: undigested DNA, digested with restriction enzyme *Bsp*1431 which can recognize both methylated and unmethylated DNA, with methylation dependent restriction enzyme *Dpn*I or with methylation sensitive restriction enzyme *Mbo*I. DNA Marker used was GeneRuler™ 1kb DNA ladder from Fermentas.

to the supply of C1 units in the cell. Previous results showed that a triple mutant which can transport exogenous SAM into the cell can divide normally in LB. So in this case the strain must be able to overcome the DNA replication defect in LB medium. In glucose minimal medium with CAA, even with SAM, the cell still cannot divide properly (Figure 23). However it forms long filaments like in LB medium. So I wondered if DNA is properly replicated and segregated under these conditions?

3.5.3 The effect of SAM on DNA segregation

Whereas the triple mutant carrying the SAM transporter makes huge misshapen cells in the presence of CAA, the cell makes long filaments with CAA and SAM, but is still not able to divide. This suggests that the triple mutant has problems segregating DNA, or making the septum, or both.

To study DNA segregation, an overnight culture of the MEW999 psamT in glucose minimal medium was diluted 10^4 fold into fresh glucose minimal medium with CAA with or without 2 mM SAM. Samples taken at four, six, eight and ten hours after subculture were then fixed and stained with DAPI.

The turbidity of the culture with SAM increased faster than that of the culture without SAM. However, these cultures were still made up of filaments, and DNA was not segregated but spread throughout the filaments. Indeed DNA had the same appearance as it did in swollen cells from cultures without SAM. By four hours, the cells incubated with SAM already looked longer than those incubated without SAM (Figure 23 4 hours), and they increased in length steadily thereafter (Figure 23, 6, 8, 10 hours). However even in

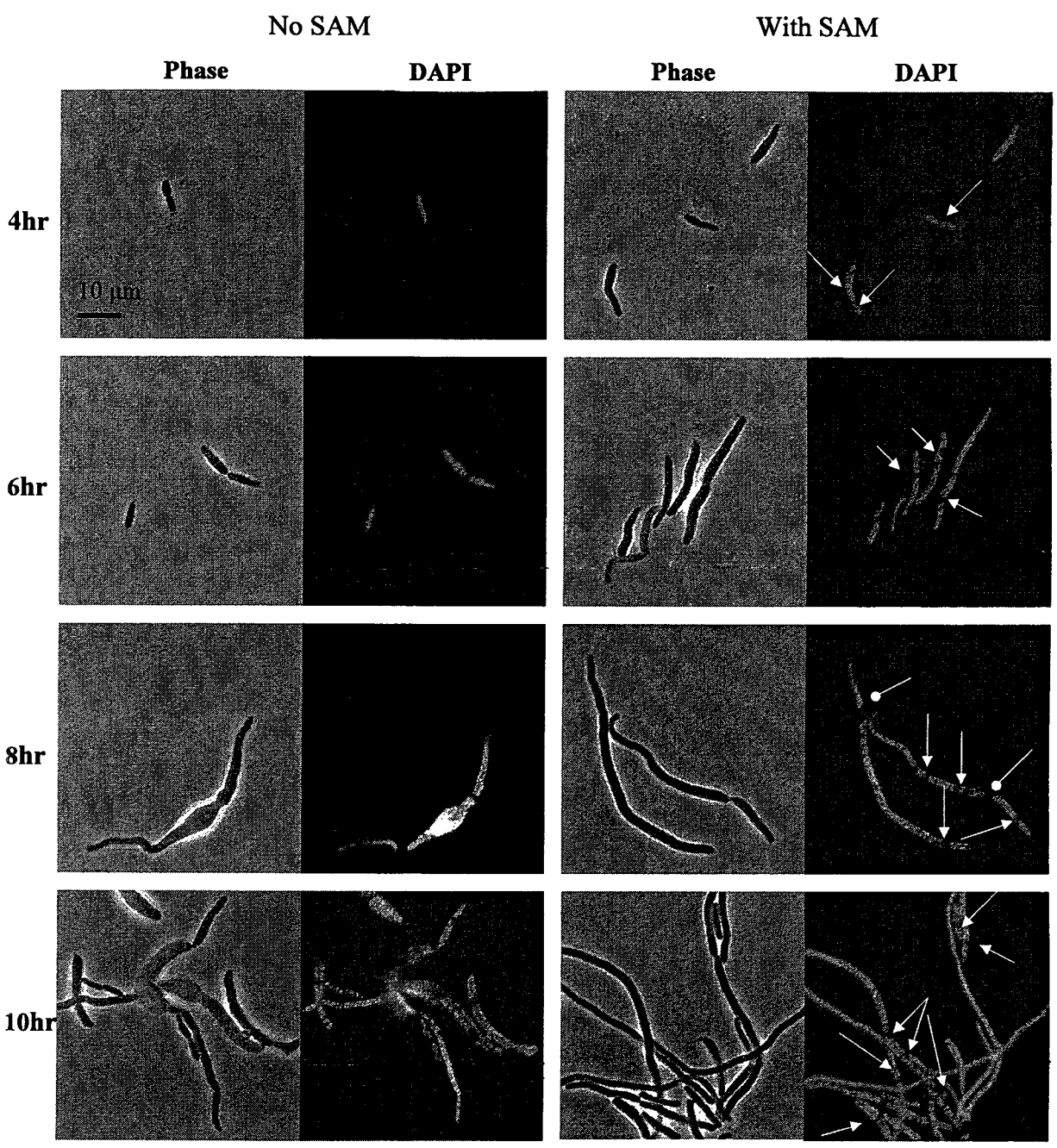


Figure 23 SAM does not help MEW999 condense or segregate DNA in glucose minimal medium with casamino acids

Triple mutant cells carrying the SAM transporter were grown overnight in glucose minimal medium and subcultured into the same medium with 0.5% CAA with and without SAM 2mM. The images were taken at 4, 6, 8 and 10 hours after subculture. The white arrows show segregated DNA and the ball-ended arrows show the big gaps between DNA in the filaments.

the longest filaments, DNA has not been condensed and has segregated only occasionally (white arrows Figure 23). At some points the filament is devoid of DNA (white ball-ended arrows Figure 23). This may indicate that there is some segregation, but no filament had regularly segregated DNA. In the pictures taken at 10 hours, there are many short filaments mixed in with the long filaments. Even in these, the DNA is uncondensed. From these data, it is clear that SAM does not improve DNA condensation and segregation deficiency of the triple mutant in glucose minimal medium with CAA.

3.6 Cell division septum assembly in MEW999

Cell division in *E. coli* involves the formation of the division septum, a protein complex assembled at the middle of the cells. Defects in the function of these proteins or incorporation process of the proteins into the ring can result in filamentation. Therefore, it is possible that the triple mutant MEW999 might have altered septal ring formation. To test this, I constructed merodiploid strains in the triple mutant background for several of the known cell division genes (as described in the materials and methods section). Assembly of the septa in the cells was monitored via green fluorescent protein (GFP) fluorescence, fused to various division proteins.

Earlier work from this lab showed that a SAM synthetase mutant *metK84* cannot recruit the cell division proteins to the septal ring because of methylation limitation (Wang *et al.*, 2005). In this study, the triple mutant has been shown to have problems with its supply of C1 units, and resulting in severe malformations in minimal medium with CAA or in LB. To investigate whether this problem is associated in some way with assembly of cell division proteins, I studied the localization of FtsZ-GFP.

3.6.1 Localization of FtsZ

FtsZ is the first protein known to localize at the septum site, where it forms the Z ring, and provides the structure for the incorporation of all the other septal proteins.

Cells of strain MEW1/*ftsZ-gfp* were subcultured into glucose minimal medium with 0.5% CAA, 25 μg/mL ampicillin and 25 μM IPTG. Samples of the parent cells taken six hours after the subculture showed bright bands of fluorescence at mid cell (>95%, Figure 24A). The filaments of *metK84*/ftsZ-gfp were sampled 16 hours after subculture in glucose minimal medium with leucine 5 μg /mL (Figure 24B). Some filaments showed a regular distribution of condensed DNA and some showed uncondensed DNA. Rings of FtsZ were seen in most filaments but there were only a relatively small number. The *metK84* filaments represent the end-point of a long incubation with suboptimal leucine and do not change much with time thereafter. The nature of the MEW999 filaments and deformed cells however changes radically with time of incubation. They may lyse, or decrease in size, eventually forming normal single cells. To monitor the change of the septa during the whole growth process, I took samples every hour from four to eight hours, and at 10 hours after the cells were subcultured into glucose minimal medium with 0.5% CAA and IPTG 25 μM.

I examined 55 cells after four hours incubation (Figure 25, Table 7), and in all of these cells the DNA was uncondensed, but 85% had at least one FtsZ ring, usually at midcell. Some cells even have three septa, one at midcell and two at the quarter parts which are destined for the subsequent division. It seems that although the triple mutant grows slower and does not divide as fast as the parental strain, the Z- ring still can be assembled

Table 7 The FtsZ-GFP ring in the triple mutant at different hours after subculture

Time	Number of Cell investigated	Cells with FtsZ-GFP	Ratio
4 hours	55	47	85%
5 hours	19	17	89%
6 hours	127	99	78%
7 hours	52	45	87%
8 hours	136	121	89%
10 hours	129	107	83%

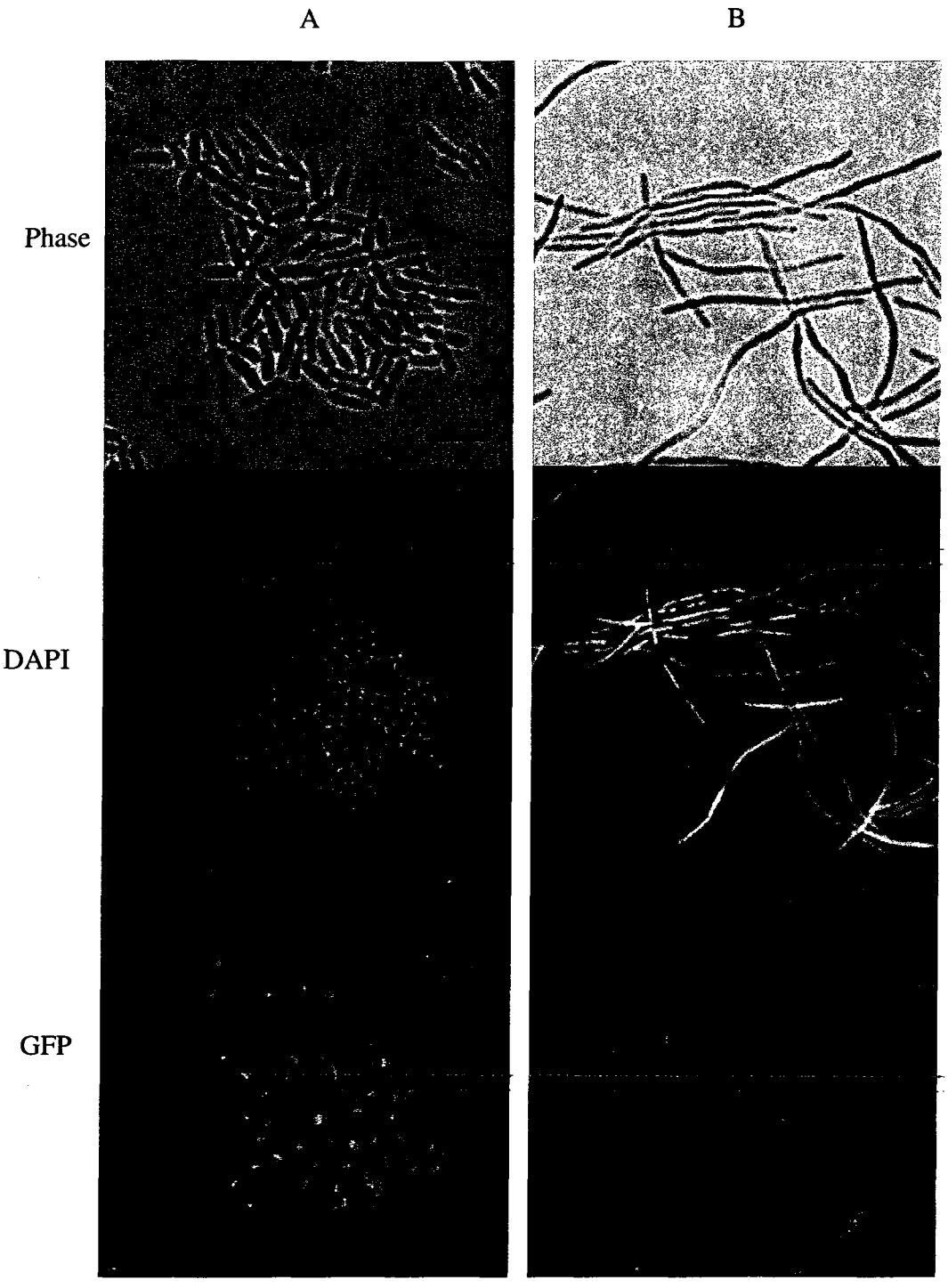


Figure 24 FtsZ localization in MEW1/*ftsZ-gfp* and *metK84/ftsZ-gfp* cells

A) Cells of strain MEW1/*ftsZ-gfp* grown in glucose minimal medium with ampicillin 25 μg /mL were subcultured for 6 hours in glucose minimal medium with 0.5% CAA and 25 μM IPTG. B). A *metK84/ftsZ-gfp* culture was collected by centrifugation for viewing with the microscope after 16 hours incubation in glucose minimal medium with 5 μg /mL leucine, and 25 μM IPTG.

at the potential division sites even at the very early growth stage when L-serine concentration is still high in the medium.

At five hours, 89% of the cells showed FtsZ-GFP, either as a ring or in some cases, spread over larger areas of the cell (Figure 26). In most of the cases, the FtsZ-GFP protein localized above the obvious cell division gap between chromosomal DNA, though it was also seen at the poles of the long cell in the absence of chromosomal DNA. The white arrows in the image show the localization of the FetZ. There were different forms of the classical "ring" apparatus and that the FtsZ-GFP protein in the triple mutant formed some wide and irregular belts on the cell envelope. If the belt formed at the poles of the cell, it even looked like a cap. The width of the septal belt corresponded to the width of the gap in the chromosomal DNA.

The situation was similar for six and seven hours images (Figure 27, 28). The FtsZ-GFP protein was seen in most of the big cells, even though DNA had not condensed and segregated normally. One long cell had several septa scattered unevenly along the cell. However in most of the cases, the septa were located at positions where the DNA was occluded. As indicated in Figure 27 by square-ended arrows, even in some cases where no distinct septum can be seen above the DNA gap the fluorescent brightness still suggests that a large amount of the FtsZ-GFP protein has accumulated.

At eight hours (Figure 29), there are many more small cells indicating that an active subdivision process has begun. Septal rings were more regularly distributed. The white ball-ended arrows in Figure 29 Biii and Ciii show long cells with uncondensed segregated DNA and septal rings.

At 10 hours, 83% of the 129 cells observed showed at least one FtsZ-GFP ring. It is

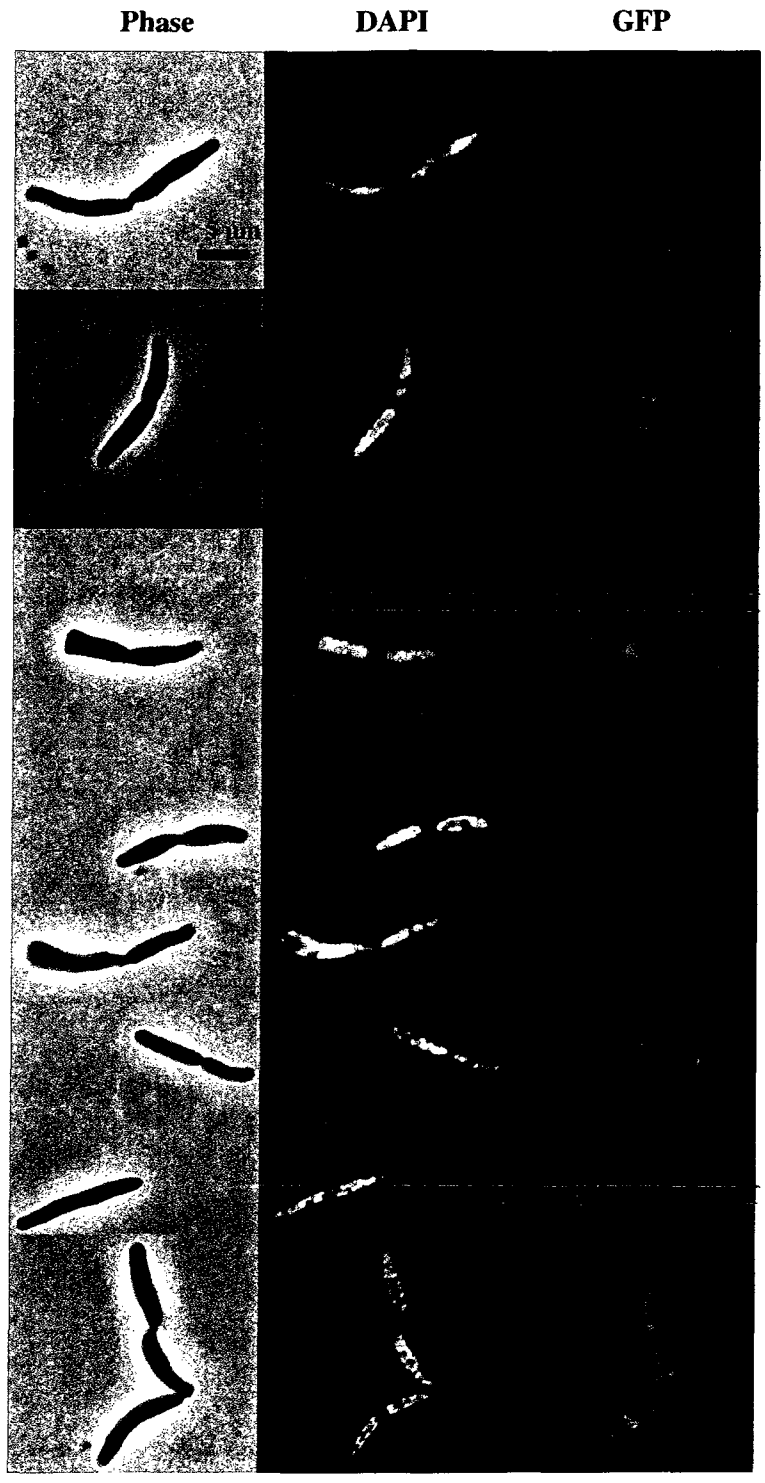


Figure 25 FtsZ localization in MEW999/*ftsZ-gfp* 4 hours

Cells of strain MEW999/ftsZ-gfp were grown in glucose minimal medium with ampicillin (25 μg/mL) and subcultured in the same medium with 0.5% CAA and 25 μM IPTG without ampicillin. 4 hours after subculture, cells were fixed, stained with DAPI and photographed. Because the cultures were quite dilute, the various images were taken from different fields.

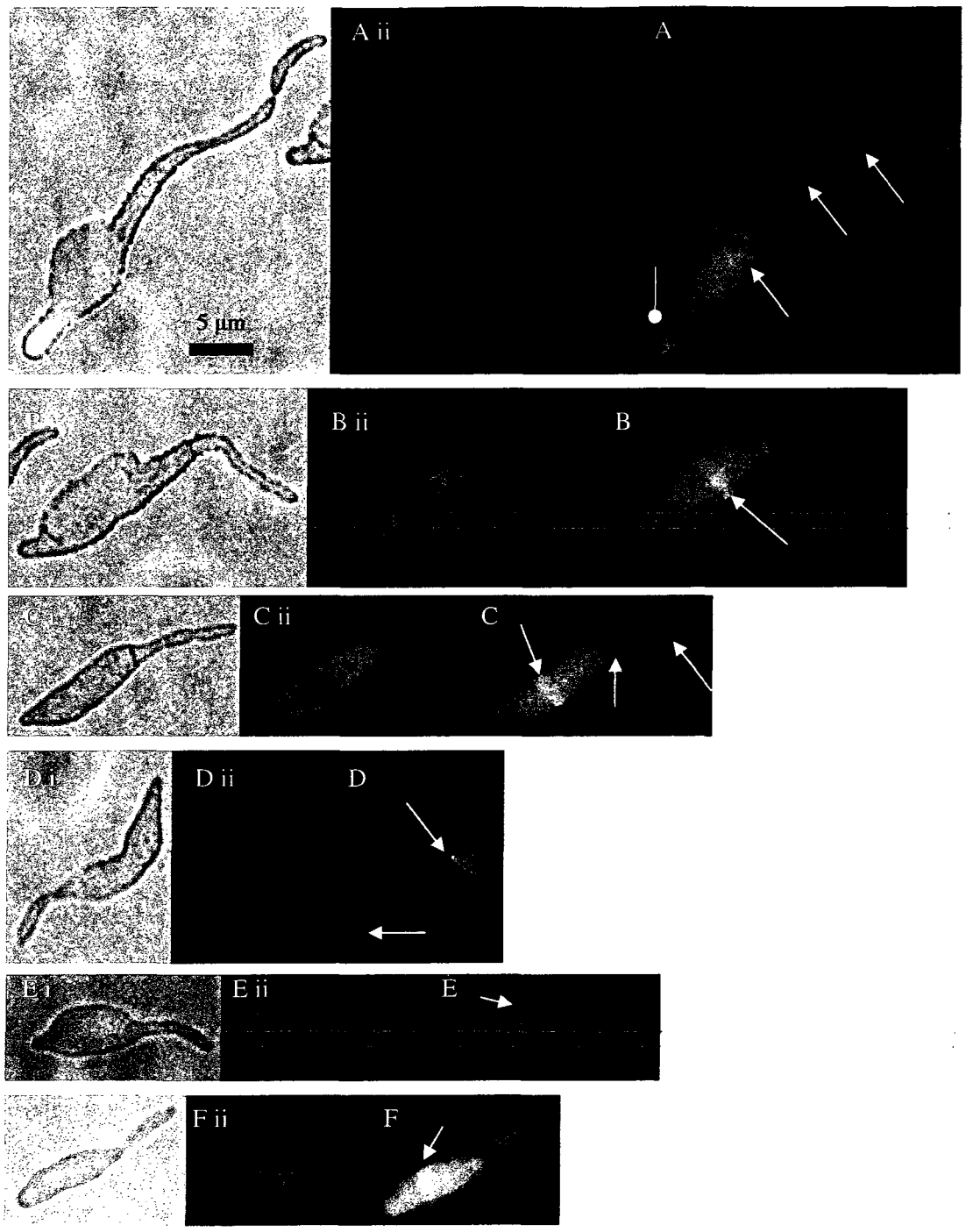


Figure 26 FtsZ localization in MEW999/*ftsZ-gfp* 5 hours

Experiment as in Fig. 25, examining a 5 hours subculture of MEW999/*ftsZ-gfp*. i, ii and iii represent phase contrast, DAPI and GFP images respectively. The white ball-ended arrow indicates the tip of a cell which has an FtsZ-GFP cap. The white arrows indicate septa.

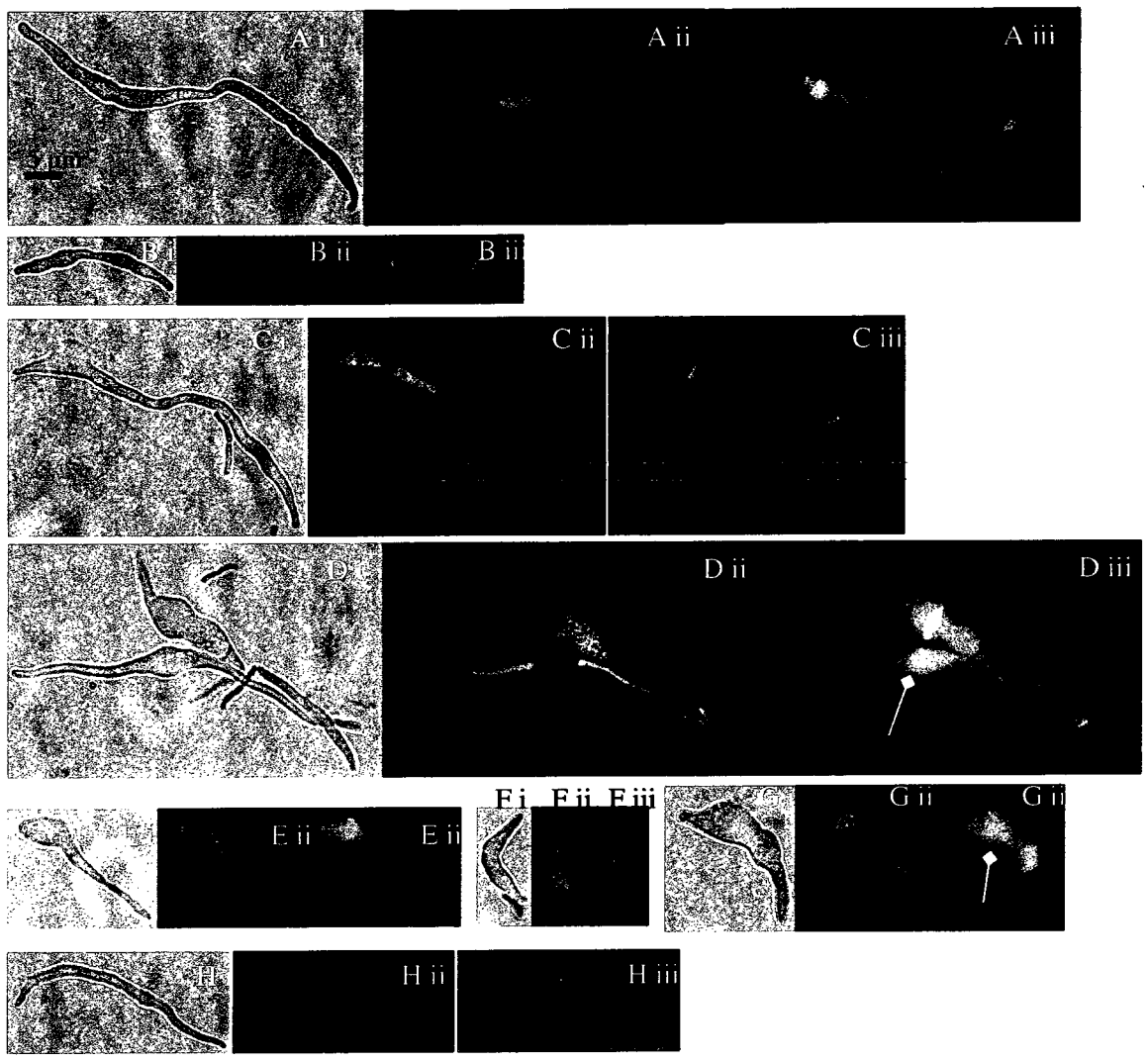


Figure 27 FtsZ localization in MEW999/*ftsZ-gfp* 6 hours

Experiment as in Fig. 25, 26, examining a 6 hours subculture of MEW999/*ftsZ-gfp*. i, ii and iii represent phasecontrast, DAPI and GFP images respectively. The white square-ended arrows indicate cells with concentrated FtsZ-GFP.

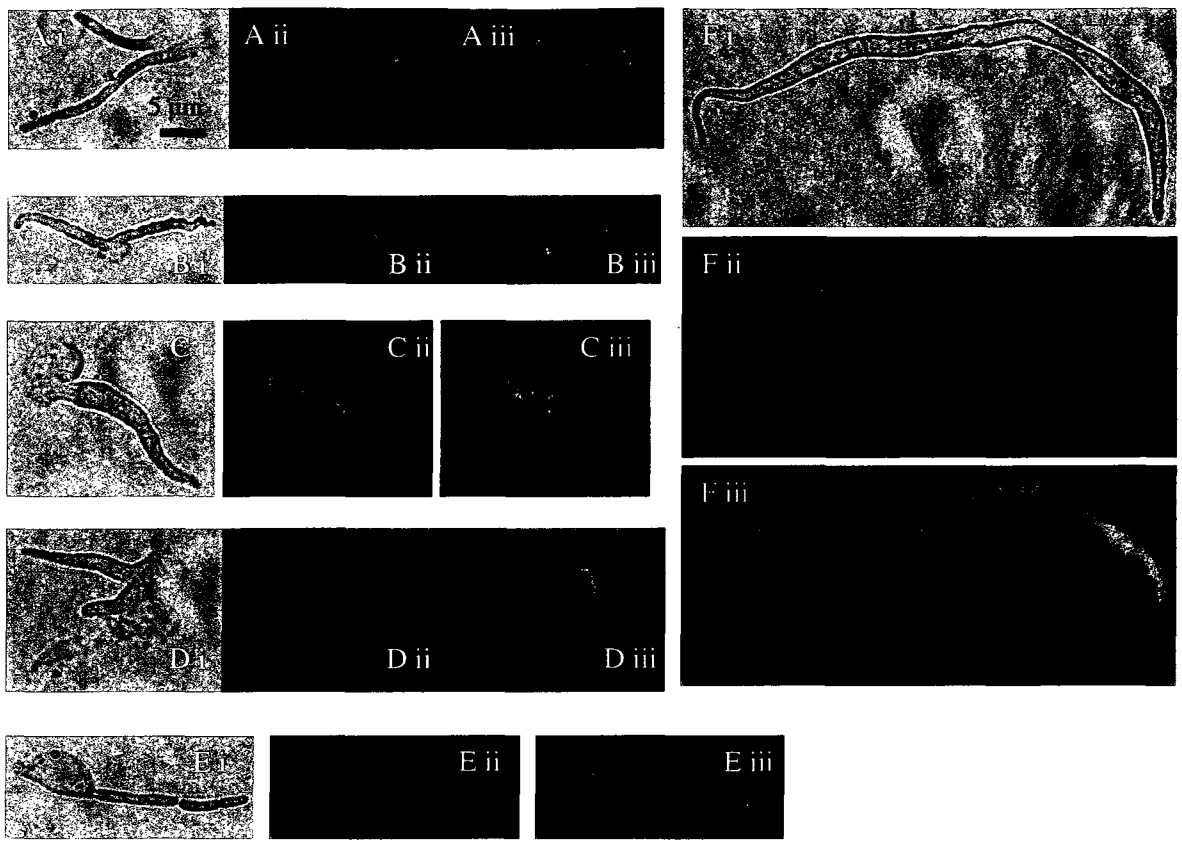


Figure 28 FtsZ localization in MEW999/*ftsZ-gfp* 7 hours

Experiment as in Fig. 25-27, examining a 7 hours subculture of MEW999/*ftsZ-gfp*. i, ii and iii represent phasecontrast, DAPI and GFP images respectively.

clear from this that the cell division deficiency in most of these misshapen cells was not caused by a lack of FtsZ rings. Swelling of the cell did not stop the assembly of septal rings and on the contrary, most of the bulgy cells showed clear septum in the mid ball (Figure 30). The 10 hours sample of the same MEW999/*ftsZ-gfp* culture seen in Figure 30 is pictured at a 400X magnification and showed in Figure 31. The white arrows show some of the swollen cells with Z rings at mid cell. More than 94% of big bulgy cells (32 cells in a total number of 34) had septal rings or obvious FtsZ-GFP signals gathering. As discussed in Chapter 2, the misshapen triple mutant cell was osmotic sensitive. Since the large bulgy cells usually lyse at the middle of the bulge, the cell wall deficiency could be the reason for the bulgy cells. One hypothesis is that the septum forming at a weak point in the cell wall, and this leading to swelling to a point where the wall could not withstand the osmotic pressure and lysed.

Of 111 FtsZ rings which I examined closely, 89% were located within visible gaps between nucleoids or at the poles of the cell, even though sometimes the gaps may vary in size. The remaining 11% were located in positions with no detectable gaps.

From the preceding images, FtsZ can form Z rings, though the localization of the septal ring is much less precisely regulated than usual. Different from the obvious deficiency in DNA condensation and segregation, Z ring formation did not show obvious delay after the DNA segregation. Thus my data support the hypothesis that Z ring assembly is not significantly affected in the triple mutant.

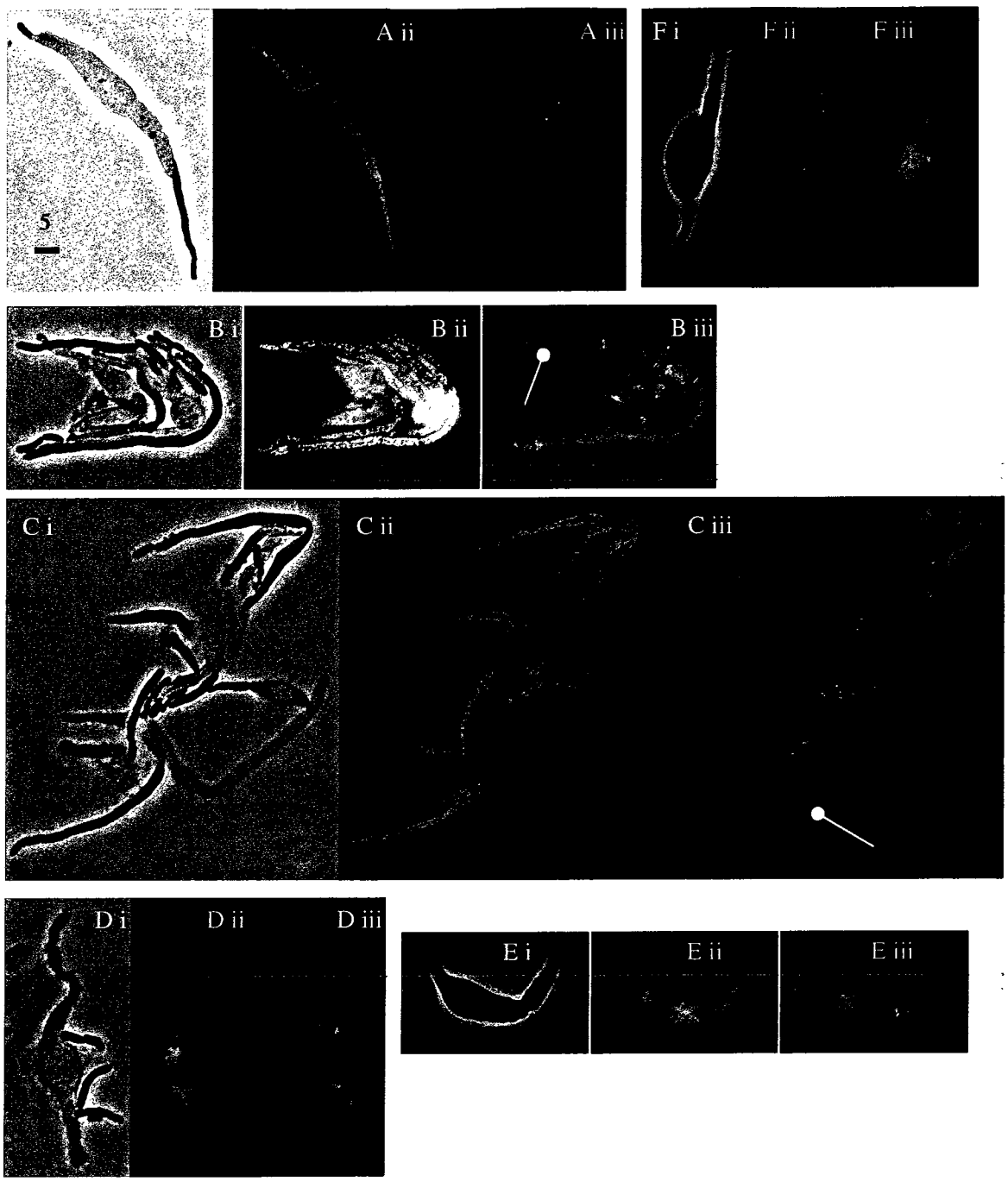


Figure 29 FtsZ localization in MEW999/*ftsZ-gfp* 8 hours

Experiment as in Fig. 25-28, examining an 8 hours subculture of MEW999/*ftsZ-gfp* cells. i, ii and iii represent phasecontrast, DAPI and GFP images respectively. The white ball-ended arrows indicate the cells with evenly localized FtsZ rings.

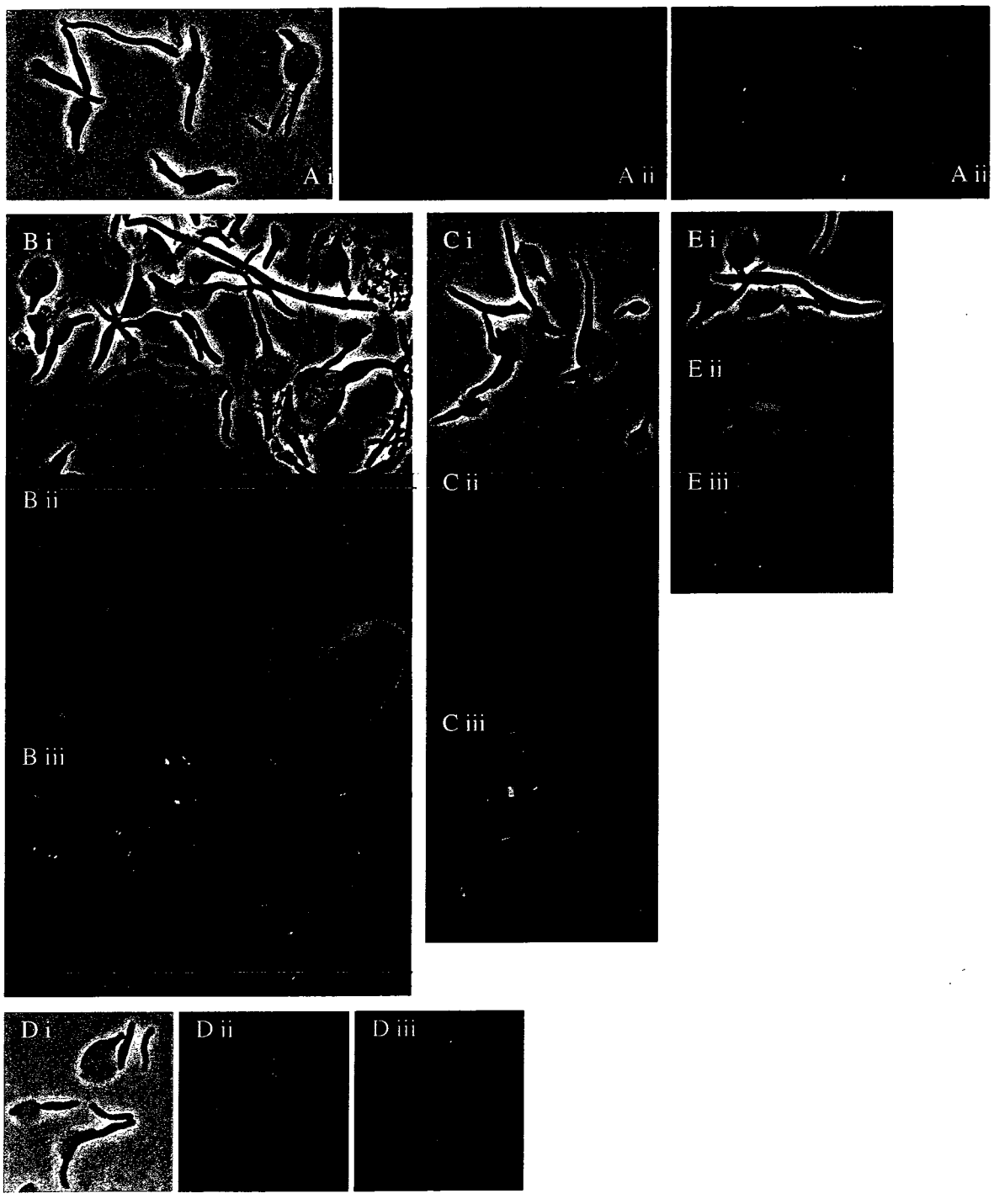


Figure 30 FtsZ localization in MEW999/*ftsZ-gfp* 10 hours

Experiment as in Fig. 25-29, examining a 10 hours subculture of MEW999/*ftsZ-gfp* cells. i, ii and iii represent phasecontrast, DAPI and GFP images respectively.

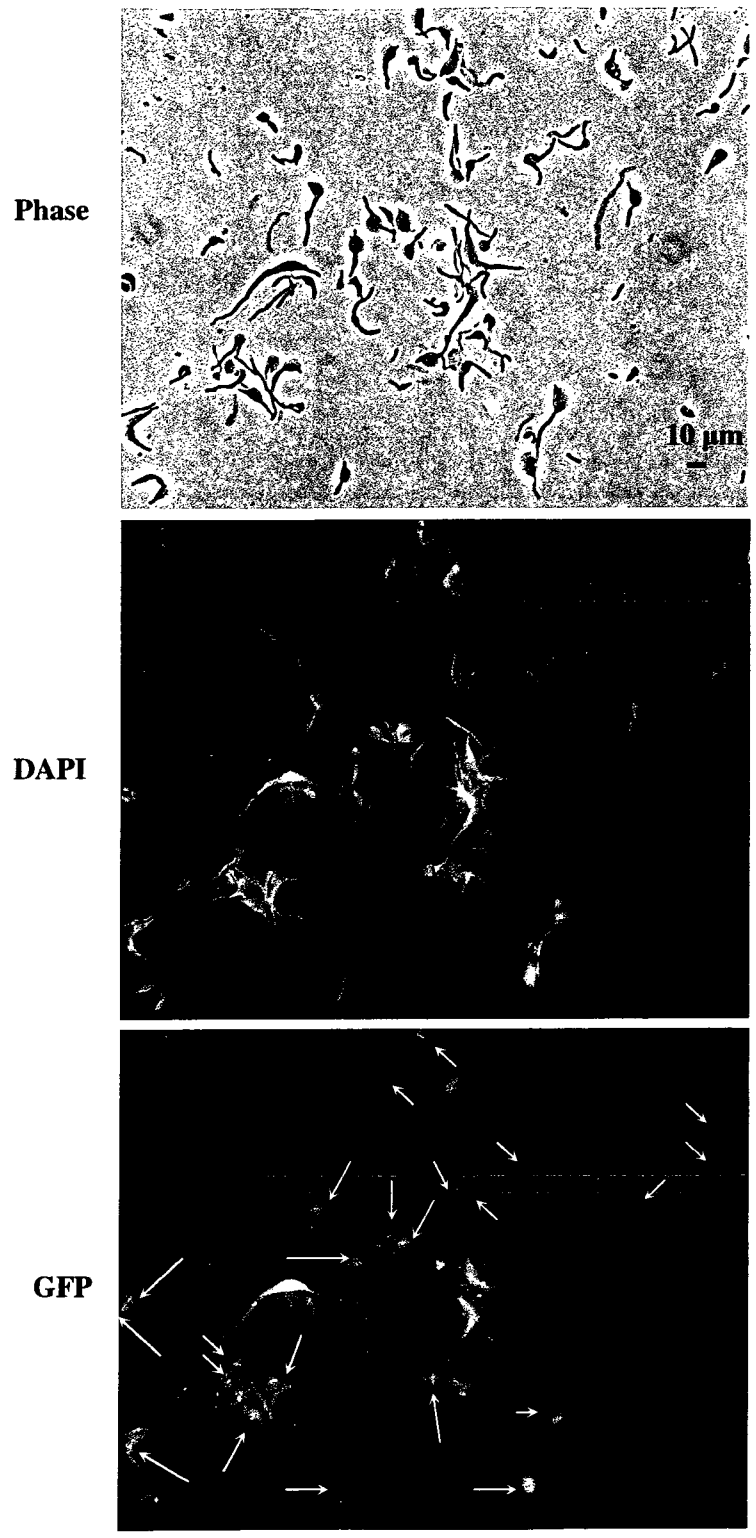


Figure 31 FtsZ-GFP localization in swollen cells

The 10 hours sample of the same MEW999/*ftsZ-gfp* culture seen in Fig. 28 is presented at a 2.5 fold lower magnification. The white arrows show swollen cells with Z rings at mid cell.

3.6.2 Localization of the GFP-FtsW protein

The localization of FtsZ-GFP into rings in the misshapen triple mutant cells suggests that the division apparatus initiates assembly. However, other ring proteins might not be recruited into the Z ring. As previously shown by the Newman laboratory, *metK*84 cannot localize the later division proteins to the septa even though it can localize FtsA and ZipA (Wang *et al.*, 2005). Therefore, I investigated the localization of a later protein FtsW fused to GFP.

Cells of parental strain MEW1/*gfp-ftsW* were subcultured into glucose minimal medium with 0.5% CAA, 1 mM IPTG. Cells taken at five hours after subculture showed septa at the middle of most of the cells (>70%, Figure 32). All the cells were of normal length, indicating FtsW overproduction was not harmful to the cells.

A GFP-ftsW band was observed in 90% of MEW999/*gfp-ftsW* cells seven hours after subculture Figure 33. The pattern of septal ring localization was similar to the FtsZ-GFP pattern, with rings coinciding with gaps in the DNA. It is clear that both FtsZ and FtsW localize without much problem.

3.6.3 The aberrant GFP-FtsI localization in triple mutant cells

During incubation with CAA, many cells swell into large bulges, often with narrower filaments at one or both ends. This is related to the osmotic sensitivity described earlier. Where 94% of big bulges showed FtsZ rings near the center, i.e. at the widest point, these were difficult to study because of their fragility. Indeed they often lysed while the slides were being prepared for microscopy. This suggests that the bulges form at points of

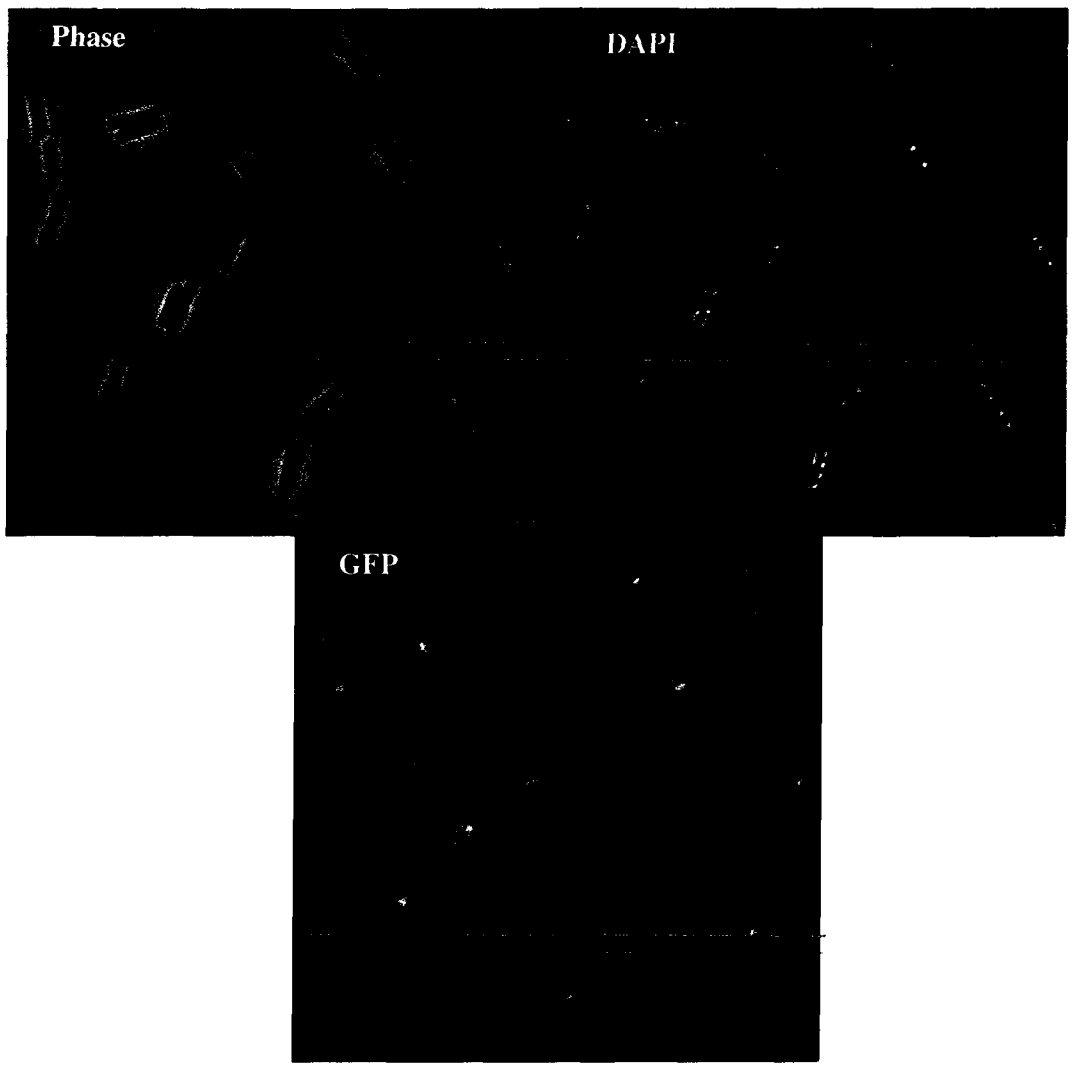


Figure 32 GFP-FtsW localization in MEW1/*gfp-ftsW*

Cells of strain MEW1/*gfp-ftsW* were grown in glucose minimal medium with ampicillin (25 μg/mL) and subcultured in the same medium with 0.5% CAA and 1 mM IPTG without ampicillin. 5 hours after subculture, cells were fixed, stained with DAPI and photographed.

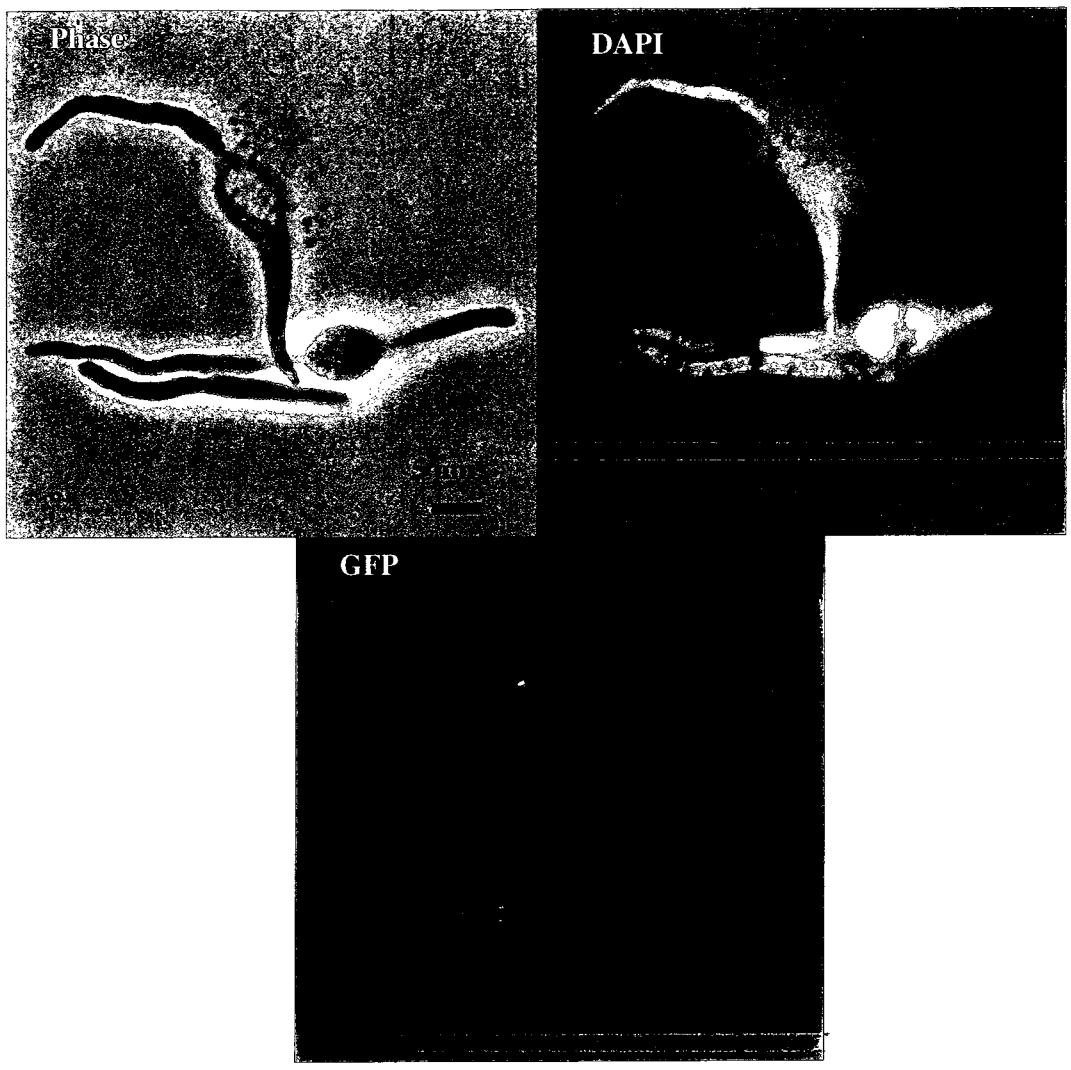


Figure 33 GFP-FtsW localization in MEW999/ *gfp-ftsW* at 7 hours

Cells of strain MEW999/*gfp-ftsW* were grown in glucose minimal medium with ampicillin (25 μg/mL) and subcultured in the same medium with 0.5% CAA and 1 mM IPTG without ampicillin. 7 hours after subculture, cells were fixed, stained with DAPI and photographed.

weakness in the cell wall, perhaps specifically at the point where the septum is forming. Because this is the widest point, these septal rings are enormous compared to those usually formed.

FtsI, also known as PBP3, is a protein that is later recruited to the septum. It functions in peptidoglycan synthesis as a transpeptidase. It is involved in septal peptidoglycan synthesis rather than in elongation of the cell wall (Adam *et al.*, 1997; Bowler and Spratt, 1989; Nakamura *et al.*, 1983). Using MEW999/*gfp-ftsI*, and 25 μM IPTG, I investigated the localization of GFP-FtsI.

As shown in Figure 34, bright bands of fluorescence at the mid cell were seen in the parent, MEW1/*gfp-ftsI,* by five hours after subculture, with 75% of the cells showing septal rings. This indicated this IPTG concentration (25 uM) did not affect the growth of the cell and did show the location of the septum.

As shown in Figure 35, at 4.5 hours after subculture, most of the triple mutant cells showed one or more clear septal rings, and 93 % had GFP-FtsI localized to at least one potential division site (Table 8). After six hours in the presence of IPTG, the number of cells with septal rings dropped to 60%. However, a lot of clumped GFP-FtsI could be observed inside the cells especially in the swollen parts (Figure 35). The white arrow in Figure 35 six hours indicates clumps of GFP-FtsI inside the cell. In the same cell a clear FtsI ring is visible, as indicated by the square-ended arrow.

At eight and 10 hours (Figure 36), only 53% or 30% had GFP-FtsI bands at eight or 10 hours respectively (Table 8). The GFP-FtsI was not localized, but rather was scattered through the cytoplasm, and was especially dense in the swollen cells (as indicated by white arrows).

Table 8 The GFP-FtsI localization in the triple mutant at different hours after subculture

Time	Number of Cell investigated	Cells with GFP-FtsI	Ratio
4.5 hours	28	26	93%
6 hours	30	18	60%
8 hours	43	23	53%
10 hours	86	30	35%

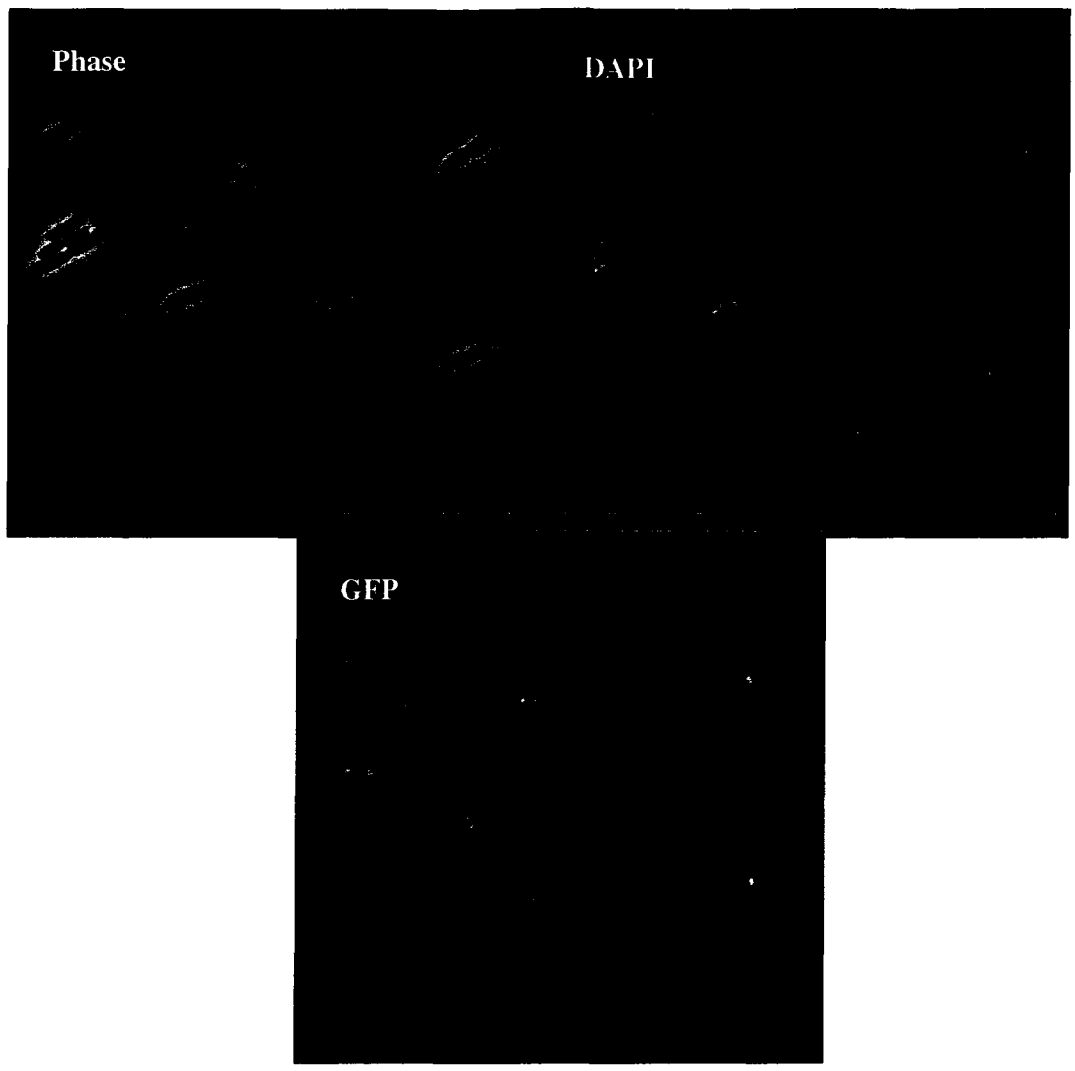


Figure 34 GFP-FtsI localization in MEW1/*gfp-ftsI*

Cells of strain MEW1/*gfp-ftsI* were grown in glucose minimal medium with ampicillin (25 μg/mL) and subcultured in the same medium with 0.5% CAA and 25 μM IPTG without ampicillin. 5 hours after subculture, cells were fixed, stained with DAPI and photographed.

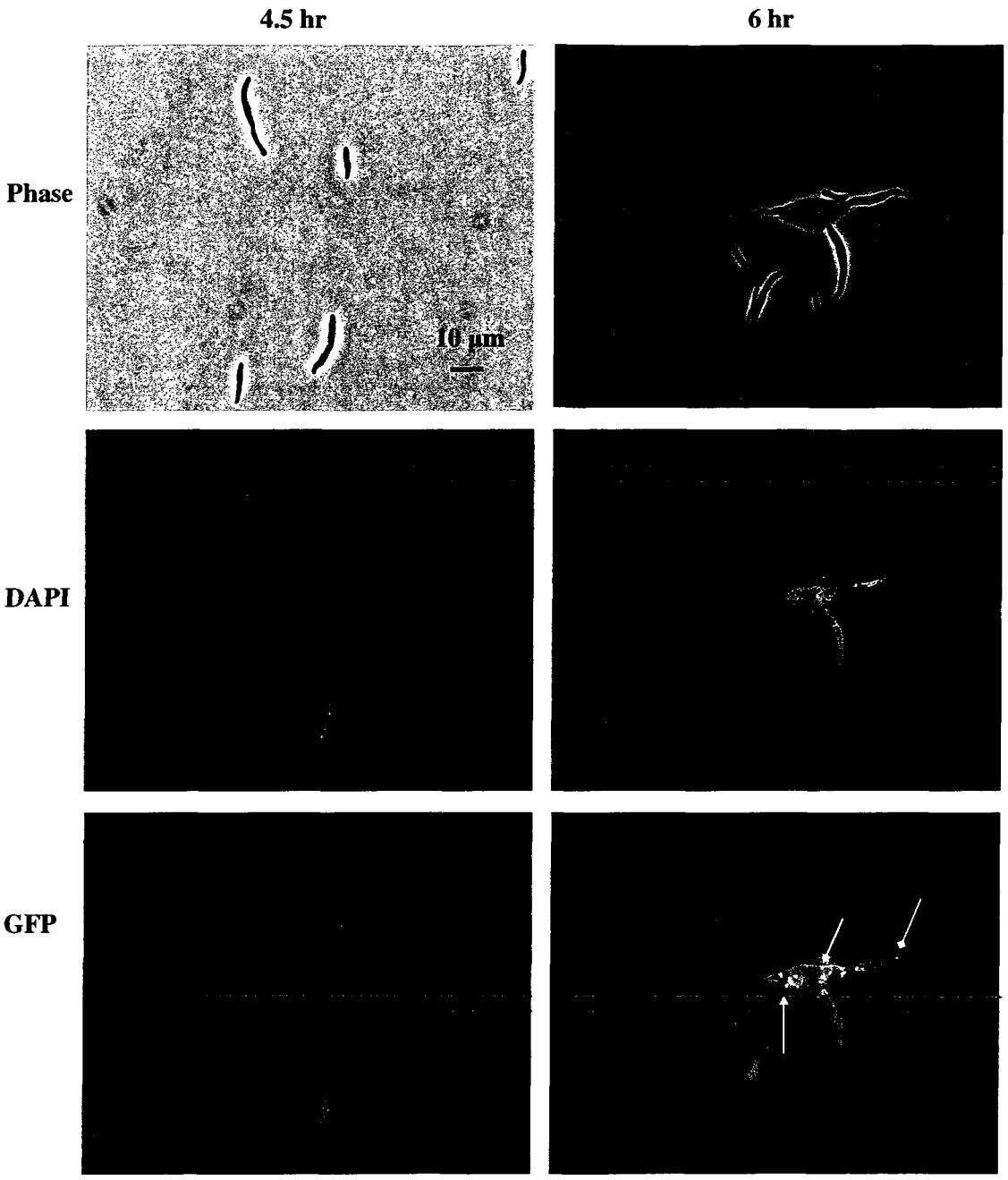


Figure 35 GFP-FtsI localization in MEW999/ *gfp-ftsI* at 4.5 and 6 hours

Cells of strain MEW999/gfp-ftsI were grown in glucose minimal medium with ampicillin (25 µg/mL) and subcultured in the same medium with 0.5% CAA and 25 µM IPTG without ampicillin. 4.5 and 6 hours after subculture, cells were fixed, stained with DAPI and photographed. The white arrows indicate areas of GFP-FtsI aggregation. The square-ended arrows indicate the GFP-FtsI belt.

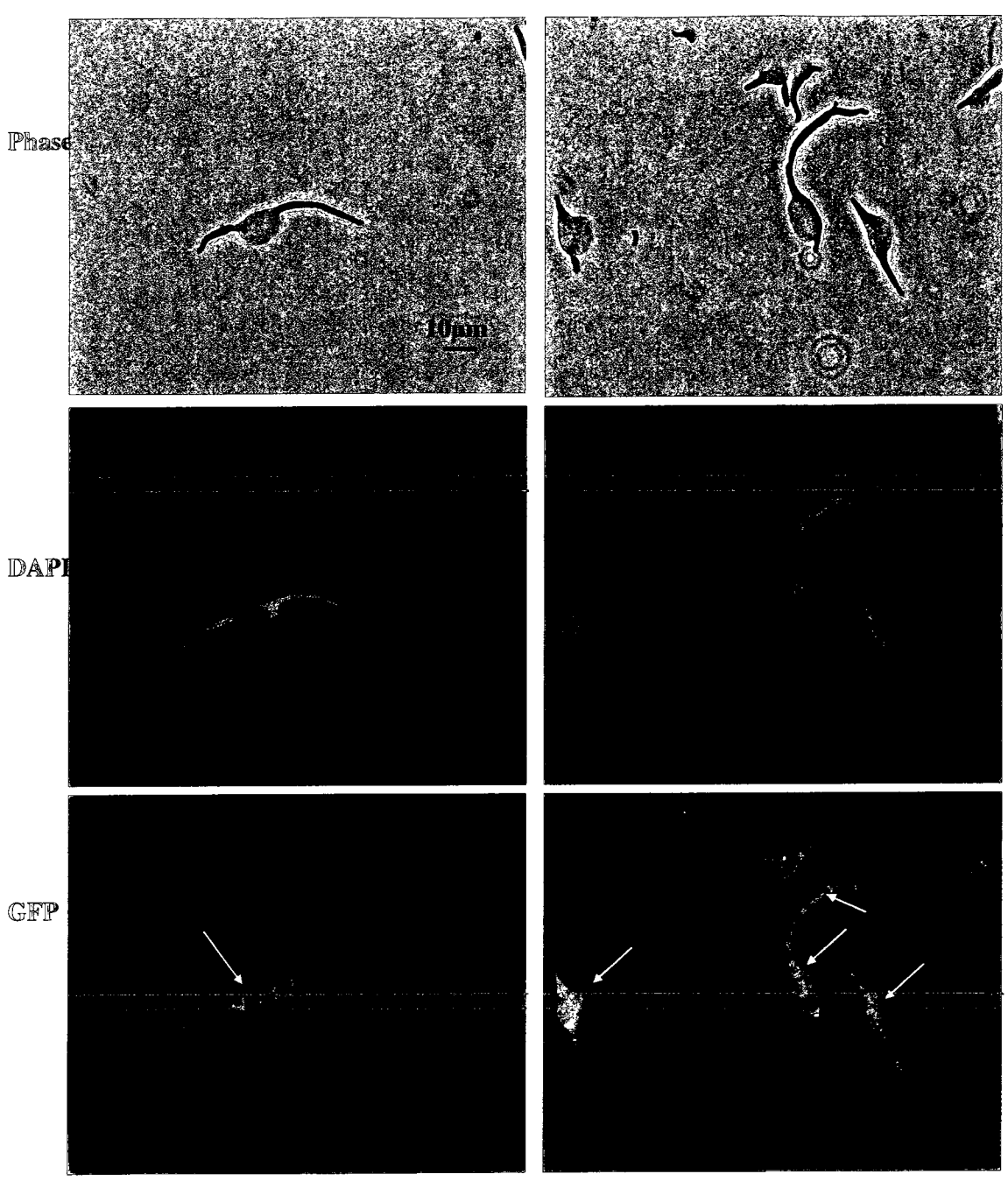


Figure 36 GFP-FtsI localization in MEW999/ *gfp-ftsI* at 8 and 10 hours

Experiment as in Fig. 34, examining 8 and 10 hours subcultures of MEW999/*gfp-ftsI*. The white arrows indicate areas of GFP-FtsI aggregation.

FtsI failed to localize to potential division sites in triple mutant, and could be linked to the cell wall synthesis failure at the septal site.

3.6.4 Septal ring synthesis in MEW999 *ftsZ-gfp* strain capable of using exogenous SAM

As previously mentioned, adding SAM to LB restores the growth of the triple mutant, so that the cells divide normally and do not form filaments. SAM does not restore normal growth to the triple mutant growing in glucose minimal medium with CAA but it allows the formation of long filaments of even width, which ressemble the filaments formed by cells growing in LB. Therefore, I studied the growth of MEW999/ *ftsZ-gfp* with psamT in glucose minimal medium with CAA.

As shown in Figure 37 and 38, the DNA was not condensed in the presence of SAM. However, many regularly localized FtsZ-GFP rings were observed in the long filaments. Most of the rings were seen between the visible gaps of DNA. This is very different from the observations of swollen cells without SAM in which FtsZ assembly was abnormal and some very broad FtsZ rings were seen. Filaments had double rings (Figure 38, 8 and 10 hours GFP image). Therefore, the cells may have some problems in localizing FtsZ.

These results indicate that SAM improves FtsZ ring assembly, though it does not fully restore localization. However, it does not restore DNA condensation and segregation.

3.6.5 The effect of exogenous SAM on the localization of FtsI in the triple mutant

The triple mutant *gfp-ftsI* carrying psamT was studied in the same way. The cells of MEW999/*gfp-ftsI* psamT grown overnight in glucose minimal medium were subcultured into glucose minimal medium with 0.5% CAA, with and without 2 mM SAM. When the

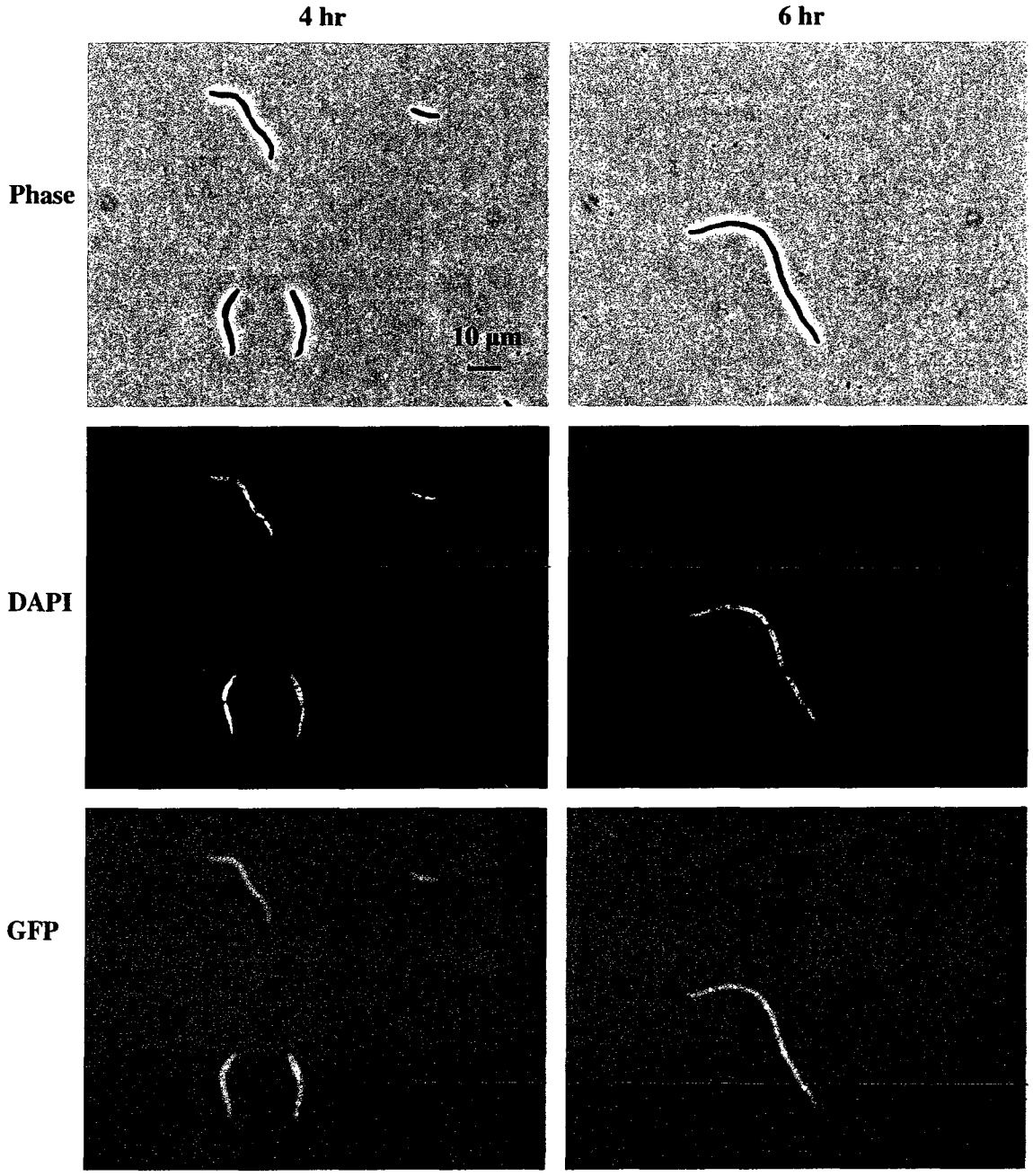


Figure 37 FtsZ-GFP localization in MEW999/*ftsZ-gfp* carrying psamT at 4 and 6 hours

Cells of strain MEW999/*ftsZ-gfp* carrying psamT were grown in glucose minimal medium with 25 μg/mL ampicillin and chloramphenicol and subcultured in the same medium with 0.5% CAA, 2 mM SAM and 25 μM IPTG without ampicillin. 4 and 6 hours after subculture, cells were fixed, stained with DAPI and photographed.

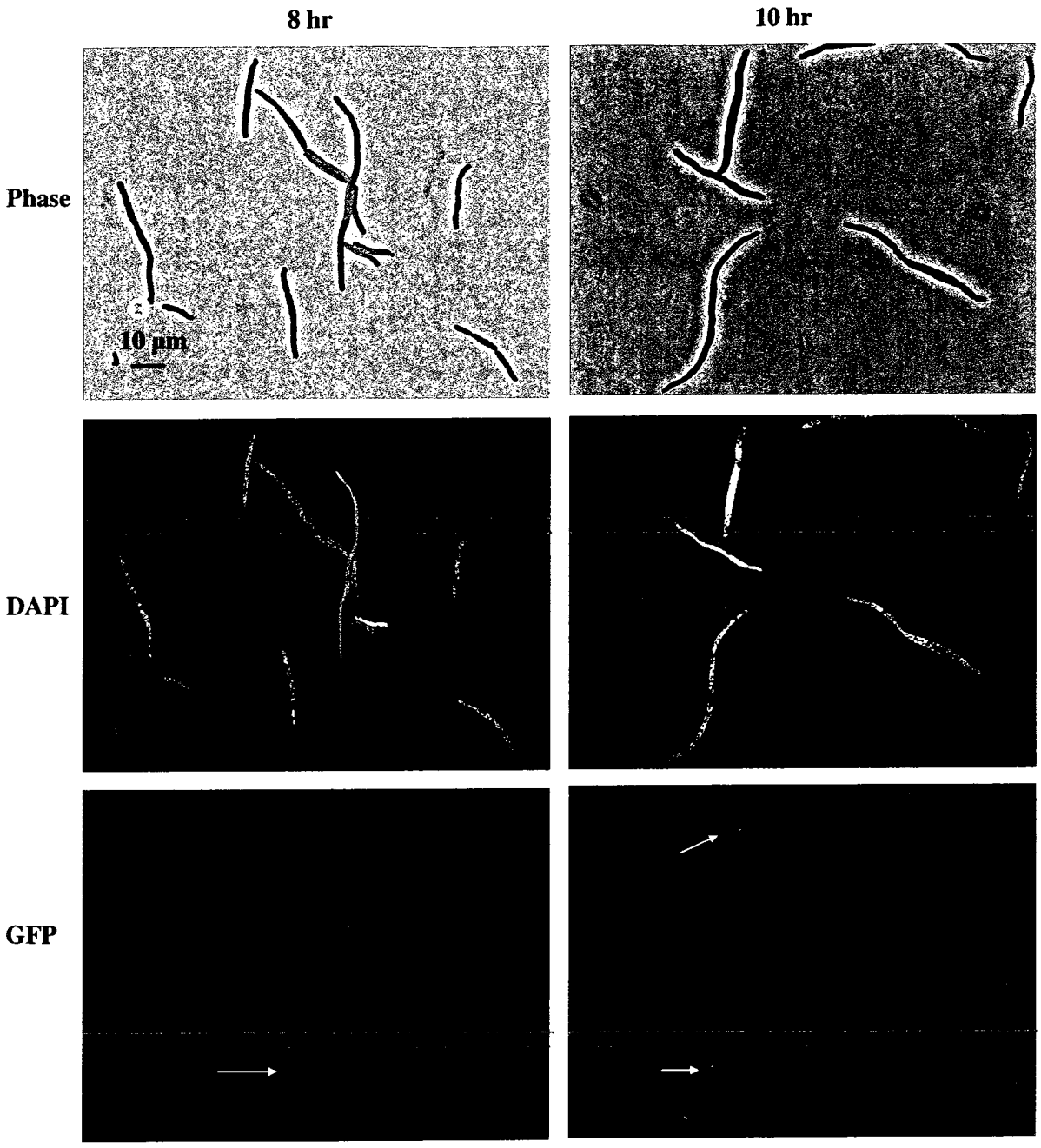


Figure 38 FtsZ-GFP localization in MEW999/*ftsZ-gfp* carrying psamT at 8 and 10 hours

Experiment as in Fig. 36, examining 8 and 10 hours subcultures of MEW999/*ftsZ-gfp* carrying plasmid psamT. The white arrows indicate multiple or double rings localized to potential division sites

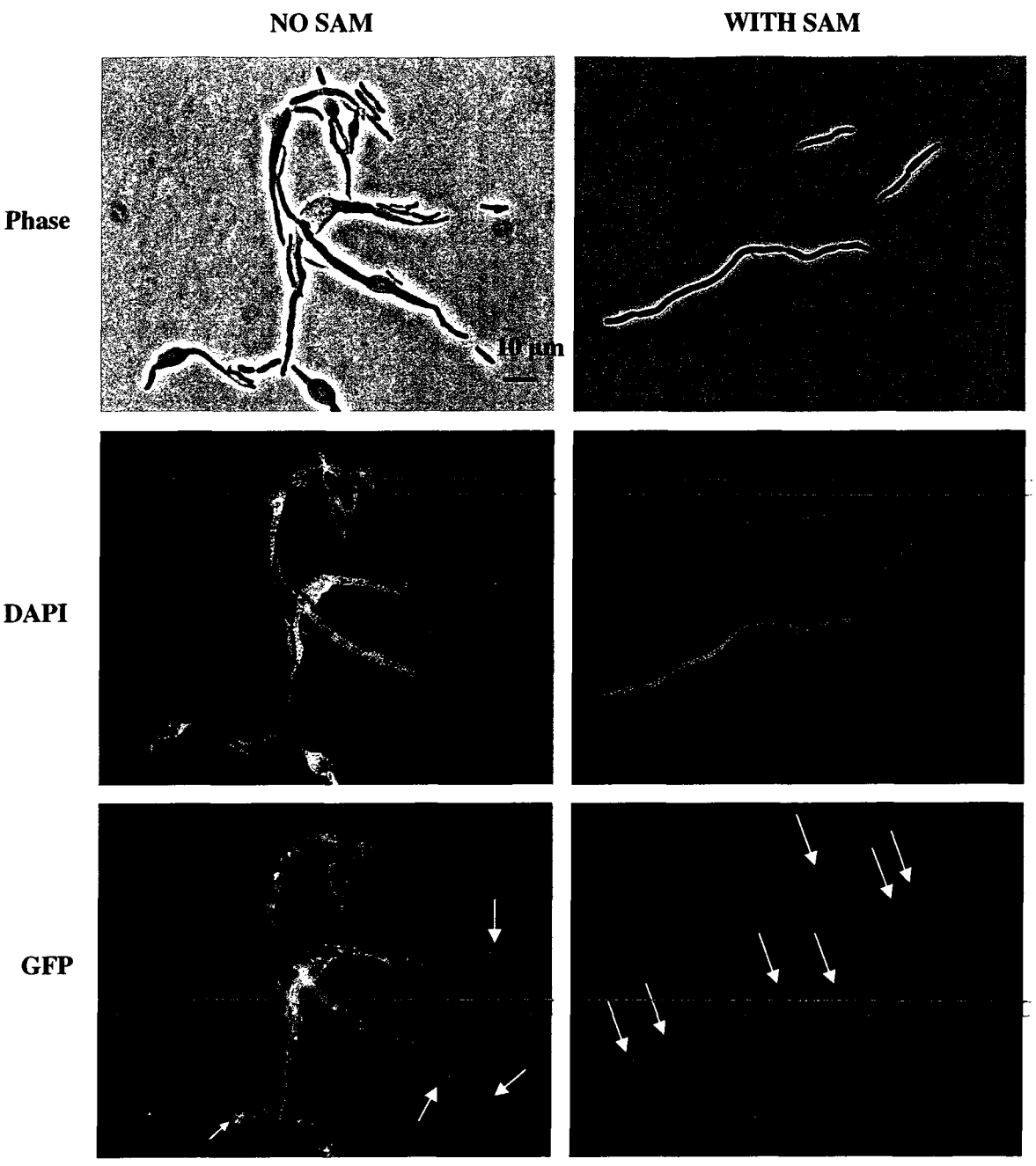


Figure 39 GFP-FtsI localization in MEW999/ *gfp-ftsI* with psamT at 10 hours

An overnight culture of MEW999/*gfp-ftsI* carrying psamT grown in glucose minimal medium with 25 μg/mL Amp and Cm was subcultured into glucose minimal medium with 0.5% CAA, 25 μg/mL Cm with and without 2 mM SAM. 10 hours samples were fixed and stained with DAPI. The white arrows indicate the division rings in the cells.

culture was examined after 10 hours, the filaments could recruit FtsI-GFP into the divisome. Without SAM, the triple mutant formed misshapen cells in glucose minimal medium with CAA as usual. The GFP-FtsI formed clumps scattered throughout the cytoplasm. A few septa could be seen in these misshapen cells (white arrows, Figure 39). In the culture with SAM, a lot of long filaments formed as expected. However, in these long filaments, the GFP-FtsI was recruited to the speta nicely. There was no clumped GFP-FtsI visible in the cytoplasm. In a total of 69 filaments recorded, 88% had at least one septum.

3.7 The effect of two min cluster on the growth of MEW999

Since the septa are not properly formed in strain MEW999 subcultured into CAA, I wondered if the loss of L-SD affected expression of the cell division proteins. At two min on the *E. coli* chromosome, there is a large cluster of genes, (from *mraZ* to *envA*) known as *dcw* for division and cell wall. This cluster, beginning with the p*mra* promoter, codes for proteins involved in cell envelope biosynthesis and cell division (Ayala, 1994; Flardh *et al.*, 1997; Vicente and Errington, 1996; Vicente *et al.*, 1998).

To study the *mra* promoter Hara *et. al.* (1997) constructed a strain named JE7968 which disrupted P*mra* on the chromosome and replaced it with the inducible *lac* promoter (Figure 40).

To confirm that MEW999h carries the P*mra*::P*lac* promoter insertion, I tested the IPTG dependence of this strain (Figure 41). While the strain grew well with IPTG, without IPTG, all the cells of MEW999h lysed about three hours after subculture on a slide culture with glucose minimal medium with CAA.

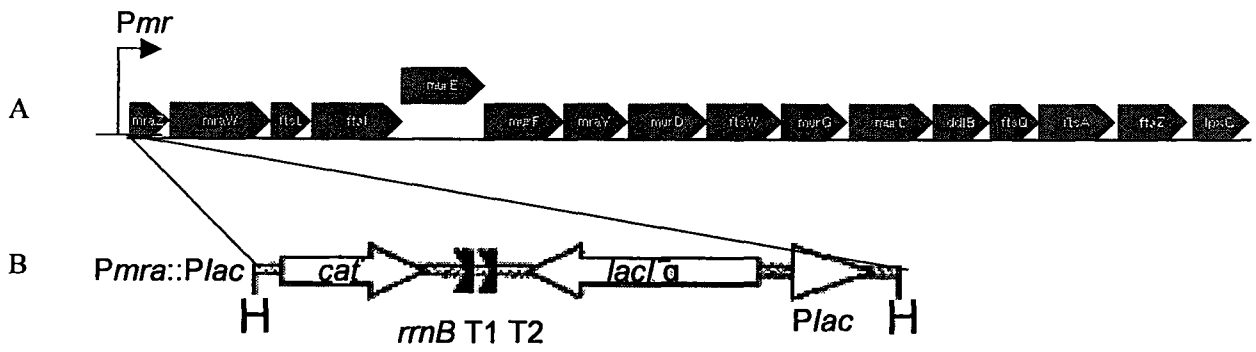


Figure 40 P*mra*::P*lac* in two min *dcw* cluster of JE7968

A) The *dcw* cluster at two min of the *E. coli* chromosome, adapted from Ecocyc and Hara *et al.*(1997) B) Expansion of the *mra* promoter showing the insert constructed by Hara *et al.* (Hara *et al.*, 1997; Keseler *et al.*, 2005)

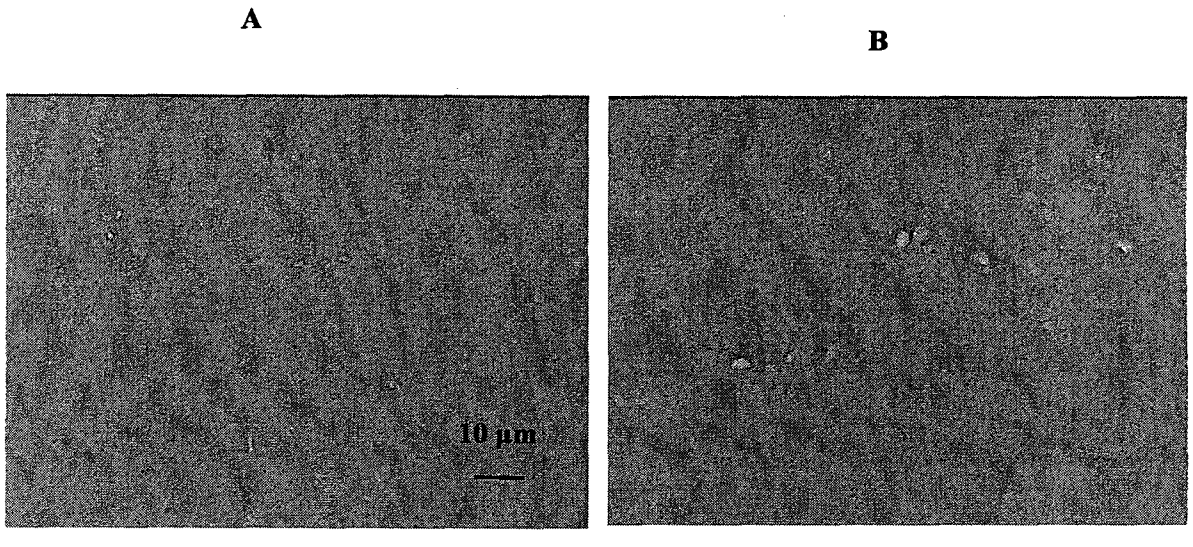


Figure 41 Micrographs of MEW999h incubated for 3 hours in the absence of IPTG

Cells of strain MEW999h, grown in glucose minimal medium with 25 µg/mL chloramphenicol and 1 mM IPTG were subcultured in the same medium without IPTG but with CAA and photographed immediately (A) and after 3 hours (B).

The growth of strain MEW999h was tested on a slide culture with a layer of glucose minimal medium agar with CAA 0.5% and 1 mM IPTG. As seen in Figure 41, the morphology of MEW999h on the slide culture was similar to that of the triple mutant itself, though the cells divided earlier and were therefore smaller at most time points. After three hours subculture, the cells were larger than the original cells and swollen (Figure 42) but after another two hours, most of the cells on the slide culture had already divided into small round or square cells, and some cells had lysed. This division into small colonies occurs noticeably earlier than in the triple mutant itself, suggesting that removal of the *dcw* cluster from control of p*mra* facilitated this cell division. However, the cells still became deformed and lysed. Thus loss of L-SD has some effect on expression from the *mra* promoter but most of the altered growth of MEW999 is independent of the *dcw* cluster.

From these data, I conclude that the regulation of the two min cluster from P*mra* might have some effect on the growth of the triple mutant but it is not a critical factor for the cell division problems of MEW999.

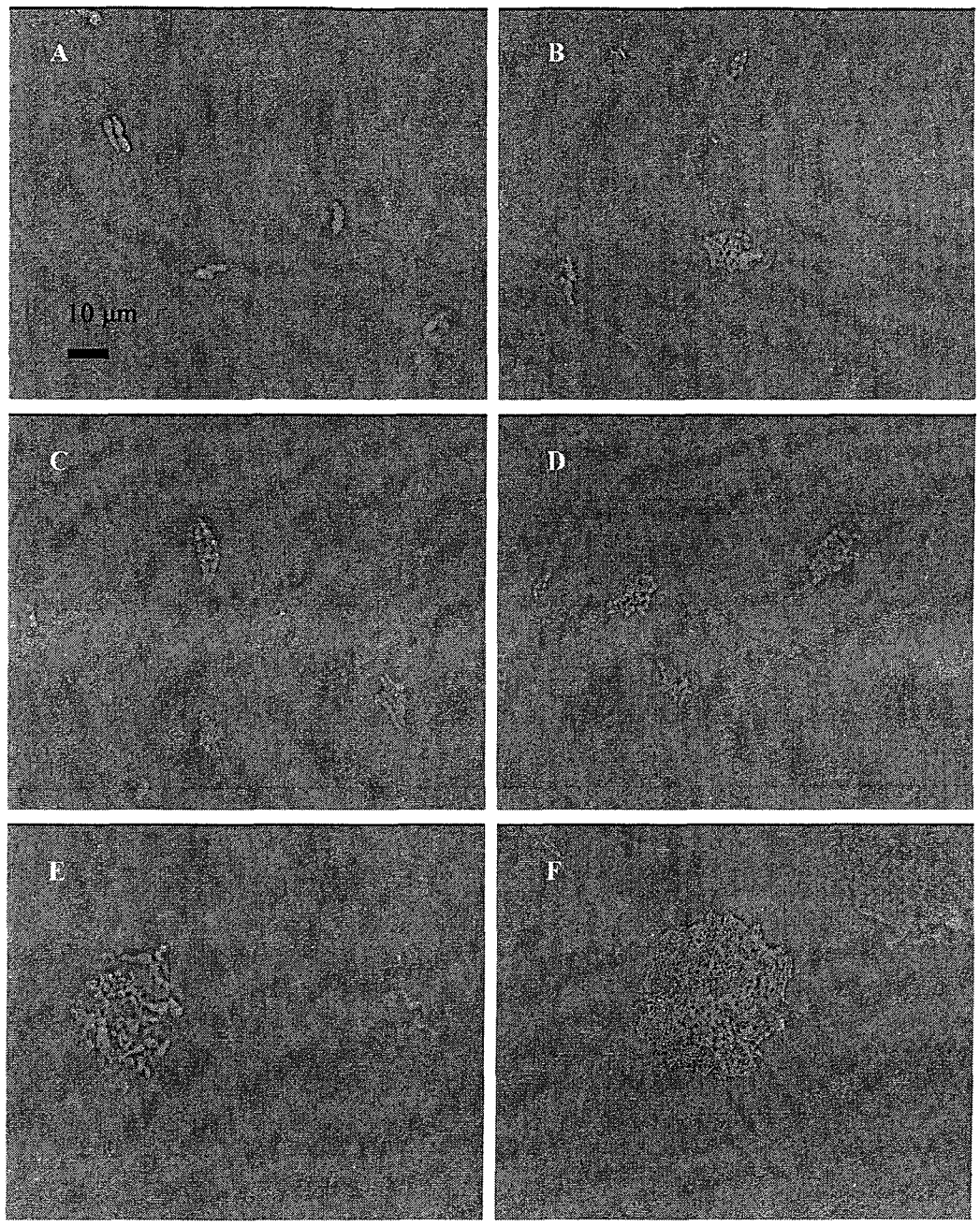


Figure 42 Micrographs of MEW999h on glucose minimal medium with casamino acids slide culture in the presence of IPTG.

Cells of strain MEW999h grown in liquid glucose minimal medium with 1mM IPTG and 25 µg/mL chloramphenicol were streaked on slide cultures with IPTG and 0.5% CAA and photographed after 3 (A), 5 (B), 6 (C), 7 (D), 9 hours (E), and overnight (F).

Chapter 4 Discussion

I constructed the L-SD coding gene triple deletion mutant MEW999 that lacks L-SD activity. The physiological study of this strain (MEW999) showed a cell division problem in rich medium LB or in glucose minimal medium with CAA. Further studies suggested that MEW999 is deficient in C1 metabolism. This deficiency likely causes cascade defects in DNA condensation, DNA segregation, cell division, cell wall synthesis and cell shape maintenance of the triple mutant cell growing in glucose minimal medium with CAA. In this discussion, the questions arising from the experiments are discussed in detail.

4.1 Creation of the triple mutant and study of its physiological property

Previous work with L-SD showed that no single one of the three L-SD coding genes was essential for the growth of *E. coli*. This suggested that it might be possible to construct a strain devoid of all three L-SD coding genes, allowing characterization of a strain totally deficient in L-SD, and investigating its physiological differences from the parent strain.

The availability of the the Keio collection (Baba *et al.*, 2006) made the triple mutant construction work much easier than it would have been using the traditional methods to delete the genes from the genome. The Keio collection includes deletions of all of the nonessential *E. coli* genes, and they are freely available from the Functional Genomic Analysis of *E. coli* in Japan and from the *E. coli* Genetic Stock Center at Yale University (Baba *et al.*, 2006). These deletion mutants were constructed in the *E. coli* K-12 strain W3110, which is similar to the parent strain MEW1 used in this lab. Further, the

construction of the Japanese strains allows for sequential transfers of deletions.

The use of these strains is not without pitfalls. The quality control on such a vast production scheme is good but not perfect. In particular, there are cases in which deletion of the gene from its usual site is accompanied by its insertion elsewhere in the genome (H. Mori, in discussion at IECA conference, 2008). However, the deletions are easily verified by PCR, and a lack of enzyme activity indicates that no active form of the enzyme is made. These verifications were included in this work.

The construction of strains MEW991 *ΔsdaB*, MEW992 *ΔtdcG* and MEW995 *ΔsdaB ΔtdcG* presented no particular problems. However, the *sdaA* gene deletion was very difficult to transduce. To solve the problem, I transformed the cells with an *sdaA* gene expression plasmid p*sdaA*, to complement the growth of the cell before I did the P1 transduction but this still did not help. Finally, the original P1 transduction worked, i.e., on a strain without p*sdaA*. Once the *sdaA* transduction worked for one strain, the same phage was used for other transductions and they all worked. I constructed the single, double, and triple deletion mutants with the *sdaA* gene deleted.

It is not clear why *sdaA* is difficult to transduce, especially since insertion mutants in *sdaA* have been transduced in our laboratory for years. The P1 phage titer on the *ΔsdaA* was not very different from that on any other strain I tested. Moreover the phage preparation that finally worked showed the same titer as the ones that did not! One can imagine that the methods or media presented problems but they worked for other transductions in the lab. In view of the growth problems later described for the triple mutant, it now seems reasonable that it might be hard to transduce because the recipient became osmotically sensitive. However, this would not explain the problems of the other

sdaA transductions.

The gene deletions were verified by PCR on chromosomal DNA (Figure 3). Two sets of PCR primers were designed for each gene. One set of external primers was designed according to the sequences upstream and downstream of the corresponding L-SD coding genes. The size of the three L-SD structural genes is ~1.4 kbp, so that the upstream and downstream primers were designed to produce a product with a total size around 1.9 kbp (including the L-SD structural gene) for the parent strain, and around 600 bp for deletion mutants. This allowed an unambiguous verification by PCR on agarose gels. I also used internal primers designed according to each L-SD structural gene sequence to give a band around 600 bp for *sdaA* and around 700 bp for *sdaB* and *tdcG*. Obviously, for the deletion mutants without the respective gene, no band would be produced after the PCR. All the single, double and triple deletion mutants used in this study were verified by PCR according to these criteria.

4.2 L-SD activity analysis

I showed that the strains constructed had the L-SD activity predicted from earlier studies. This was done by *in vivo* L-SD activity assays for all seven deletion mutants. As shown in Table 4, the L-SD activity of each strain in LB or in glucose minimal medium with glycine and L-leucine, was consistent with previous studies on regulation of expression regulation of the three L-SD coding genes (Burman *et al.*, 2004; Shao and Newman, 1993; Su and Newman, 1991; Su *et al.*, 1989).

With cells grown in glucose minimal medium, the deletion of the *sdaA* gene eliminated L-SD activity almost completedly. The three strains MEW990, MEW993 and MEW999, all of which carried *sdaA* deletions, showed no significant L-SD activity even

when grown with the inducers. This results from the fact that the expression of *sdaB* gene is under the regulation of CRP protein and cAMP, and thus cannot be expressed in the presence of glucose. MEW990 which carries only *tdcG* has no expression because *tdcG* is an anaerobically expressed gene (Hesslinger *et al.*, 1998). Furthermore, the *tdc* operon is also regulated by cAMP and the CRP protein, and so would be repressed in glucose minimal medium. On the other had, the deletion mutants which still carried the *sdaA* gene but lost one or both of the other L-SD coding genes did not show a deficiency in L-SD activity in glucose minimal medium with glycine and L-leucine, consistent with the view that *sdaA* was the only L-SD coding gene expressed under this condition.

Lastly cells carrying the *sdaA* deletion still showed L-SD activity when grown in LB, though less than the parent strain. This verifies that both *sdaA* and *sdaB* can be expressed in LB (Shao and Newman, 1993; Su and Newman, 1991).

4.3 Physiology study of triple mutant

The construction of the triple mutant was intended to elucidate the role of L-serine deamination in the cell. The drastic effects on cell function were surprising as discussed below.

4.3.1 Cell division deficiency of the triple mutant in glucose minimal medium with CAA

MEW999 is extremely sensitive to the presence of CAA in glucose minimal medium. The strain grew normally in glucose minimal medium. However with 0.5% CAA added to the medium, the cells became not only longer (filamenting) but also wider (swelling) which is much more rarely seen in growing *E. coli*. The usual uniform rod shape was

replaced with a variety of irregular shapes. Many of the misshapen cells lysed, indicating problems with membrane and/or cell wall synthesis.

The triple mutant MEW999, when grown in the presence of amino acids, does not divide when it doubles in length (Figure 7). In its first divisions, it divides only after reaching more than double its length and thus increases in length at each division. On a solid medium, the cells maintain a shape which at least resembles the parent cell. In liquid, or on wet slide cultures, the cells enlarge more, and take on very diverse shapes, suggesting that they may have osmotic problems, which is also indicated by the large number of cells which lyse. It seems that the larger the cell gets, the more difficulty it has in maintaining its regular form.

This can be explained simply if cell division is normally triggered by an L-SD dependent mechanism at 40 minute intervals in glucose-CAA medium. Without L-SD, cells may lack the usual metabolic or structural septation signal. The division machinery might assemble more slowly, or the cells might simply grow until a backup mechanism is triggered. In either case, the cells would continue to enlarge. Were this to continue, the cells would presumably reach a size at which they could not function. However, though the cells look moribund after a few generations, the many cells which survive in fact suddenly begin to divide more rapidly, and to reduce in size. I attribute this reversal to changes in the concentration of some critical (but as yet undetermined) medium component(s).

The reduction in size takes place rapidly- perhaps more rapidly than *E. coli* can usually divide (Figure 8). This suggests that the large cells had produced some or all of the machinery and organization to divide more than once well before they were able to

actually divide. The resultant cells, smaller even than the parent cells grown in the same medium, accumulate at the periphery of microcolonies (Figure 9). This might be due to inhibitory metabolites formed within the microcolony, or, more likely, to motile cells finding the path of least resistance.

4.3.2 L-SD deficiency leads to larger than normal, and smaller than normal, cells

The large cells which quickly accumulate in the presence of CAA are viable, but lyse easily, suggesting that the cell wall is weakened. The problems of the cell wall are confirmed by the sensitivity of the cells to dilution and the formation of protoplasts in protective media (Figure 10). The triple mutant likely has a high internal L-serine concentration which may affect the cell in many ways.

The weakening of the wall might be due to a direct effect of L-serine on D-alanine metabolism, such that L-serine or a derivative might interfere with D-alanine synthesis or D-alanine D-alanine ligase.

In any case, the cells in liquid medium are bigger and often swollen and show a bewildering variety of sizes and shapes somewhat reminiscent of the oddities described in elegant reviews of cell shape (Young, 2003, , 2006). Moreover, unlike cases in these reviews, this alteration in form affects the entire population. The osmotic protection by solid medium is particularly dramatic in moist slide cultures where we see monster cells (Figure 9A) in moist areas, and more regular ones (Figure 8) in drier areas. Agar is thought to support the growth of L-cells, though it may not be strictly necessary (Joseleau-Petit *et al.*, 2007).

It is also surprising that *E. coli* can organize its metabolism even in these very odd-looking cells. Most are able to produce progeny. Moribund though the cells appear, after

five hours of increasing size, the large cells (grown with liquid or solid media) suddenly altered their growth and rapidly produced small cells (cf.Figure 8). The rate at which this occurred was often much faster even than the usual growth rate of *E. coli.* This suggests that the large cells had produced some or all of the machinery and organization to divide more than once well before they were able to make their first division.

It is clear that a very small change in the metabolic armament of *E.coli* changes its function dramatically, and that the usually efficient *E. coli*, dividing regularly making exact copies of itself, can also form astonishingly irregular cells which are nonetheless still viable.

4.3.3 The growth difficulty of MEW999 was much more alleviated in LB

The triple mutant has much less trouble with amino acids when other components found in yeast extract are also provided, i.e. in LB. It cannot divide but otherwise its metabolism functions more or less normally. Its biomass increases but it does not swell, instead lengthening into filaments which are not subject to lysis. This suggests that the cell wall problems are avoided by some components in LB but the division problems are not. Of course, the effects of both LB and amino acids depend on the presence of L-serine, but L-serine is not sufficient in itself to cause these problems.

When MEW999 was subcultured into LB to assay the growth curve. The turbidity of the MEW999 culture increased more slowly than that of the parent strain, even after the culture entered log phase. Colser examination in a light microscope showed most of the cells was filamenting. The apparent doubling time of the triple mutant in LB was about 84 min, close to three times longer than the parent strains. Although the cell forms filaments at the early growth stage, the stationary phase culture was composed of normal

small rod-shaped single cells. This means that the cells have division problems at the beginning of growth in LB, and these problems were overcome later so that the long filaments are subdivided into single cells.

I suggest two possible explanations for this phenomenon. The first hypothesis is that the medium contains some chemical which blocks the division of MEW999. As the cell mass gradually increases, the chemical is consumed by the cells thus detoxifying the medium, permitting the filaments to subdivide into single cells. A second possibility is that the cells produce a product required for cell division that is secreted into the medium. As the cell mass increases, the concentration of the chemical reaches a point that allows normal cell division. This may be thought of as a form of quorum sensing.

The first explanation seems more likely. The L-SDs are deleted from the genome so that the cell cannot deaminate the L-serine in the medium, therefore, the most possible reason for the filamentation of the triple mutant is that the L-serine in the medium is essential (though not sufficient) to block the cell division. This is supported by the fact that addition of L-serine (600 ug/mL) to LB delayed the break up of the filaments (Table 5). The fact that when more cells are inoculated, recovery from filamentation is faster (Figure 5) also suggests that the medium is detoxified, but does not provide information as to the nature of the toxic compound.

4.3.4 L-serine toxicity

L-serine is known to be toxic to the cell (Cosloy and McFall, 1970; Uzan and Danchin, 1978). One reason for inhibition is that L-serine inhibits homoserine dehydrogenase and thus prevents L-isoleucine biosynthesis (Hama *et al.*, 1990). This is not involved in the present work since isoleucine is provided to all cultures to counteract the *ilvA* deletion

carried by our strains. The only other specific inhibition described is an inhibition of aromatic amino acid biosynthesis (Tazuya-Murayama *et al.*, 2006).

L-serine toxicity is not apparent until the concentration of the exogenous L-serine is quite high in the medium. Su showed that adding 2 mg/mL L-serine to glucose minimal medium, did not alter the doubling time of strain MEW1. However when 10 mg/mL L-serine was provided to the cells, the doubling time increased from 58 min in the glucose minimal medium to 89 min (Su, 1991).

As shown in Figure 12, the triple mutant is much more sensitive to L-serine than the parent strain. The highest concentration of L-serine on this gradient plate was 600 μg/mL which is not toxic to strain MEW1 (Su, 1991). This suggests that without L-SD activity, the strain is more sensitive to L-serine. The intense effects described in results section 3, may be due to a combination of L-serine with glycine and a variety of other amino acids.

4.3.5 Dilution factor of subculture is important to show the cell division problem

The cell division problem of the triple mutant became apparent when a high enough dilution 10^5 was used for subculture. This turned out to be critical to show the cell division inhibition efficiently especially in rich medium LB which affects the cells much less than glucose minimal medium with CAA.

This is probably to be expected if the inhibition is due to an exhaustible component of the medium, L-serine and/or whatever else. If there were too many cells subcultured, the relevant material might be exhausted before the inhibition could be established. A higher dilution exaggerates the effect of L-serine on a single cell and also prolongs this effect so that the phenotype can be observed clearly and easily.

It is also worth noting that in most of the physiological experiments done in this lab, in

the last decades, and elsewhere, overnight cultures were subcultured to midlog phase in order to assay something or other. If I had done the same with this mutant, I would not have noticed the problems in LB glucose, and perhaps not those in CAA either.

4.4 C1 metabolism deficiency in MEW999

I concentrated on the cell division deficiency of MEW999 in glucose minimal medium with CAA because the effects on the physiology are overwhelming and easier to study than the lesser changes seen in LB. Furthermore, compared to the rich medium LB, CAA in glucose minimal medium is much easy to mimic and decipher.

4.4.1 Six amino acids involved in inhibition of MEW999 growth

When the triple mutant MEW999 was subcultured into glucose minimal medium with L-serine, it had a long lag in growth but its morphology was not affected. This suggests that L-serine alone does not induce deformed cells. The 20 minus one amino acid experiment showed that another five amino acids, glycine, L-leucine, L-methionine, L-lysine, and L-threonine were also involved in causing the cell division problems of MEW999. The metabolic pathways for these five amino acids except L-leucine are shown in Figure 1 (L-serine and glycine) and in Figure 43 (L-methionine, L-threonine and L-lysine). L-leucine is involved in this process as a co-regulator of the Lrp protein.

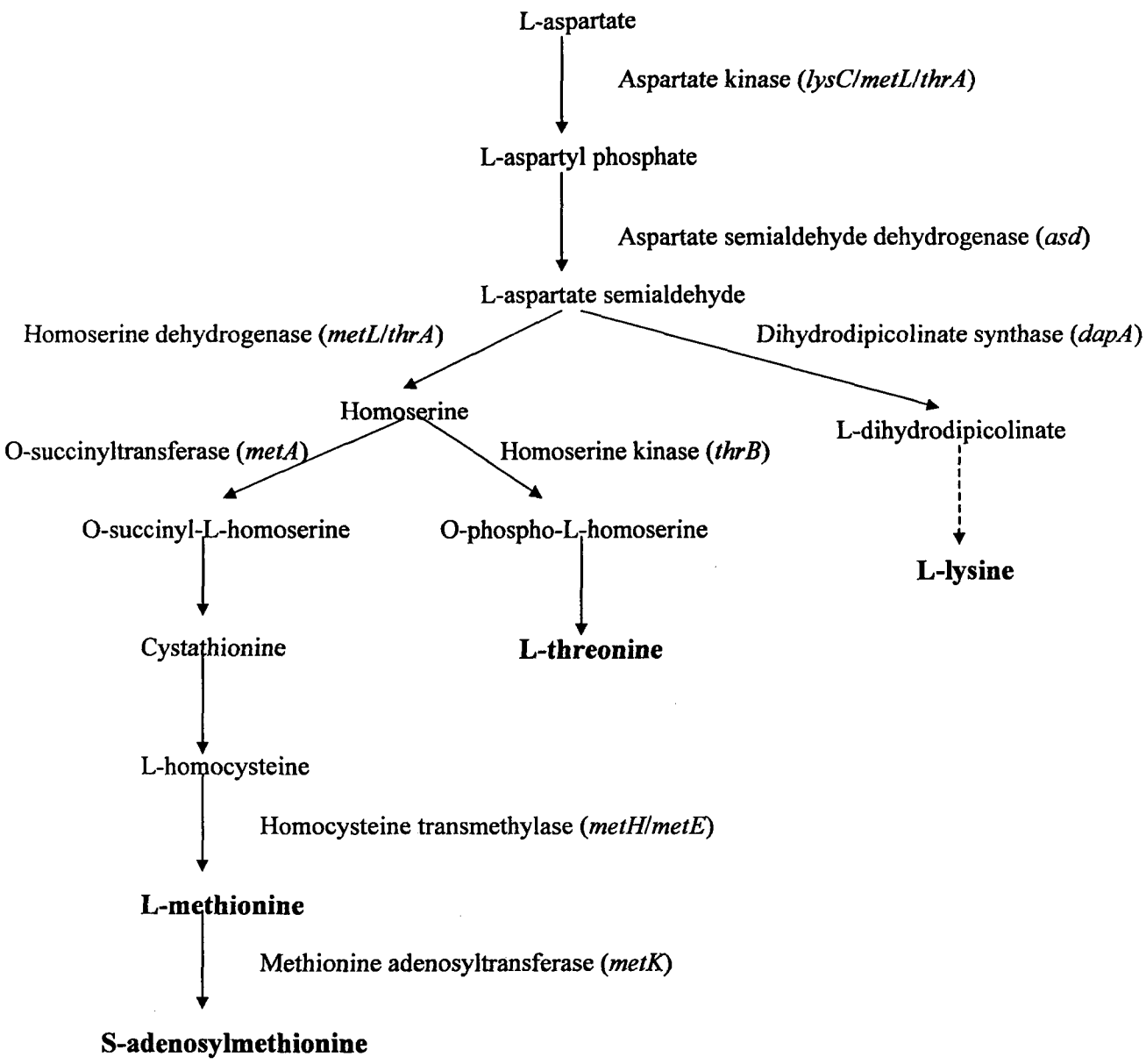


Figure 43 L-methionine, L-lysine, L-threonine and S-adenosylmethionine biosynthesis pathways in *E. coli* K-12

Adapted from Ecocyc (Keseler *et al.*, 2005). The dashed arrows indicate the enzymatic reactions which need more than one enzyme.

4.4.2 Cell division deficiency of MEW999 is related to deficiency in C1 unit production

Four pieces of evidences suggest that L-SD deficiency caused a deficiency in C1 units.

First, the six amino acids most involved in the distortion of MEW999 growth in glucose minimal medium are all involved in C1 metabolism directly or indirectly. L-serine and glycine are the main donors of C1 units in *E. coli*. L-lysine, L-threonine and L-methionine itself are involved in the regulation of the L-methionine synthesis pathway, while L-methionine is the precursor of SAM. L-leucine is the co-regulator of Lrp which is essential for the expression of the *gcv* operon, and also regulates L-serine and glycine biosynthesis (Keseler *et al.*, 2005).

Secondly, addition of SAM alleviated the growth problems of strain MEW999 caused by CAA or LB. Addition of SAM to CAA resulted in the triple mutant making long filaments instead of bulgy cells. In LB, the effect of SAM was even more dramatic as adding SAM to LB rescued the division problems of MEW999, allowing the cells to multiply as small rod-shaped units just like the parent strain.

The third and perhaps the most important observation, is that expression of a plasmid carrying the *gcv* operon rescued all of the cell division problems. Strain MEW999 pGS146 grew well from the start of the subculture into glucose minimal medium with CAA, with no sign of filaments, deformed cells or lysis.

The importance of *gcv* activity is also indicated by the fact that hypoxanthine causes total inhibition of growth when provided L-serine in glucose minimal medium (Figure 17). With just hypoxanthine (40 μg/mL) or L-serine (300 μg/mL), the triple mutant can grow as well as the parent strain on the plates. When hypoxanthine was provided with L-

serine, a dramatic growth repression was observed for the triple mutant but not for the parent strain. Expression of both the *glyA* and the *gcv* operons are regulated by the PurR-hypoxanthine transcriptional repressor (Matthews, 1996). Addition of hypoxanthine decreases production of both the SHMT and GCV enzyme systems, and thus restricts the synthesis of C1 units. The presence of L-serine causes this phenotype because a high internal L-serine concentration decreases SHMT expression and synthesis.

4.4.3 A tentative biochemical explanation as to how L-SD deficiency might lead to a deficiency in C1 units.

In glucose minimal medium, *E. coli* relies on a single pathway for L-serine biosynthesis from phosphoglycerate (using the *serA*, *serB* and *serC* gene products), and a single pathway for producing glycine (from L-serine using SHMT) (Figure 1) (Newman and Magasanik, 1963; Pizer, 1963). C1 units can be made either from L-serine cleavage to glycine, or from glycine cleavage by the GCV enzymes which produces C_1-THF from glycine. Threonine conversion to glycine is also possible, and is induced by leucine (Fraser and Newman, 1975). These enzyme reactions are regulated so as to permit either L-serine or glycine to be the precursor of the other.

This system might break down in the presence of exogenous amino acids when L-serine deamination cannot occur and other amino acids are also present. Glycine inhibits SHMT (Miller and Newman, 1974) so that the cell relies more heavily on the GCV enzymes for its C1 synthesis when glycine is present. Transcription of the GCV operon is regulated by a variety of factors (Stauffer and Stauffer, 1994). For instance, the GCV system depends on the Lrp and is virtually unexpressed in the absence of Lrp (Lin *et al.*, 1990). Lrp concentration is much lower in LB than in minimal medium (Lin *et al.*, 1992).

If a very high internal L-serine pool also results in the inhibition of glycine cleavage, the cell would find itself starved for C1-THF and SAM when faced with an external supply of amino acids which included both L-serine and glycine.

Uzan and Danchin (1978) described a temporary arrest in growth produced by a mixture of L-serine, methionine and glycine. The same mixture totally inhibited growth of a *relA* mutant. This was ascribed to isoleucine starvation which cannot be a problem here since the strains in the Newman laboratory carry a deletion in *ilvA* and so are always grown with isoleucine in excess (to avoid L-serine deamination by the *ilvA*-encoded threonine deaminase). Shape or division phenotypes were not described in that work. Nonetheless, it seems that these phenomena must in some way be related to those described here even though our strain is not a *relA* mutant.

4.4.4 Possible explanation for SAM deficiency in MEW999

As shown in results section four, SAM can alleviate deformation of MEW999 cells due to CAA, though it cannot prevent filamentation, and it has even dramatic effect on the growth of MEW999 in LB by correcting the entire growth deficiency problem.

SAM is made from L-methionine, which is made from L-aspartate, which is also the precursor of L-lysine and L-threonine (Figure 43). The first step is common to all three pathways, the conversion of aspartate to aspartyl phosphate, which is carried out by three homologous enzymes, the three L-aspartate kinases, coded by the genes: *thrA*, *metL* and *lysC* (Cohen, 1969). Each of the aspartyl kinases is inhibited by the corresponding endproduct, threonine, methionine and lysine. The aspartyl phosphate so formed is converted by aspartate semialdehyde dehydrogenase to aspartate semialdehyde, the last compound which is common to the three pathways and this can be acted on by any of the

three enzymes. The biosynthesis of lysine diverges at this point, and takes aspartate semialdehyde to dihydrodipicolinate, the first specific step in lysine biosynthesis, and this step is inhibited by lysine. If dihydrodipicolinate synthase (DapA) was overproduced and overactive, the cell would be starved of methionine and threonine.

The biosynthesis of methionine and threonine can pose reciprocal problems for lysine synthesis. Aspartate semialdehyde is converted in both cases to homoserine, using the *metL* and *thrA* gene products, which also catalyze the conversion of aspartate to aspartyl phosphate. Addition of threonine when it inhibits aspartyl kinase, thus stops production of homoserine too. The same is true of methionine. Thus, in cells given methionine and threonine, aspartate semialdehyde derives mainly from *lysC* and is directed to lysine biosynthesis.

The complications do not end there. Homoserine is the precursor of both methionine and threonine. The first step in the methionine pathway is inhibited not only by methionine but also by SAM. Provision of SAM thus inhibits formation of methionine. Provision of methionine inhibits its own biosynthesis but neither SAM nor methionine interferes with threonine biosynthesis, nor does threonine interfere with methionine and SAM synthesis.

From this one can see that a combination of lysine, methionine and threonine, would prevent formation of aspartyl phosphate and homoserine. However, this should not present any difficulty for the cells because the endproducts are present and the biosynthetic pathways are not needed. Eliminating any one of the three amino acids from the 20-amino acid mix would remove the inhibition of one of the aspartate kinases. This would allow aspartylphosphate to be made, but it is not clear why this would help the

triple mutant.

Removal of threonine or methionine would also remove an inhibition on homoserine dehydrogenase, so that the aspartyl phosphate made could be diverted to homoserine. Increased availability of homoserine could increase either threonine or methionine synthesis depending on which was present.

Both SAM and methionine inhibit the first step in methionine biosynthesis and SAM starves the cell of methionine (Reyes, 2005). Methionine is made by the methylation of tetrahydrofolic acid, which might also vary depending on the amino acid composition of the medium.

Intuitively methionine, threonine and lysine are likely involved in the regulation of C1 and SAM biosynthesis. This hypothesis is supported by my finding that omitting any of the three somewhat counteracts the effects of amino acids on the triple mutant. However it is difficult to specify exactly how this works.

The fact that adding SAM allows the triple mutant to grow (more) normally suggests that the SAM supply is insufficient in the triple mutant.

From another point, a combination of six amino acids causes the mutant difficulties in cell division (Figure 13). Removing any of L-serine, glycine, threonine, methionine, lysine or leucine from a mixture of the six allows normal growth. To synthesize methionine, cell needs methylTHF, which would be in short supply in the triple mutant. If this resulted in the conversion of methionine to methylTHF, the strain might be starved for SAM. However, this should be alleviated by providing more methionine. Alternatively, if the SAM synthetase was inhibited to some extent, the mutant might not synthesize SAM as efficiently as the parental strain and it might need a higher L-

methionine concentration to push this enzymatic reaction. The concentration of amino acids in this test was 100 ug/mL for each. It could be that at this concentration, there might be little methionine to make SAM, but more than enough to inhibit further methionine synthesis. Omitting any one of these three amino acids could release the inhibition, thus giving the cell enough L-methionine for SAM synthesis.

4.5 The metabolic function of L-SD

In most databases like Eco-cyc, L-SD is considered to be involved in energy provision, a role for which there is little evidence. Though the conversion of L-serine to pyruvate is an obvious first step in the use of L-serine as a carbon and energy source, *E. coli* K-12 does not actually use L-serine as the sole carbon source (Newman and Walker, 1982). Deamination could also serve as a bypass of the Embden-Meyerhof pathway (phosphoglycerate to L-serine to pyruvate) but in fact it does not (Newman and Walker, 1982).

The work in this thesis suggests that rather than usng L-serine as an energy provider, L-SD, together with L-serine, could be a monitor of the state of cell metabolism. This is the first demonstration of a metabolic role for the 4Fe-4S L-SD. I hypothesize that L-SD is a defense mechanism used by *E. coli* to grow in the presence of amino acid mixtures containing L-serine and glycine along with other amino acids- particularly methionine, lysine, leucine and threonine. Indeed L-serine deamination is sufficiently important for the cell to code for three homologous L-serine deaminases and to regulate them so as to have at least one present in any environment. I suggest that L-serine inhibits some metabolic reaction which is essential to amino-acid challenged cells. This does not cause problems for the parent cell which rapidly deaminates it. Indeed in *E. coli* cultures

growing in LB, L-serine and L-alanine are the first amino acids to be used (Pruss *et al.*, 1994).

The production of C1 units is important for cells. N_5, N_{10} methyleneTHF, the major source of C1 units in the cell, is involved in the biosynthesis of purines, thymidine, methionine, choline and lipids (Keseler *et al.*, 2005). The methylation reaction is involved in many important metabolic processes, so that the concentration of C1 units in the cell can be used as a sign of the metabolic period or metabolic requirement to the cell. L-serine is a good choice for a metabolic signal for the following reasons: first, L-serine is the only precursor for glycine biosynthesis, so that whatever controls the concentration of L-serine subsequently controls the concentration of glycine. Second, it is the biosynthetic precursor for L-cysteine, L-methionine, L-tryptophan, glycine and phospholipids (Pizer and Potochny, 1964). As L-serine concentration varies, so will these compounds, influencing the reactions in which they are involved.

The cell requires a lot of L-serine. However, a high concentration of L-serine may present the cells with problems. This is probably the reason why all the three L-SDs have a very high Km (2.5-5 mM),(Burman *et al.*, 2004; Cicchillo *et al.*, 2004), which assures that the intracellular L-serine pool will not be depressed to detrimental levels. This concentration ensures that normal cell metabolic activities such as protein synthesis, C1 metabolism, and the synthesis of other products from L-serine are carryied out normally. When the cell accumulates too much L-serine, L-serine inhibits some reactions (probably the GCV enzyme system in particular) and slows down cellular metabolism. In the parent strain, L-SD would react to this high intracellar L-serine concentration and degrade L-serine to ensure the fluent integration of cell metabolism. In summary, an attractive

hypothesis is that L-SD functions in metabolic defense.

4.6 L-serine deamination deficiency and cell division

High L-serine concentration could result in a shortage of C1 units may be due to inhibition of SHMT by glycine and of GCV by higher L-serine. However, it is not clear why high intracellular L-serine, and C1 deficiency, should interfere in particular with *E. coli* growth and cell division, resulting in an unusual variety of sizes and shapes of *E. coli* cells. Since an exogenous supply of the cell's major methyl donor, SAM, overcomes many of the problems of the triple mutant, I suggest that cell division is dependent on one or more methylation reactions, and the starvation for C1 units that results from high intracellular L-serine levels prevents these methylation reactions from occurring.

This hypothesis is consistent with earlier reports from this lab that a deficiency in SAM results in the formation of long filaments (Newman *et al.*, 1998; Wei and Newman, 2002) in which only FtsZ, FtsA and ZipA are found in the septum (Wang *et al.*, 2005). Of course the hypothesis is also consistent with the results presented here that SAM prevents filamentation in LB glucose and the formation of large deformed cells in glucose minimal medium with CAA.

4.6.1 Misshapen cells do not produce condensed DNA

As shown in Figures 7 and 8, MEW999 has two growth stages on the slide with glucose minimal medium agar with 0.5% CAA. For the first five hours, the cell has a generation time of one hour and forms large deformed cells. Thereafter, for the following two hours, the cells divide quickly in some cases with an apparent doubling time of about 20 min. DAPI stained images of cells incubated for four hours (Figure 20) showed that

cells had unsegregated DNA which had dispersed throughout the cytoplasm. However, by eight and 11 hours (Figure 21), many long filaments showed multiple areas of unevenly segregated DNA. This result suggests that loss of L-SD interrupts the chromosome assembly system in MEW999. In *E. coli*, usually for the entire cell cycle, the chromosome is highly folded and compressed into a nucleoid that occupies less than 25% of the intracellular cell volume (Zimmerman, 2006). It has been suggested that the DNA is much more diffuse in cells that are committed to lysis, as during autolysis (Zimmerman, 2006). The fact that the DNA is diffuse in cells of strain MEW999 could then be related to their tendency to lyse.

Several factors considered to be involved in DNA condensation are DNA supercoiling, the concentration and nature of nucleoid-associated proteins and the total mass of cytoplasmic material including ribosomes, mRNA and whatever else (Woldringh and Nanninga, 2006). The deficiency of DNA condensation in MEW999 might be due to problems with any of these factors as well as, changes in RNA polymerase, DNA-associated binding proteins, macromolecular crowding, supercoiling, concentration of polyvalent cations, and confinement by the cell envelope (Zimmerman, 2006). It was also reported that exposure to chloramphenicol could cause the fusion of otherwise separated nucleoids in *ftsZ* filaments into single nucleoids. The effect of chloramphenicol might be due to its inhibition of protein synthesis and decrease in the concentration of DNA-binding proteins. Of course, chloramphenicol is not added during the experiments described here. However, any other factor which affected the supply of nucleoid-associated proteins might result in dispersion of the DNA.

Zimmerman and Murphy (1996) suggested that the huge number of macromolecules

in the cell (about 340 mg/mL of RNA and protein) generated a macromolecular crowding force that pushes the DNA into about 20% of total cell volume. If that is true, perhaps the concentration of the macromolecules in the much larger deformed cells of MEW999 could be lower than in the normal *E. coli* cell. The DNA might then not become compact, but it is in any case distributed along the filament on either side of developing septa. This suggests that even uncondensed DNA can be segregated.

A deficiency in C1 units might result in problems of DNA methylation. However, there appears to be no DNA methylation deficiency detected in the cells of MEW999 as judged by restriction enzyme digestion (Figure 22).

A wide range of prokaryotic and eukaryotic proteins are methylated, including histones, actin, ribosomal proteins, translation factors, heterogeneous nuclear ribonucleoproteins (hnRNPs) and translation factors (TFs) (Clarke, 1993; Paik and Kim, 1990). Loss of methylation at a specific protein (though as yet unknown) might influence DNA folding. Furthermore, inefficiency in assembling the ribosome due to the ribosomal protein methylation deficiency might cause not only growth problems but also a low macromolecular mass in the cytoplasm, a direct consequence of which would be an inadequate crowding force driving DNA compacting.

Whether DNA replication initiates or continues normally in the triple mutant is unknown. However, since most of the misshapen cells can subdivide into smaller cells in a very short period after five hours on the slide culture (Figure 8), it is very likely that the DNA replicated normally. The rapid production of small cells may represent the rapid partitioning of previously synthesized DNA. This would be similar to the demostration of Van Helvoort *et al.* (1998),that the fused nucleoids in filaments of *E. coli pbpB* (Ts)

generated by treating cells with chloramphenicol resegregated after the removal of chloramphenicol. Therefore, the segregation of DNA can disconnect from cell elongation and division, with cell division largely postponed until resegregation had taken place. Thus, this is a possible explanation for the rapid subdivision of the MEW999 filaments. The chromosomal DNA of strain MEW999 can be replicated during the slow growing stage, but the DNA cannot be condensed, or segregated. Thereafter, when the inhibitory condition due to the medium is overcome, the DNA can be segregated quickly, though still uncompacted, and the long filaments can divide into many single cells.

4.6.2 DNA segregation deficiency of MEW999

It is not clear whether DNA segregates correctly in the filaments. A septum forms at a point where there is no DNA, leaving uncondensed DNA on either side. The septum may be complete enough to prevent further movement of DNA across it. Alternatively, the septum may be prevented from completing due to the failure of the DNA segregation.

In *E. coli*, the structure of DNA is influenced by a variety of different binding proteins. MukB is one of the earliest proteins considered to be involved in the establishment and/or maintenance of *E. coli* DNA structure. When grown in glucose minimal medium supplemented with 0.4% CAA, some *mukB* mutants are temperature sensitive and generate cells with decondensed and improperly arranged chromosomes and anucleate cells during exponential growth (Niki *et al.*, 1991). This is very similar to the phenotype of the triple mutant in the same medium with a slightly different CAA concentration (i.e. 0.5%). However, the triple mutant is not temperature sensitive and does not produce as many anucleate cells as the *mukB* mutant.

The problems of strain MEW999 might involve the malfunction of several factors.

Histone-like proteins HBsu, and HU, IHF and H-NS which interact with the nucleoid could play an role in the chromosome diffusion in MEW999 cell too (Guo and Adhya, 2007; Nash, 1996).

4.6.3 Assembly of the Z ring in the triple mutant cells

In *metK84* filaments, the cell division septum formed only partially (Wang *et al.*, 2005). To compare MEW999 to this, I constructed the merodiploid strain MEW999 *ftsZ-gfp*. According to the GFP images at four hours (Figure 25), the FtsZ-GFP protein can be observed in 85% of the cells examined. This suggested that at least the FtsZ protein could be expressed and localized in the presence of CAA. However, while the *metK84* filaments had evenly distributed FtsZ rings, the Z rings formed in the big cells of MEW999 were totally irregular. Variously formed rings were observed anywhere along the length of the cell and 89% of the rings were assembled at the sites devoid of DAPI-staining material (Figure 24-30). That is, even when the DNA did not condense properly, it prevented the formation of FtsZ rings. Nucleoid occlusion did not depend on (all) the DNA being condensed! This is consistent with my observation of nucleoid occlusion in both misshapen cells and filaments.

In *E. coli*, nucleoid occlusion ensures the cell center and the cell poles competent for Z ring formation (Graumann, 2007). However, the Z ring does not usually form at the poles, likely due to a second form of regulation by the Min system (Hu *et al.*, 1999). Among triple mutant cells expressing FtsZ-GFP, I found 22 which did not have DNA at its poles. 18 of 22 had GFP at the poles, perhaps indicative of ectopic septa. This may indicate that the Min system does not work properly in the deformed triple mutant cells. MinCD oscillates between the cell poles in *E. coli* inhibiting division at the poles while allowing

it to occur at midcell (Lutkenhaus, 2007). However, *minCDE* are nonessential genes of *E. coli*. The *min* mutant still can divide though it produces small anucleate minicells from the poles of the mother cells. No minicells were observed during the growth of MEW999. All of the cells examined had chromosomal DNA. That is, even if there were FtsZ caps at the poles of the cell, these did not lead to cell division at those sites, suggesting the Min system is active.

Nonetheless, the MinCD system might not work efficiently in the large cells. The oscillation of MinCD has a periodicity on the order of ~40s at 20 °C (Raskin and de Boer, 1999b). When cell division is inhibited in a long filament, the oscillation of MinCD switches from a pole-to-pole pattern to pole-to-midcell and the maximum distance of the oscillation is about 7-8 μm (Lutkenhaus, 2007). In the huge and misshapen cells of MEW999, the oscillation could be delayed by the cell shape or the cell width, so that the FtsZ can polymerize at points where there is not (frequent) enough MinCD to inhibit it. Moreover, MinCD can disperse polymerized FtsZ if its concentration rises.

The *dcw* cluster at two min of chromosomal DNA of *E. coli* includes many essential genes involved in cell division and peptidoglycan synthesis. I created a cell in which these genes were expressed under the control of IPTG, and not under their usual promoter. If growth of the triple mutant in CAA decreased synthesis from the *mraA* promoter, that cell should not be sensitive to CAA, but it was. This shows that overexpression of the *dcw* cluster does not overcome the growth problems of the triple mutant. It also suggests that even if the triple mutant growth resulted in inhibition of *mraA*, this cannot be the sole reason for the triple mutant phenotypes.

4.7 Cell lysis and rod shape maintenance deficiency of MEW999

4.7.1 Osmotic sensitivity of the triple mutant cell

Cells of strain MEW999 showed a huge variation in morphology at various places on a slide culture. It seemed that this was related to the availability of water, cells growing in a wetter area forming much bigger and odder-shaped cells than those in a drier area. In particular, monster cells (Figure 9) were observed at the wetter but not drier areas. This suggests that MEW999 cells grown in glucose minimal medium with CAA were much more osmotically sensitive than normal cells. The filaments formed in LB were quite uniform in width but in CAA were swollen, and irregular and often huge, compared to normal *E. coli*.

While cells are known to lyse due to problems in their cytoplasmic membranes or cell walls, I hypothesize that the osmotic problems of the triple mutant are due to defective cell wall synthesis. *E. coli* cells exposed to penicillin will lyse in liquid medium unless protected by a medium of high osmotic pressure (20% sucrose), in which protoplasts form (Lederberg, 1956). These protoplasts are formed because the cell membrane is intact, but the cell wall is broken. A similar phenotype was observed when inactive PBP1B was overproduced in the wild-type cells (Meisel *et al.*, 2003).

Clearly, if the triple mutant cell wall was defective and the membrane intact, it should be possible to form triple mutant protoplasts. These were formed when I added 20% sucrose to a liquid culture of deformed cells produced after seven hours incubation with CAA. The culture then began to accumulate protoplasts. Presumably cells had already started to lyse, but those which began to lyse after sucrose was added were preserved as protoplasts. That is, cells incubated in glucose minimal medium with CAA form cells

with intact membranes and weakened cell walls. The protoplasts were also formed by plating the seven hours cells on the slide with a layer of glucose minimal medium agar with 0.5% CAA. Agar protected the cell membranes as the sucrose did.

4.7.2 FtsI localization and the lysing of the triple mutant cells

The product of the *ftsI* gene is a transpeptidase, which is involved in peptidoglycan biosynthesis at the septum site (Adam *et al.*, 1997; Bowler and Spratt, 1989; Nakamura *et al.*, 1983). In general, lysis of the large swollen cells formed in the CAA experiments took place at the widest diameter, despite the fact that over 94% of those cells have localized at least FtsZ-GFP to a septum at that point (Figure 31). Indeed the triple mutant can localize both FtsZ and FtsW without any problems, and therefore presumably everything in between. However, GFP-FtsI localization was different, the percent of cells with GFP-FtsI incorporated into the septal ring varied with time (unlike the other two which were consistently above 80%). At 4.5 hours, 95% of the cells examined had visible septal rings. This percentage dropped to about 35% at 10 hours. Furthermore, there were many visible intracellular clumps of GFP-FtsI, unassociated with any visible or known cellular structure (Figure 35, 36). These clumps were not observed with either the FtsZ-GFP and GFP-FtsW samples. It is clear that GFP-FtsI was made, but could not be efficiently recruited to the septum. The lack of FtsI in the septum might result in an unstable structure subject to lysis precisely at the septal sites.

4.7.3 Provision of SAM produces filaments with properly localized FtsI

Strain *metK84*, when starved of SAM, produced long filaments (Newman *et al.*, 1998), which could not recruit FtsK and later septal proteins into the septum (Wang *et al.*, 2005).

This was the first time that SAM was found to be involved in cell division.

The triple mutant forms huge cells in liquid glucose minimal medium when incubated with CAA, but if also provided with SAM, it instead forms long filaments. Without SAM, the cells assemble Z rings at potential division sites, even when this involves making an enormous Z ring with 10-20x the usual circumference. However, most cells do not incorporate FtsI, and instead precipitate it in clumps around the cytoplasm. This suggests that without SAM, the proteins up to and including FtsW can be incorporated, and FtsI cannot.

In the filaments made by MEW999/*gfp-ftsI* carrying the SAM transporter, there were no visible clumps of FtsI precipitated in the cytoplasm. The filaments looked clean with unevenly localized FtsI belts at the potential division sites. From this data, FtsI can sometimes be localized in SAM filaments, either because SAM prepares a site or because it prepares FtsI itself. SAM might relieve the inhibition of cell division in some other way so that FtsI can localize properly. The filaments formed with SAM were not obviously osmotically sensitive, but I did observe some lysis. One explanation is that SAM is easily degraded in the medium. SAM was added to the medium at the beginning of the incubation, and most of the SAM might already be degraded by the end of 10 hours.

When SAM was provided, the cells still could not subdivide. However, they were able to produce more complete septa. This seems to indicate that SAM, and presumably a methylation, is needed to produce a stage of the septum to which FtsI can be incorporated. This would mean that there are at least two methylation 'check point' in cell division, one after the addition of FtsZ, ZipA and FtsA, and one after ftsW and before FtsI.

The synthesis of peptidoglycan requires factors other than FtsI. PBP1 and PBP2 are

also involved in the synthesis and elongation of the cell wall. Even if SAM allows localization of FtsI to the septum, it might not suffice for the other two, or other proteins.

4.7.4 MEW999 cell shape

Although bacteria have different shapes, most of them keep a uniform and symmetrical profile. This is important for the distribution of DNA and cytoplasmic material between daughter cells at division (Errington *et al.*, 2003). Rod-shaped and spherical bacteria are most commonly seen in the environment and among these bacteria *E. coli* is the best-studied. In *E. coli*, many genes have been identified as being involved in rod-shape maintenance (Pichoff and Lutkenhaus, 2007). The most interesting and well studied of these include the *fts* genes required for septation and *mreB*, *mreC*, *mreD*, *rodA* and *pbpB* required for elongation.

The localization of inert peptidoglycan determines the gross shape of normal cells (Young, 2003). Many of the genes just mentioned code for products involved in the regulation of peptidoglycan synthesis. Among these genes *ftsZ* is the one directs the positioning of peptidoglycan-synthesizing enzymes at the septal ring (Carballido-Lopez and Errington, 2003; Kroos and Maddock, 2003). Aberrantly localized FtsZ will lead to a similarly incorrectly localized FtsI (penicillin-binding protein (PBP) 3), which in turn fails to direct peptidoglycan synthesis at the developing septum (Holtje, 1998; Nanninga, 1998). In this work, I saw many diverse forms of Z rings in the various misshapen cells in the swollen areas or near the sites of lysis (Figure 25-31). The aberrantly located Z ring interferes with the synthesis of functional inert peptidoglycan (iPG). It has been suggested that partial Z rings or incomplete FtsZ arcs could initiate iPG synthesis at abnormal positions and these spots of misplaced or extended iPG may develop into

aberrant protuberances and inflection points (Young, 2003). Many ectopic poles and inflection points were observed in the misshaped cells of MEW999 in glucose minimal medium with CAA.

It is also possible that C1 deficiency could decrease protein methylation and interfere with ribosome assembly. This could slow down protein synthesis, decrease the concentration of various enzymes, leading to a shortage of cell wall synthesis subunits and insufficient iPG synthesis. The inner cell pressure pushes the cell wall and the cell swells at the fragile cell division points. When the unhealthy peptidoglycan cannot resist the cytoplasm pressure, the cell will lyse.

The swelling of MEW999 cells usually does not happen at the poles. Thus most of the cells look like a spindle with two tapered poles and a swollen midsection. This is probably because that the polar peptidoglycan is either not recycled or is recycled at an extremely low rate after the cell division. So the stable peptidoglycan is very unlikely to be distorted by the inner pressure.

PART 2 IDENTIFICATION OF A MUTATION AFFECTING POST-TRANSLATIONAL ACTIVATION OF L-SERINE DEAMINASE

Chapter 5 Introduction

The activity of L-SD from *E. coli* K-12 has been studied extensively in the Newman laboratory. The enzyme is sensitive to air exposure and is unstable in both crude extracts and purified preparations. However, it was shown that inactivated enzyme can be reactivated by incubation with iron and dithiothreitol (DTT) under aerobic conditions *in vitro* (Newman and Kapoor, 1980; Newman *et al.*, 1985a).

Two mutants MEW128 and MEW191 in the Newman laboratory are known to be physiologically devoid of L-SD activity and unable to grow with L-serine, glycine and leucine as carbon sources in minimal medium (Newman *et al.*, 1985b). However, crude cell extracts do show considerable activity when incubated with iron and DTT (Newman, personal communication). This suggests the existence in the cell extract of an inactive form of L-SD, which can be activated by iron and DTT *in vitro*. This implies that L-SD is synthesized in a form which requires post-translational activation modification. Alternatively, it is made in an active form that is easily inactivated. In either case, the inactive protein can be activated enzymatically *in vivo*, by an unknown mechanism, or it can be activated chemically *in vitro* by incubation with iron and DTT (Cicchillo *et al.*, 2004; Newman *et al.*, 1985b).

This part of the thesis reports on my studies of one of the two mutants, MEW128. I have identified the site of the relevant point mutation in the chromosomal DNA of strain MEW128. The possible function of the mutated protein in the post-translational

modification of the L-SD protein was also studied. Here I will review studies on the iron-sulfur proteins and their activation.

5.1 The function of Iron-sulfur clusters

Iron-sulfur clusters are simple inorganic prosthetic groups that are present in proteins among all organisms, and are probably one of the earliest iron cofactors used by living organisms (Beinert *et al.*, 1997; Beinert, 2000; Wachtershauser, 1992). Fe-S clusters were first noticed by researchers because of their involvement in nitrogen fixation. The metallocluster of nitrogenase in *Azotobacter vinelandii* is essential for enzyme function (Yuvaniyama *et al.*, 2000). The major functions of Fe-S clusters have been reviewed by Johnson *et. al.* (2005). There are three main roles of iron-sulfur clusters in metabolism. First, because the electron density over both the iron and sulfur atoms can be delocalized, electron transfer is a major function of Fe-S clusters. Different Fe-S clusters consist of iron and elemental sulfur at various molar ratios and have physiologically relevant redox potentials. Secondly, Fe-S clusters are also found in the substrate binding sites of enzymes and are involved in substrate binding and activation in dehydratases and radical-S-adenosylmethionine enzymes. Thirdly, Fe-S clusters are involved in gene regulation at both the transcriptional and translational levels as "molecular switches". Apart from these three major roles, the Fe-S cluster in some Fe-S proteins such as ferredoxins in *Clostridia* and many other anaerobic bacteria and archaea can also serve in iron storage. The involvement of Fe-S clusters in the regulation of enzyme activity has also been documented. Glutamine phosphoribosylpyrophosphate amidotransferase can either be activated by assembling a [4Fe-4S] cluster or be deactivated by degrading the Fe-S cluster by O_2 with concomitant proteolysis.

The most common cluster forms are [2Fe-2S] and [4Fe-4S]. The simplest Fe-S proteins, such as rubredoxins, contain a [2Fe-2S] cluster coordinated to proteins by four cysteine residues. Other forms of Fe-S cluster include [2Fe-2S], [3Fe-4S], [4Fe-4S], [7Fe-8S], [8Fe-8S] clusters (Beinert *et al.*, 1997; Beinert, 2000). In *E coli*, about 90% of the [Fe-S] proteins are of the [4Fe-4S] type (Fontecave and Ollagnier-de-Choudens, 2008).

5.2 Fe-S cluster assembly pathways in *E. coli*

The *nif*, *isc* and *suf* operons encode proteins essential for Fe-S cluster biosynthesis, are conserved universally in all organisms (Johnson *et al.*, 2005).

The *isc* operon was the first site of genes identified as responsible for Fe-S cluster biosynthesis, by facilitating Fe-S cluster assembly into nitrogenase in *A. vinelandii* (Zheng *et al.*, 1998). It is also the major system for Fe-S cluster assembly in *E. coli*. This system is not limited to prokaryotes but is also found in the mitochondria of yeast and higher eukaryotes (Lill *et al.*, 1999).

In *E. coli* the Isc complex is the main builder of Fe-S clusters and the Suf system serves in stressed circumstances, the preference between the different operons is different in other organisms. Furthermore, the exact components of the two systems can vary among different organisms. For instance in cyanobacteria the Suf pathway appears to be the dominant system (Lang and Kessler, 1999). Furthermore, only the Suf pathway, is found in *Mycobacterium tuberculosi* and some archaea (Ayala-Castro *et al.*, 2008). This is not quite consistent with the proposal by Ayala-Castro *et. al.*, that the three known pathways can be characterized by physiological roles. According to this, the Isc system is mostly used for housekeeping cluster assembly, while the Suf pathway functions under

stress conditions, and the Nif enzymes are used to assemble complex or specialized clusters for specific enzyme (Ayala-Castro *et al.*, 2008).

Although the different operons are used under different circumstances, they all have genes coding for cysteine desulfurase and scaffold proteins (Fontecave and Ollagnier-de-Choudens, 2008). The mechanism for synthesizing the Fe-S cluster is similar for different machineries: first, the scaffold protein obtains sulfur atoms from a cysteine desulfurase and then it receives iron atoms from an iron donor and generates a Fe-S cluster. Secondly, the Fe-S cluster is transferred to an apo-protein (Fontecave and Ollagnier-de-Choudens, 2008).

The two Fe-S cluster biosynthesis operons of *E. coli*, Isc and Suf, are illustrated in Figure 44. The Isc operon includes genes for molecular chaperones (*hscA* and *hscB*), an electron transferring [2Fe-2S] ferredoxin (*fdx*), and three *isc* genes, *iscS*, *iscU*, and *iscA* (Keseler *et al.*, 2005). The *iscS* gene encodes a cysteine desulfurase, which catalyzes sulfur donation of a persulfide to the scaffold protein IscU for Fe-S cluster assembly (Agar *et al.*, 2000). The function of IscA is unclear, though some reports suggested that IscA is involved in [2Fe-2S] cluster assembly and activation of ferredoxin (Ollagnier-de-Choudens *et al.*, 2001). More recently, experiments demonstrating the Fe-S cluster transferring from IscA to various apo-Fe-S proteins suggested a role for IscA as scaffolds for Fe-S cluster assembly (Johnson *et al.*, 2005). However, the functions of IscU and IscA are not interchangeable. Recent work suggested IscA might act as the secondary cluster donor after IscU (Ollagnier-de-Choudens *et al.*, 2004). The transcription of *isc* genes is under the control of the IscR repressor (Giel *et al.*, 2006).

The second machinery, the SUF system, usually is active in conditions of iron

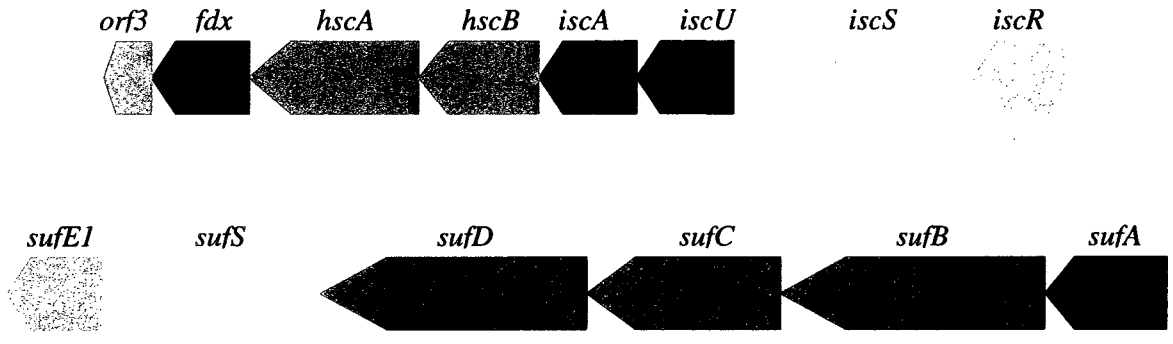


Figure 44 The Fe-S cluster biosynthesis operons in *E. coli*

The figure is adapted from Figure 3 (Johnson *et al.*, 2005)

As the authors described, genes from different clusters encoding products having the same function are depicted with the same color, (e.g. *iscS*, and *sufS* in yellow). Genes located within a single cluster that encode subunits of a macromolecular complex also have the same color, e.g. *hscB* and *hscA* (light purple); and *sufB*, *sufC*, and *sufD* (orange).

limitation and oxidative stress (Nachin *et al.*, 2003; Outten *et al.*, 2004). It belongs to the oxidative stress OxyR-dependent regulon (Zheng *et al.*, 2001). SufS encodes a cysteine desulfurase which functions much as IscS (Loiseau *et al.*, 2003; Mihara and Esaki, 2002). The *suf* operon also codes for the scaffold protein, SufA, which is homologous to IscA providing an intermediate assembly site for Fe-S cluster (Ollagnier-de Choudens *et al.*, 2003). Besides their possible role as the Fe-S scaffold, IscA and SufA have also been suggested to have a regulatory role, with SufA involved in the regulation of *sufBCD* expression (Balasubramanian *et al.*, 2006; Ollagnier-de Choudens *et al.*, 2003). SufC is a soluble ATPase, which interacts with SufB and SufD to form the SufBCD complex. SufB is homologous to SufD. However, their functions are unknown, except that the SufBCD complex can enchance SufS cysteine desulfurase activity (Eccleston *et al.*, 2006; Nachin *et al.*, 2003; Outten *et al.*, 2003). SufE also stimulates SufS activity and can transfer S atoms from SufS to SufA (Sendra *et al.*, 2007).

Besides these two operons, other genes scattered on the chromosome are also involved in Fe-S cluster synthesis. These are homologous to one of the genes belong to Isc and Suf operons. The function of these genes is not clear and will not be discussed here.

5.3 L-SD activity and the Fe-S cluster

As reviewed in the introduction of the first part of this thesis, L-serine can be deaminated by a variety of enzymes that have different levels of specificity. According to Swiss-Prot database, these enzymes belong to the ammonia-lyase class [EC 4.3.1.x]. They are put into two families according to their catalytic mechanisms: the L-serine/threonine deaminases (EC 4.3.1.19), and the bacterial L-serine deaminases (EC 4.3.1.17). Most of the enzymes of type EC 4.3.1.19 use pyridoxal phosphate catalysis.

These include mammalian liver L-serine deaminase (Mudd *et al.*, 1965; Ogawa *et al.*, 1989), and bacterial threonine deaminase (TdcB), D-serine deaminase (DsdA), and the biosynthetic threonine deaminase (IlvA). They use pyridoxal 5'-phosphate as a cofactor to facilitate the removal of the α-proton of the target amino acid, and generate water from the hydroxyl group. Following this the α-aminoacrylate is tautomerized forming 2-iminopropionic acid, which is hydrolyzed to ammonia and pyruvate (Davis and Metzler, 1972).

The second family, the bacterial L-serine deaminases (L-SD), uses protein bound to Fe-S clusters in catalysis. The enzyme activities and Fe-S clusters of L-SDs from *Peptostreptococcus asaccharolyticus*, *Clostridium sticklandii*, *C. propionicum*, *C. acidiurici* and *E. coli* are very similar (Carter and Sagers, 1972; Cicchillo *et al.*, 2004; Grabowski and Buckel, 1991; Hofmeister *et al.*, 1993; Hofmeister *et al.*, 1994; Hofmeister *et al.*, 1997; Zinecker *et al.*, 1998).

Detailed studies on L-SD in *E. coli* confirmed that L-SD has a [4Fe-4S] cluster (Cicchillo *et al.*, 2004). When purified anaerobically, L-SD1 (SdaA) behaved as if it contained a [4Fe-4S] cluster. Anaerobic treatment of the protein with $FeCl_3$ and Na_2S in the presence of DTT generated the cluster, and as well as dramatically increased the L-SD activity of the protein (Cicchillo *et al.*, 2004). TdcG, the anaerobically expressed *E. coli* L-SD, was characterized by Burman *et al.* (2004) UV-visible spectroscopy, iron and labile sulfide analyses suggested that the homodimeric enzyme had two oxygen-labile $[4Fe\text{-}4S]^{2+}$ clusters. Exposure of the active enzyme to air resulted in the loss of the $[4Fe\text{-}4S]^{2+}$ cluster.

Electron paramagnetic resonance spectra (EPR) were also done on L-SD from *P.*

asaccharolyticus purified under anaerobic condition by Hofmeister *et al.* (1994) The data revealed that the enzyme contained a $[3Fe\text{-}4S]^{+}$ cluster in the inactive enzyme. Incubating the enzyme under air resulted in an increased $[3Fe\text{-}4S]^{+}$ signal, which was accompanied by the loss of enzymatic activity. It was proposed that the active L-SD probably contained a diamagnetic $[4Fe\text{-}4S]^{2+}$ cluster which was converted by oxidation and loss of one iron ion to a paramagnetic $[3Fe\text{-}4S]^{+}$ cluster, resulting in inactivation of the enzyme.

A model for the deamination of L-serine by L-SD1, similar to the catalytic mechanism of aconitase, was proposed by Cicchillo *et. al.* in 2004 (2004). According to this model, the [4Fe-4S] cluster facilitates loss of the hydroxyl group by acting as a Lewis acid. The unique Fe of the Fe-S cluster coordinates the hydroxyl group of L-serine and eliminates it from L-serine. A 2-amino-2-propenoic acid is formed and tautomerizated to 2-iminopropionic acid, which then is hydrolyzed to ammonia and pyruvate.

The enzyme activity of L-SD is sensitive to oxygen and is unstable both in crude extracts and after purification. Indeed after purification under aerobic conditions, the preparation has no activity at all. Inactive L-SD from different organisms can be activated by Fe and DTT. DTT alone or DTT with other kind of ions such as Cu^{2+}, Mg^{2+}, Mn^{2+}, and Ni^{2+} does not activate. L-SD from *C. acidiurici* and *C. sticklandii* can be activated by Fe^{2+} and DTT under anaerobic conditions (Carter and Sagers, 1972; Zinecker *et al.*, 1998); extracts of both *P. saccharolyticus* and *C. propionicum* could be reactivated by incubating with Fe^{2+} and DTT (Grabowski and Buckel, 1991; Hofmeister *et al.*, 1993).

5.4 L-SDs from *E. coli* and several L-SD post-translational modification deficient mutants

The Fe-S cluster usually is assembled by recruiting S atoms from the cysteine residues

of a protein. The three L-SDs in *E. coli* K-12 contains nine (SdaA), ten (SdaB) and eight (TdcG) cysteine residues, respectively. Previous work by Tang (2004) showed that three cysteines at positions 339, 381 and 392 in SdaA of *E. coli* were essential for its enzyme activity, since site-directed mutagenesis of any of these three residues caused an almost total loss of L-SD activity. This suggests that these three cysteines form part of the structure of the [4Fe-4S] cluster. The cysteines of many bacterial L-SDs and found the three essential cysteines to be widely conserved with the same spacing, suggesting the motif C-X41-C-X10-C was found in most L-SDs (Tang, 2004).

Wild type *E. coli* cannot use L-serine as a sole carbon source, while it can use a combination of L-serine, glycine and leucine as carbon source (SGL). Various mutants which cannot grow on SGL have been isolated. Mutants in the L-SD coding genes should be deficient in L-SD activity and would not be expected to grow on SGL medium. However in other mutants, the L-SD protein could be made, but the strain was nonetheless deficient in L-SD activity probably due to a deficiency in post-translational modification.

One of these, strain MEW191, is a SGL$^-$ derivative made by Mu::dX insertion into MEW1. It does not grow on SGL plates and shows very little L-SD activity *in vivo*. However, *in vitro* addition of Fe and DTT to the enzyme assay restored 60% of L-SD activity. The location of the Mu::dX insertion is not known, but it is clearly not in *sdaA* since extracts have activity, and since the insert-encoded β-galactosidase activity was not induced by the inducers of L-SD. This suggests that MEW191 had a Mu:dX insert in a gene which is involved in L-SD post-translational modification (Newman *et al.*, 1985a; Newman *et al.*, 1985b).

Strain MEW128 was isolated by penicillin selection from strain MEW1. Strain MEW128 has a strong requirement for thiamine in addition to its inability to grow on SGL. In both LB and glucose minimal medium with L-SD inducers, MEW128 produces less than 15% of the parental level of L-SD. The presence of thiamine in the growth medium allows growth but does not restore L-SD activity. The L-SD deficiency and thiamine auxotrophy was restored simultaneously (Newman *et al.*, 1985b). Just as in strain MEW191, L-SD activity of MEW128 could be restored to about 60% of the parental level *in vitro* by incubating with Fe and DTT (Newman *et al.*, 1985a). Since L-SD activity requires a [4Fe-4S] cluster, the mutation in MEW128 may affect a gene involved in Fe-S cluster synthesis in the L-SD protein.

L-SD protein activation has been of interest for a long time in the Newman laboratory. An *iscS*$^{-}$ mutant has very little L-SD activity, suggesting that the *isc* genes are involved in L-SD maturation (Newman, personal communication). However, it is not clear as to what genes are needed for Fe-S cluster formation in L-SD, which makes it very interesting to identify the mutation in either MEW191 or MEW128.

In this part of the thesis, I will present my work on MEW128. I located the mutation site in MEW128 in the *ygfZ* gene. Not much is known about the *ygfZ* gene or its encoded protein. The function of the YgfZ protein is unknown but its crystal structure was published in 2004 by Alexey *et al.* (Teplyakov *et al.*, 2004). Its structure is similar to that of dimethylglycine oxidase (DMGO) from *Arthrobacter globiformic* and GcvT of GCV. They all have a folate-binding site in the central cavity (Teplyakov *et al.*, 2004).

YgfZ has a concave surface covered mostly by the basic residues, thus it is very likely that this protein is a nucleotide-binding protein, which is involved in the regulation of C1

unit metabolism (Scrutton and Leys, 2005; Teplyakov *et al.*, 2004). This is very interesting and relates to the first part of my thesis which showed that the metabolic function of L-SD is involved in the C1 units metabolism in *E. coli*.

Chapter 6 Materials and methods

* This section just includes the materials and methods which have not been addressed in part one.

6.1 Strains, and plasmids

The strains, and plasmids used in this study are listed in Table 9.

6.2 Experiment methods

6.2.1 β-galactosidase assay

β-galactosidase activity was assayed in whole cells according to the method described by Miller (Miller, 1972) and expressed in Miller units.

6.2.2 DNA Sequencing

DNA was sent to McGill University and the Genome Quebec Innovation Centre for sequencing.

6.3 Construction of strains

6.3.1 Construction of MEW128 tetR

TetR was transduced into MEW128 using P1 phage lysate made on DH5αZ1. The transductants were selected on LB plates with 100 μg/mL streptomycin.

Table 9 *E. coli* strains and plasmids used in part 2

Strains and plasmids	Genotype and/or relevant characteristics	Source or reference
Strains		
MEW128	Mutant SGL-, unable to grow with L-serine, glycine, L-leucine; isolated by penicillin selection from strain Cu1008	Newman *et al.*,1985b
DH5αZ1	DH5α *tetR lacI* Sp^R	LutZ et *al.*, 1997
MEW128 tetR	MEW128 tetR, Sp^R	This study
JW2866	Keio collection *E. coli* K-12 BW25113*ΔygfZ* Km^R	Baba *et al.*, 2006
MEW1*ΔygfZ*		This study
Plasmids		
psd*lacZ*	Plasmid carrying *lacZ* gene under the regulation of *sdaA* promoter, generated from p*sdaA*, Amp^R	This study
pHindI	pBR322 carrying DNA fragment which complements the growth of MEW128 on SGL plates, Amp^R	This study
pHindII	pBR322 carrying DNA fragment which complements the growth of MEW128 on SGL plates, Amp^R	This study
pHind2	pBR322 carrying gene *yqfB*, *bglA* and partial *yqfA*, generated from plasmid pHindI, Amp^R	This study

Cont.

pHind3	pBR322 carrying gene *ygfZ*, *ygfX* and *fldB*, generated from plasmid pHindI, Amp^R	This study
pHind4	pBR322 carrying gene *ygfX* and *fldB*, generated from plasmid pHindI, Amp^R	This study
pLtet	PltetO-1 promoter, p15A replicon, MCS-1, Cm^R	Wei thesis, 2001
pMES22	pBR322 carrying 2.6 kbp insert including *sdaA* and partial upstream and downstream sequences of *sdaA*	Su *et al.*, 1991
pLtet*ygfZ*	pLtet carrying *ygfZ* gene, Cm^R	This study
pACYC*lacZ*	pACYC184 carrying *lacZ* gene, Te^R	This study
pygfZ*lacZ*	pACYC184 carrying *lacZ* gene under the regulation of *ygfZ* promoter, Te^R	This study

I: A IPTG dependent strain whose *mra* promoter was disrupted by inserting the *lac* promoter

6.3.2 Construction of MEW1 *ΔygfZ*

The methods used to construct MEW1 *ΔygfZ* are the same as the methods used for constructing the single, double and triple L-SD deletion strains as described in part one materials and methods section 5.1. The donor strain is JW2866 which carries the *ygfZ* deletion from the Keio collection (Baba *et al.*, 2006). Following the same steps, *ygfZ* deletion was introduced into MEW1 by P1 phage transduction.

6.4 Construction of plasmids

6.4.1 Construction of plasmid psd*lacZ*

To construct an in-frame fusion of *sdaA* to *lacZ* at the unique *Hpa*I site on plasmid pMES22 (Su *et al.*, 1989), pMC1871, a plasmid carrying *lacZ* was digested by *Sma*I and *Pst*I and the 3.1 kbp DNA band corresponding to *lacZ* was isolated. Then pMES22 was totally digested by *Hpa*I and partially digested by *Pst*I. The 5.4 kbp DNA band, which corresponds to DNA from the *Hpa*I to the *Pst*I site (at base pair 353 bp) was isolated. The 3.1 and 5.4 kbp bands were ligated with T4 ligase. The strategy used is shown in Figure 45.

6.4.2 Construction of plasmid pLtet*ygfZ*

To construct pLtet*ygfZ*, the gene *ygfZ* was first amplified by PCR from the chromosome of the wild type strain MEW1. The strategy used is shown in Figure 46.

The primers *ygfZ*pLtetL and *ygfZ*pLtetR were designed to amplify the coding sequence of the *ygfZ* gene and contained *Kpn*I and *Sal*I sites respectively. The reaction

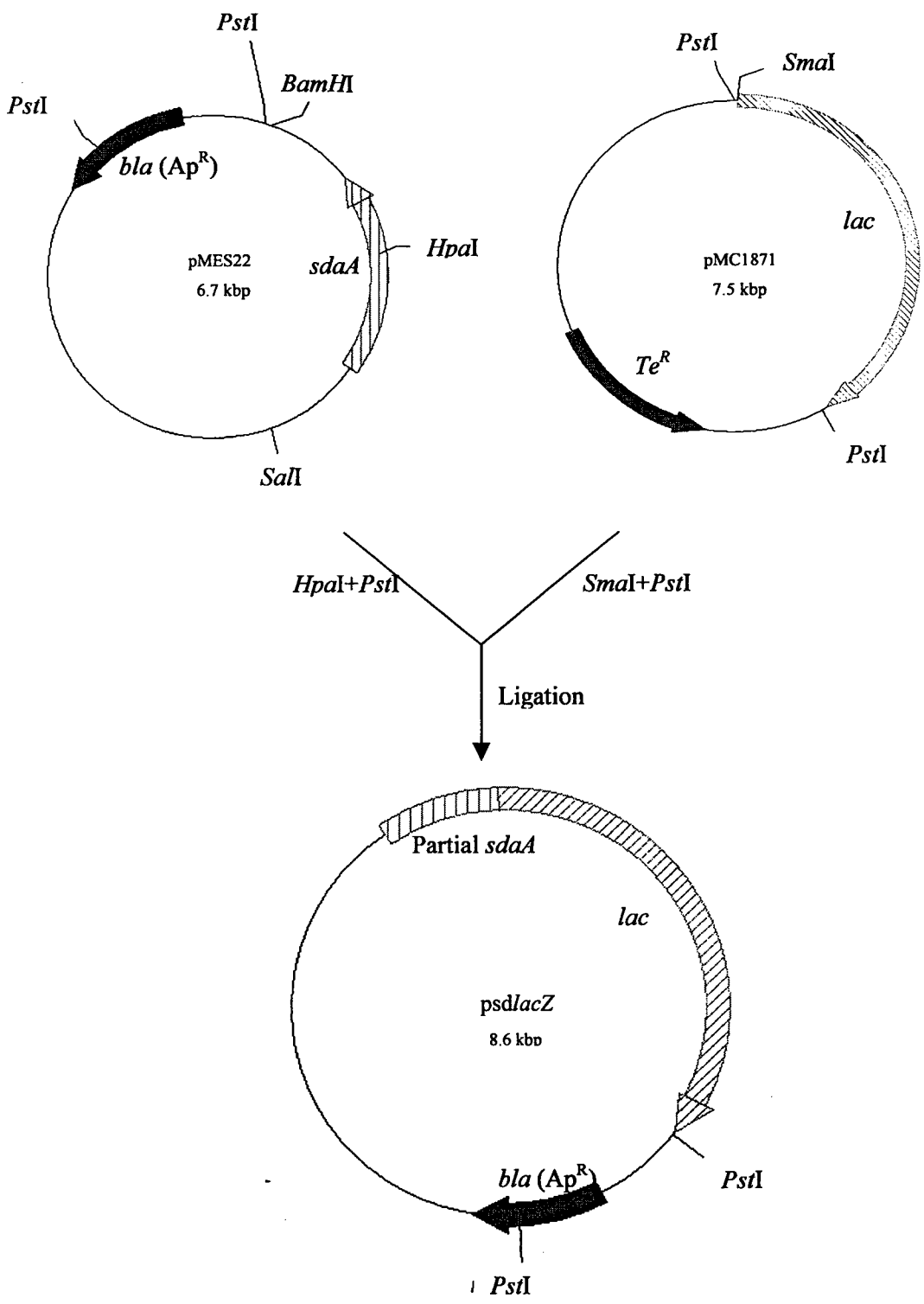


Figure 45 Construction of plasmid psd*lacZ*

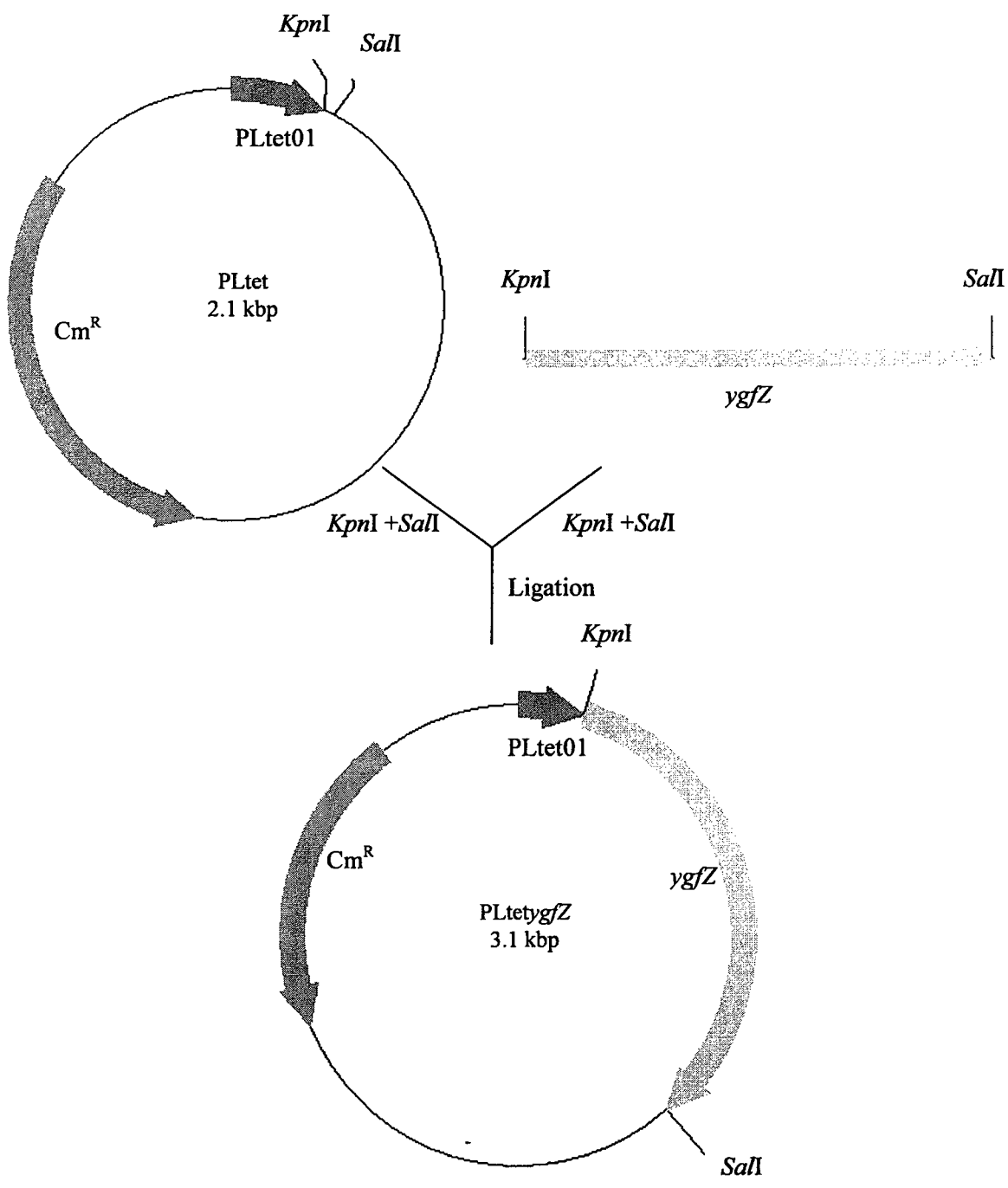


Figure 46 Construction of plasmid pLtet*ygfZ*

was carried in a volume of 100 μl and the mixture contained 2.5 μM of each primer, 50 ng of template chromosome DNA, one time's Pfu reaction buffer (Fermentas, USA), 200 μM dNTPs, 5 U of Pfu DNA polymerase (Fermentas, USA). The reaction was performed using the following program:

94 °C 4 min

35 cycles 94 °C 45 s

40 °C 45 s

72 °C 2 min 30 s

72 °C 7 min

After amplification, the 1070 bp product was analyzed on a 1% agarose gel and DNA was purified using QIAquick PCR Purification Kit (QIAGEN Montreal, Canada). The fragment was then cloned into *Sma*I site on plasmid pUC18. MEW1 competent cells were transformed by electroporation and selected on LB Ampicillin plates. Finally some colonies were picked and the ones, which had the *ygfZ* gene, were used to extract the plasmids. The plasmids were then digested with the appropriate restriction enzymes to confirm the construction. The confirmed plasmid was sent for sequencing. The primers used for sequencing were M13/pUC sequencing primer and M13/pUC reverse sequencing primer. After the *ygfZ* gene on pUC18 was confirmed by sequencing, the plasmid was digested sequentially with *Kpn*I and *Sal*I, a 1050 bp fragment was generated and then inserted into plasmid pLtet. MEW1 competent cells were transformed by electroporation and selected on LB chloramphenicol plates. Finally, some colonies were picked and their plasmids were isolated and digested with the appropriate restriction enzymes to confirm the structure of these plasmids. The final plasmid was 3122 bp and

named as pLtet*ygfZ*.

6.4.3 Construction of *ygfZ*::*lacZ* promoter fusion plasmid-p*ygfZlacZ*

To study the regulation of *ygfZ* expression in the cell, a *ygfZ*::*lacZ* promoter fusion plasmid-p*ygfZlacZ* was constructed. A DNA fragment covering -484 bp to +4 bp from the start codon ATG was amplified by PCR. Primers used were *ygfZ*paL corresponding to the region from -484 bp to -460 bp upstream of *ygfZ* and including an *Xho*I restriction site and *ygfZ*paR corresponding to +4 bp to -26 bp of the *ygfZ* coding region with a *Nco*I restriction site. The amplified fragment was cloned into pUC18 at *Sma*I site. The pUC18 plasmid with the insertion fragment was sent for sequencing. The primers used for sequencing were M13/pUC sequencing primer and M13/pUC reverse sequencing primer. After the sequence was confirmed, the promoter fragment was released from the vector pUC18 by digesting with *Xho*I and *Nco*I, and then purified and cloned into the plasmid pACYC184*lacZ* at *Xho*I and *Nco*I sites. The ligation mixture was transformed into strain MEW1 and plated on LB plate with X-gal and tetracycline. Plasmids from blue colonies were isolated and examined by restriction enzyme digestion. Figure 47 illustrated the construction of plasmid p*ygfZlacZ*.

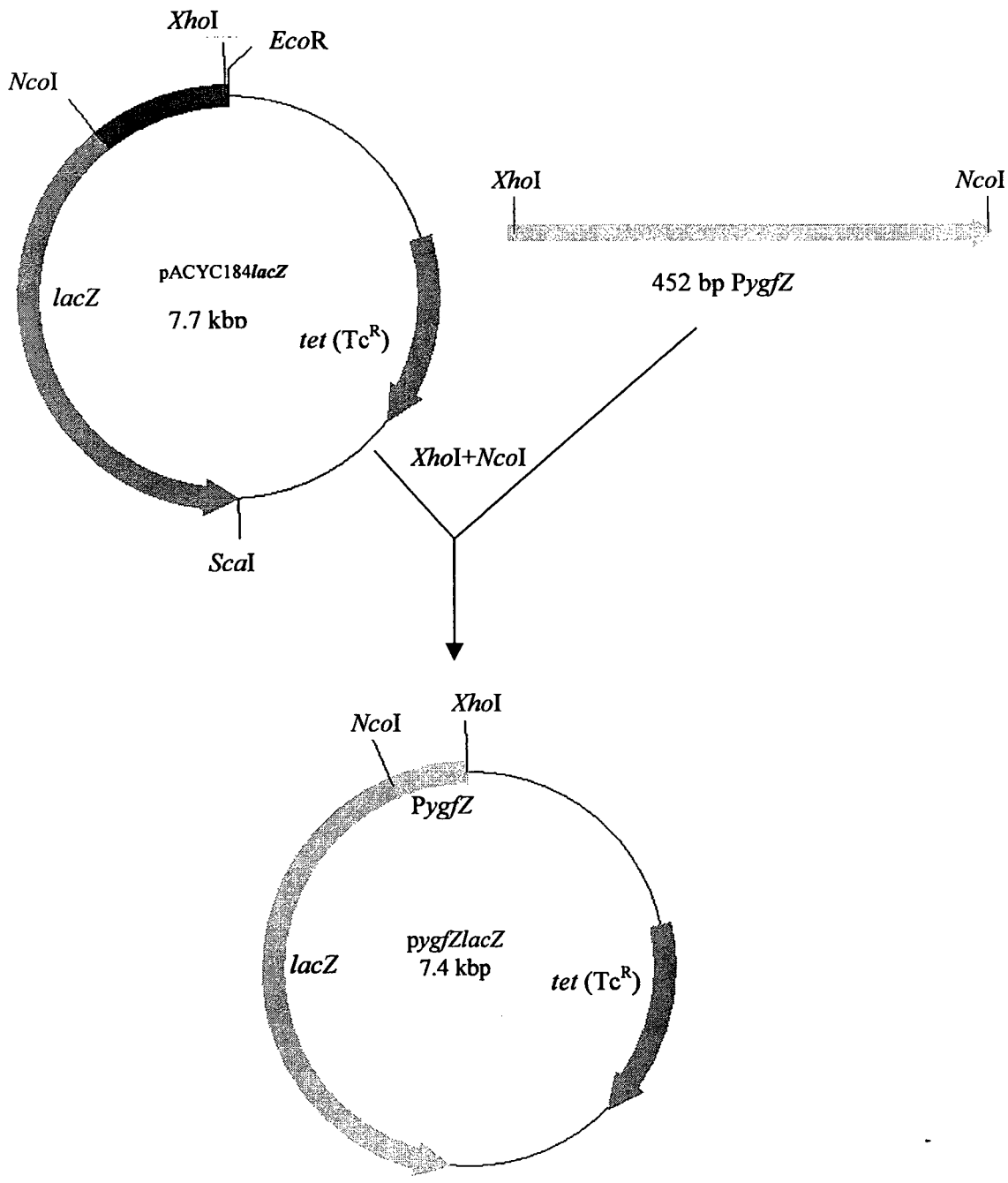


Figure 47 Construction of plasmid p*ygfZlacZ*

Chapter 7 Results

7.1 Identify mutant MEW128

7.1.1 Analysis of L-SD transcriptional regulation in mutant MEW128

Wild type *E. coli* K-12 cells cannot use L-serine as a carbon source unless they are also provided with glycine and leucine. A mutant MEW128 cannot use the combination of L-serine, glycine and leucine as a carbon and energy source (Newman *et al.*, 1985b). Strain MEW128 was shown to be deficient in L-SD activity as usually measured *in vivo* in toluene-treated cells (Newman *et al.*, 1985b). However *in vitro*, strain MEW128 synthesized significant amounts of L-SD, though less than the parental strain (Newman *et al.*, 1985b).

From previous results, strain MEW128 produces an L-serine deaminating protein, which is not activated *in vivo* (Newman et al., 1985b). That is, in MEW128, and presumably also in the parent strain, L-SD is synthesized in an inactive form, and is activated by some other gene product. Thus the gene mutated in strain MEW128 may be involved in the post-translational modification of L-SD.

To investigate the transcriptional regulation of L-SD in strain MEW128, I constructed an in-frame fusion plasmid psd*lacZ*, in which the *lacZ* gene is under the control of the *sdaA* promoter on this plasmid (see methods section), psd*lacZ*, was transformed into strains MEW128, the 'triple mutant' strain MEW999 and the parent strain MEW1. The plasmid-carrying strains were grown in glucose minimal medium with glycine (300 μg/mL) and L-leucine (300 μg/mL) and assayed at mid-log phase for L-SD and β-

galactosidase activity. The *lacZ* gene is under the regulation of the *sdaA* promoter, and since the β-galactosidase protein does not require additional activation, the activity of β-galactosidase in these three strains was directly related to the extent to which the *sdaA* promoter was transcribed

The parent strain MEW1 psd*lacZ* showed 524 units (Table 10) which is related to the strength of the *sdaA* promoter and reflects the total transcriptional ability in the medium used for this study. The other two strains showed similar activity, MEW999 psd*lacZ* with 511 units and MEW128 psd*lacZ* with 494 units. That MEW999 allows the same transcription as MEW1 is not surprising. MEW999 is deficient in chromosomally encoded *sdaA* but this should not prevent transcription of the plasmid-carried gene. Strain MEW128, which shows little or no L-SD activity *in vivo*, also transcribes the gene normally, suggesting that the effect of the MEW128 mutation is on a post-translational event.

The L-SD assay results of the same strains are consistent with this picture (Table 10). When assayed without Fe and DTT in whole cells, strain MEW999 psd*lacZ* showed no L-SD activity *in vivo*, and strain MEW128 showed very little (only 5% of the parental strain's activity). In the presence of iron and DTT, the *in vivo* L-SD activity in MEW128 increased to 39% of the parental value. Even strain MEW999 which has no gene coding for L-SD showed 11% of the parental activity, which can be ascribed to a nonenzymatic deamination of L-serine by iron and DTT (Newman *et al.*, 1990).

Table 10 β-galactosidase and L-SD activity in cells carrying psd*lacZ*

	β-galactosidase (units)*	L-SD activity **	L-SD activity Iron & DTT***
MEW 1	524	11	14.2
MEW 999	511	0	1.6
MEW 128	494	0.6	5.5

* β- galactosidase activity was measured by the method of Miller and is expressed in his units.

** L-SD activity was measured by adjusting the cell density to OD600nm equal to 0.6 and then taking 0.1 mL cell for assay. Activity is expressed as μg/mL pyruvate formed per min.

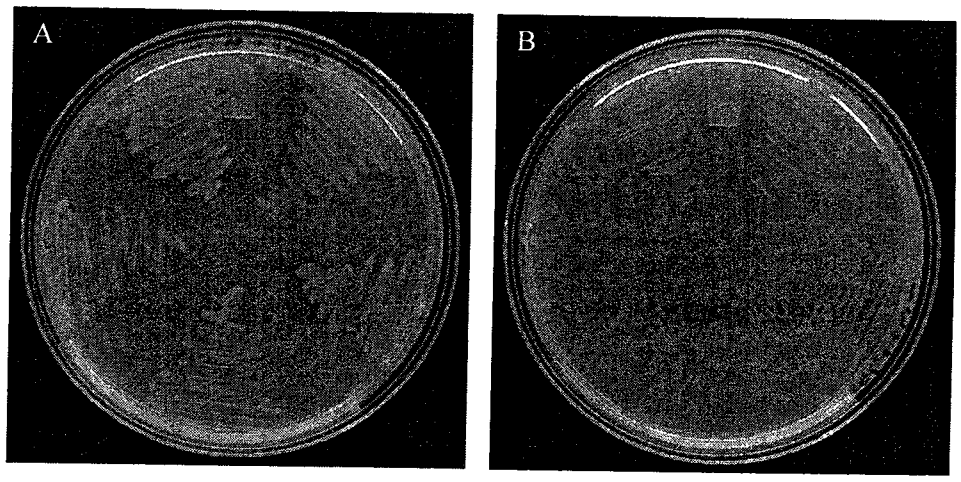
*** Additions to the assay were 10^{-3} M Fe and 10^{-2} M DTT.

7.1.2 Genetic screening for genes complementing the growth of MEW128 on SGL

Strain MEW128 clearly lacks some function needed to activate L-SD. If I could identify the gene mutated, that might help understand the mechanism by which L-SD is activated. To do this, I tried to identify genes which could compensate for the mutation and allow growth on SGL with no thiamine supplied. To do this, I created a genomic library by partial digestion of parental *E. coli* chromosomal DNA with *BamH*I, *Hind*III, *EcoR*I, *Pst*I and *Sal*I. The partially digested DNA fragments were cloned into the pBR322 plasmid cut, in each case, with the enzyme used to cut the chromosomal DNA. MEW128 cells were transformed with the genomic library, selecting transformants by their ability to grow on SGL plates. Such SGL^+ colonies were purified on glucose minimal medium and then restreaked on SGL plates to verify their growth.

Plasmid DNA was isolated from a variety of the colonies that showed stable growth on SGL, purified, and each retransformed into MEW128 to confirm its ability to support growth on SGL medium. Two plasmids, cloned from *Hind*III-digested DNA were able to restore growth of MEW128 on SGL (Figure 48). To test if these plasmids actually restored L-SD activity, I used the whole cell L-SD assay on cells grown in glucose minimal medium with glycine (300 μg/mL) and L-leucine (300 μg/mL). As shown in (Table 11), the L-SD activity of mutant MEW128 is about 17 % of that of the parent strain. Introduction pHindI brought that to 155%, and pHindII to 133% both even higher than the parent strain, perhaps because they were cloned on a high copy number plasmid. I conclude that each of these plasmids carries DNA that allows strain MEW128 to make active L-SD.

These two plasmids were sent for partial DNA sequence analysis from both ends of



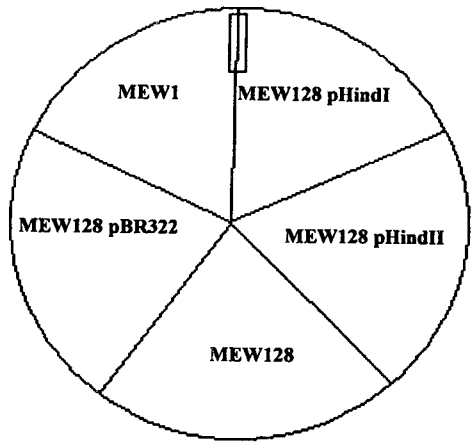


Figure 48 Introduced pHindI and pHindII complement MEW128's growth on SGL plates

MEW1, MEW128 pHindI, MEW128 pHindII, MEW128 and MEW128 pBR322 were streaked on a glucose minimal medium with thiamine (5 μg/mL) (A) or on SGL (B) and photographed after 48 hours.

Table 11 The L-SD activity of strain MEW128 carrying various plasmids

	MEW1	MEW128	MEW128 pHindI	MEW128 pHindII
L-SD activity*	13.15	2.22	20.32	17.45

* L-SD activity as in Table 10.

the inserted DNA fragments (see Methods section). A comparison of the sequences with the NCBI database using Blast identified the whole sequence of the insertion fragment. Sequencing of both plasmids of the coding region resulted in the same sequence information for 5.24 kbp, which comprised seven open reading frames (ORFs) (Figure 49), *fldB*, *ygfX*, *ygfY*, *ygfZ*, *yqfA*, *yqfB* and *bglA*. To identify which of these genes is responsible for the complementation of the MEW128 mutation, the plasmid pHindI was digested by *BstE*II, which has two digestion sites in the inserted fragments. I then rejoined the plasmid DNA fragment and transformed the new plasmid, pHind2, which includes *yqfB* and *bglA* and partial *yqfA* into MEW128. However, pHind2 did not complement the growth of MEW128.

I then digested pHindI with *Stu*I and *Eco*RI, blunted the ends of the plasmid fragment, ligated and transformed the new plasmid, pHind3, which includes *ygfY*, *ygfZ*, *ygfX* and *fldB* into MEW128. pHind3 did complement the growth of MEW128.

It is clear that one of *ygfY*, *ygfZ*, *ygfX* and *fldB* should allow MEW128 to use SGL. By digesting pHindI with *Sal*I and *Af*III, blunting the ends of the plasmid fragment, and ligating, I made a plasmid which carries *ygfX* and *fldB*. That plasmid pHind4, did not complement the growth of MEW128 on SGL plate. These results suggest strongly that it is the *ygfZ* gene that can compensate for the deficiency of strain MEW128.

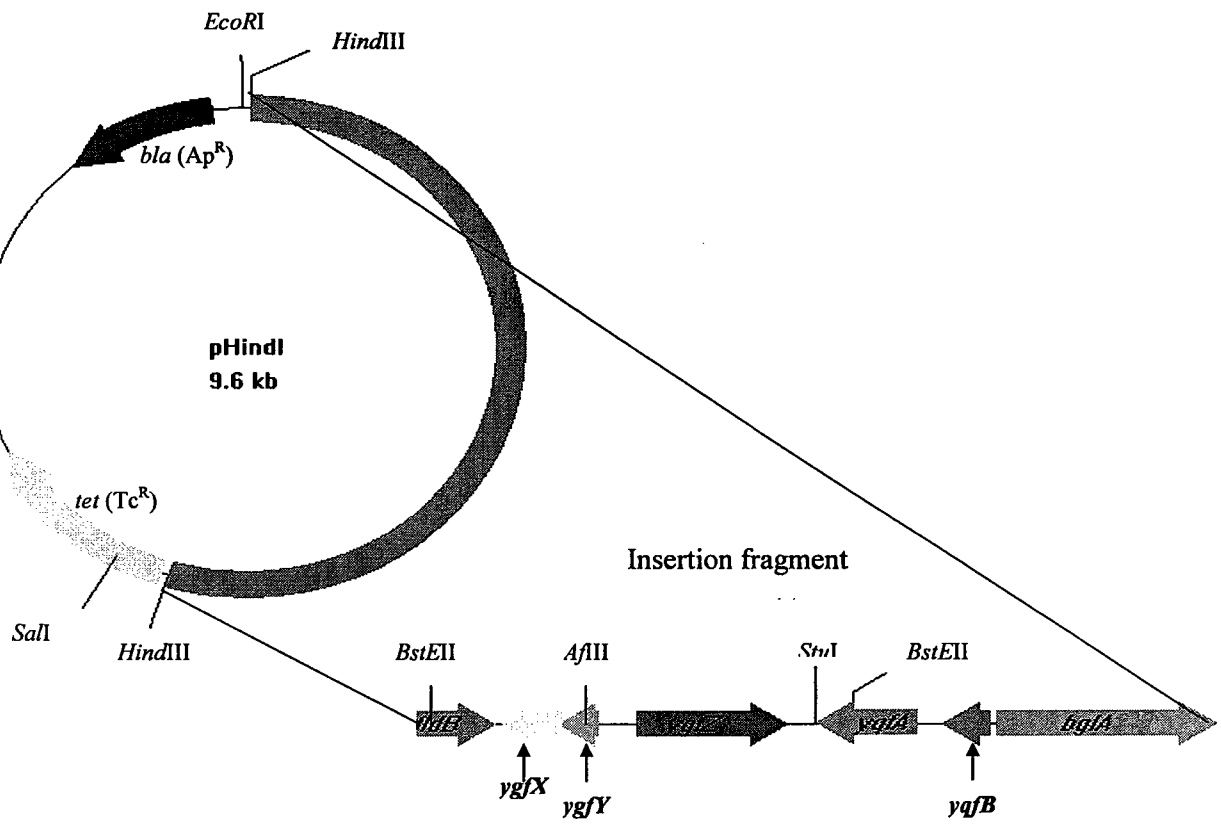


Figure 49 MEW128 Complement plasmid pHindI

7.1.3 Identification the mutation site in *ygfZ* of MEW128

If the *ygfZ* gene of strain MEW128 is in fact mutated, this should be evident on sequencing. I, therefore, used PCR to clone the *ygfZ* gene from the chromosome of MEW128 into the *Sma*I site of plasmid pBluescriptII using primers *ygfZ*F and *ygfZ*R. This amplified a fragment including the whole *ygfZ* coding region and 466 bp upstream and 201 bp downstream DNA sequences. The same primers were used for sequencing as the external primers. This identified a single nonsense mutation site at +559 bp of the *ygfZ* coding region, which changed a glutamine coding codon CAA to a stop codon TAA. Another pair of internal primers *ygfZ*InF and *ygfZ*InR was used to verify the mutation site in *ygfZ*. The sequencing result confirmed the mutation site in gene *ygfZ*. This established clearly that truncation of YgfZ protein caused the deficiency of L-SD activity in MEW128.

7.2 YgfZ restores The L-SD activity of MEW128

7.2.1 Construction of pLtet*ygfZ*

If the nonsense mutation in ygfZ results in a loss of L-SD activation, one might expect that the introduction of a complementing plasmid would restore it. To study the relation between the expression of YgfZ and L-SD activity, a regulated plasmid carrying *ygfZ* was constructed. To do this I cloned the *ygfZ* gene into pLtet, a low copy number expression vector with a p15A origin of replication (Lutz and Bujard, 1997). It carries a tightly regulated Pltet01 promoter with a convenient multiple cloning site, and, therefore, the cloned gene is transcribed when induced by tetracycline or analogues, for example anhydrotetracycline (aTc). This system was previously used in this lab for the

physiological studies of the *metK* gene (Wei and Newman, 2002).

To clone the *ygfZ* gene, the coding sequence was amplified from the chromosomal DNA of wild type strain MEW1 by PCR using a 5' primer which contains a *Kpn*I restriction site and a 3' primer with a *Sal*I restriction site to facilitate cloning into pLtet. As described in Materials and Methods, a 1060 bp *ygfZ* gene fragment was inserted into pLtet, giving a plasmid of 3122 bp. To verify the plasmid construction, it was digested with *EcoR*I, in which case the sizes of the fragments expected were 500, 1104 and 1518 bp and with *Kpn*I-*Sal*I, where the size of the fragments expected were 1052 and 2070 bp. That the fragments were of the correct size is shown in Figure 50. The plasmid containing the gene *ygfZ* regulated by a *tet* promoter was named pLtet*ygfZ* and used for subsequent studies.

7.2.2 Construction of MEW128 with Tet^R repressor

The use of pLtet requires that the host cell expresses the TetR regulatory protein, so that expression of any genes cloned under the *tet* promoter will be repressed until an inducer is added (Lutz and Bujard, 1997). The gene encoding this repressor molecule, tetR, was transferred from strain MEW1tetR to the MEW128 mutant by P1-phage transduction, and selected by spectinomycin resistance. This generated strain MEW128tetR. The plasmid pHindI (which carries seven genes) allowed growth of strain MEW128tet^R on SGL plate, just as it had done for strain MEW128.

7.2.3 Complementation of MEW128tet^R by pLtet*ygfZ*

I, therefore, introduced the plasmid pLtet*ygfZ* into the MEW128tet^R mutant to study the details of the relationship between the expression of *ygfZ* and L-SD activity, as

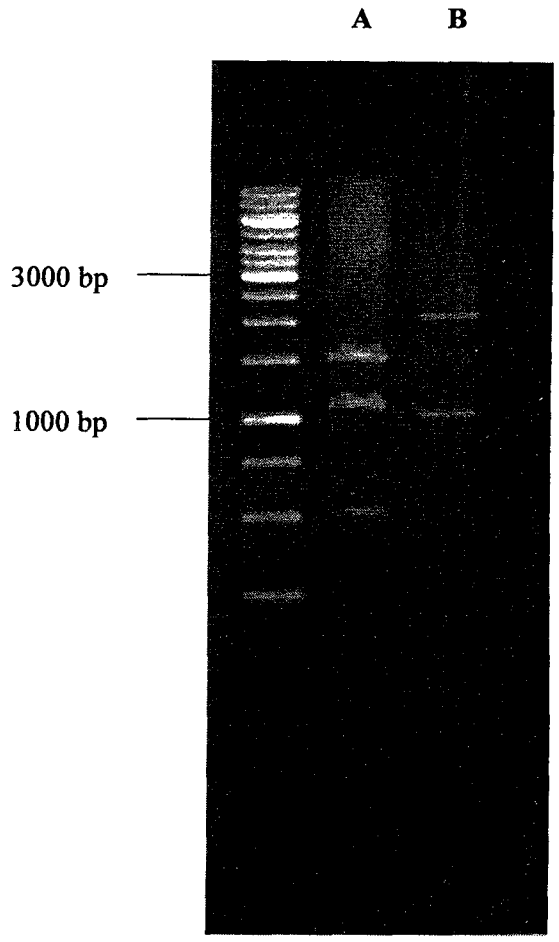


Figure 50 Verification of the construction of pLtet*ygfZ*

ygfZ gene coding sequence was amplified from MEW1 chromosome DNA. A) pLtet*ygfZ* digested by EcoRI. B) pLtet*ygfZ* digested by *Kpn*I and *Sal*I.

measured *in vivo*. Without the plasmid, L-SD, activity of MEW128 was about 8% of that seen in the parental strain MEW1 (Table 12). By comparison, strain MEW999 from which all three L-SD genes have been deleted, showed no significant L-SD activity.

Strain MEW128tetR carrying pLtet*ygfZ* and grown without inducer produced 3.3 units of activity (Table 13). When the empty plasmid pLtet was tested, there was no obvious difference found from the one without plasmid. The level of activity increased with addition of aTc. However, at the relatively high level of 100 ng/mL, the strain produced only 6.7 units, about L-SD activity increased to 51% of the parental level. MEW128 with plasmid pLtet*ygfZ* could grow on SGL with or without thiamine supplied, while the one with empty plamid pLtet could not grow (Figure 51).

I conclude from these experiments that the plasmid pLtet*ygfZ* produces a protein which activates L-SD, but how it does this is unknown.

7.2.4 MEW1*ΔygfZ* has the similar phenotype as MEW128

To verify the impact of the YgfZ mutation on the L-SD activity of the cell, I constructed a *ygfZ* deletion mutant by transferring the deletion from the Keio collection to the parental strain MEW1. The growth of the strain was tested on different plates. As shown in Figure 52, similar to MEW128, MEW1 *ΔygfZ* could not grow on glucose minimal medium without thiamine, while the parental strain grew well on it. *In vivo* L-SD activity assay also showed that MEW1*ΔygfZ* showed no significant L-SD activity as compared to the parental strain (Table 12). These results strongly support the hypothesis that the *ygfZ* gene responsibles for the physiological changes of MEW128.

Table 12 L-SD activity of MEW128 pLtet*ygfZ*

	MEW1	MEW999	MEW128	MEW128 pLtet	MEW128 pLtet*ygfZ*	MEW1*ΔygfZ*
L-SD * activity	13.1	0	1.1	0.5	6.7	0.5

* L-SD activity as in Table 10.

Table 13 L-SD activity of MEW128 pLtet*ygfZ* grown with various aTc concentrations

	aTc (ng/mL)				
	0	1	10	50	100
L-SD* activity	3.3	3.4	3.3	5.6	6.7

* L-SD activity as in Table 10.

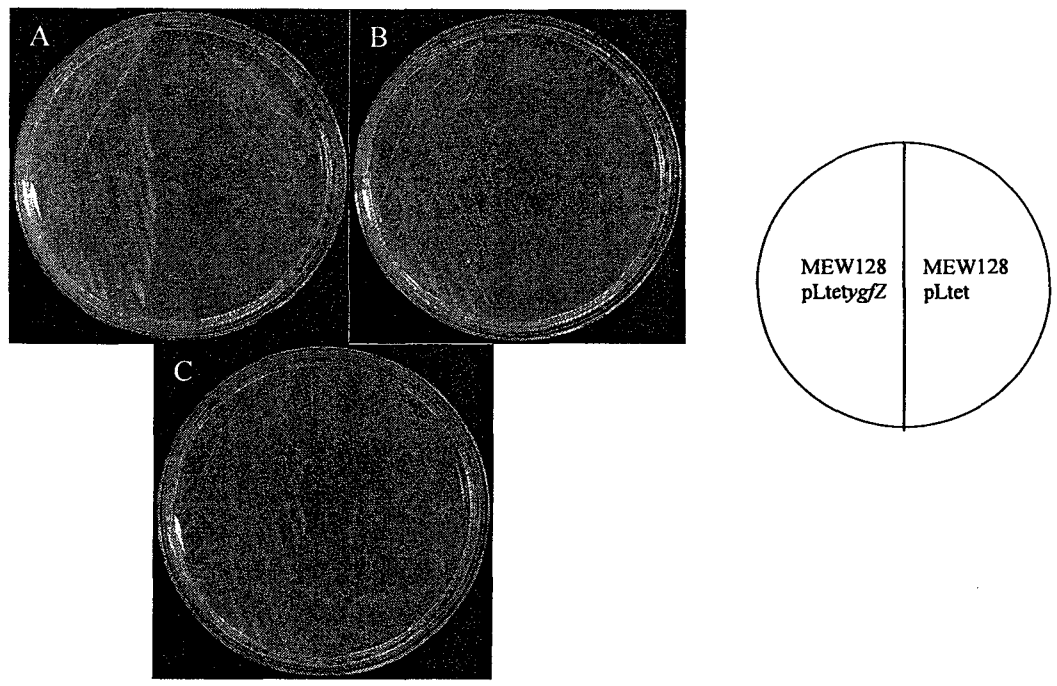


Figure 51 Plasmid pLtet*ygfZ* complement MEW128's growth on SGL

When induced by aTc (100 ng/mL), plasmid pLtet*ygfZ* could complement MEW128's growth on SGL without thiamine (B), or with thiamine (5 μg/mL) (C). However, the plasmid pLtet did not. Both could grow on glucose minimal medium with thiamine (5 μg/mL) (A). The pictures were taken after 48 hours.

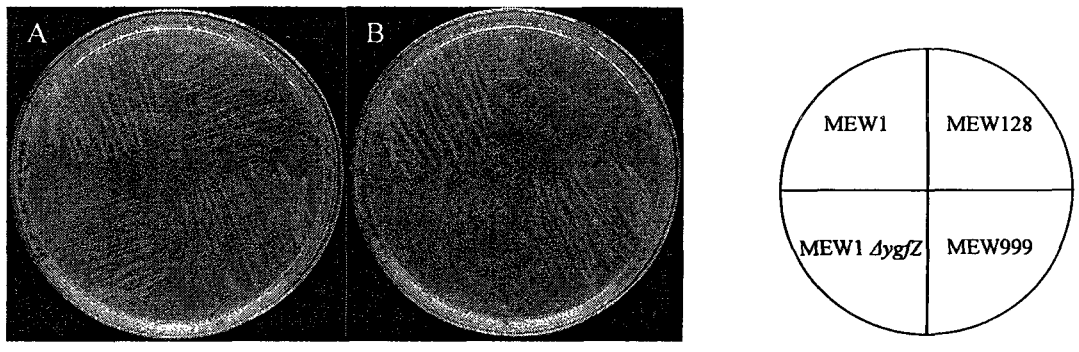


Figure 52 MEW1 *ΔygfZ* is thiamine dependent strain

The deletion mutant MEW1 *ΔygfZ* could grow on glucose minimal medium with 5μg/mL thiamine (A). It could not grow on the glucose minimal medium plate without thiamine (B). This was the same as for MEW128. On both plates, the parental strain MEW1 and the triple mutant MEW999 grew normally. The pictures were taken after 24 hours.

7.3 A study of the regulation of YgfZ expression

7.3.1 Construction of plasmid p*ygfZlacZ*

In order to determine the relationship between the amount of YgfZ and the L-SD activity, it would be useful to measure the amount of YgfZ being made. For this purpose, I constructed a plasmid carrying an *ygfZ* promoter and *lacZ* open reading frame. A promoterless *lacZ* gene missing its translation initiation site was fused in frame to the *ygfZ* promoter right after the start codon ATG on plasmid pACYC184. Since the *lacZ* gene is under the control of the *ygfZ* promoter and translated from the *ygfZ* translation start codon, I can use this construct to estimate the extent of *ygfZ* expression simply by measuring β-galactosidase activity under different conditions.

7.3.2 Effect of amino acids on production of β-galactosidase from p*ygfZlacZ*

On the one hand, a deficiency of YgfZ in *E. coli* K-12 results in a loss of L-SD activity. On the other hand, L-leucine and glycine induce L-SD. Would the inducers of L-SD also induce expression of *ygfZ*?

To study the expression of *ygfZ*, I took advantage of strain MEW999, the triple L-SD encoding gene deletion mutant from part one. That strain did not grow normally in minimal medium with six amino acids: L-serine, glycine, L-leucine, L-methionine, L-lysine and L-threonine, instead forming filaments. If the function of YgfZ is to activate L-SD in the cell, what might happen to YgfZ synthesis in cells deficient in the lack of L-SD?

β-galactosidase activity was assayed in cells grown in glucose minimal medium with L-serine (600 μg/mL), glycine (600 μg/mL), L-leucine (600 μg/mL), L-methionine (60

μg/mL), L-lysine (600 μg/mL), L-threonine (600 μg/mL) and CAA (0.5%), respectively. An overnight culture of MEW999 with p*ygfZlacZ* in glucose minimal medium with tetracycline was diluted 10^4 fold into the glucose minimal media with various additions and β-galactosidase activity was assayed after eight hour's growth (Table 14).

In all media tested, the β-galactosidase activity of MEW999 was about 20% higher than of strain MEW1. However, the level was low in both cases, consistent with the idea that YgfZ could function as a catalytic or regulatory protein. The highest level of β-galactosidase was seen in CAA where MEW999 p*ygfZlacZ* had 55% more activity than the parental strain and about twice the level it showed in glucose minimal medium without additions. Whether these relatively small changes have physiological significance is unclear.

Table 14 The effects of different amino acids on synthesis of β-galactosidase from the plasmid p*ygfZlacZ* in strain MEW999 and MEW1

Amino acids	β-galactosidase		
	MEW1	MEW999	Increasing rate %
None	13.8	17.8	**29.8**
L-serine	15.5	18.8	**21.3**
Glycine	14.9	18.4	**23.5**
L-leucine	13.0	18.6	**43.1**
L-methionine	14.1	17.3	**22.7**
L-lysine	14.5	18.3	**26.2**
L-threonine	15.5	18.4	**18.7**
Casamino acids	23.6	36.6	**55.1**

Enzyme activity as in Table 10.

Chapter 8 Discussion

Part two of my thesis presents studies done to characterize the L-SD activity deficient mutant MEW128. A genomic library was used to screen for genes which could complement the growth of MEW128 on media with L-serine, glycine and leucine as significant carbon sources. Thereafter, the complementing gene was identified. The corresponding gene of MEW128 was cloned and sequenced identifying in this gene a point mutation which causes a nonsense mutation. Expression of this gene in MEW128 could restore the cell's L-SD activity.

The following sections are devoted to a discussion of these finding and their implications.

8.1 Normal transcription of the *sdaA* gene in strain MEW128

The previous work done in the Newman laboratory showed that MEW128 is a thiamine requiring strain which shows very little L-SD activity *in vivo* and can not grow with L-serine, glycine and L-leucine as carbon source. Nonetheless, the crude extract shows a great deal of L-SD activity *in vitro*, when iron and DTT were added, as does the parent strain. This suggested that MEW128 produces L-SD in an inactive form and that the inactive protein can be activated *in vitro* by iron and DTT (Newman *et al.*, 1985a). The mutation in strain MEW128 was roughly mapped to a location away from the L-SD genes i.e. *sdaA* and *sdaB* (Newman, personal communication). Therefore, one lypothesis is that the mutated gene product is involved in the post-translational activation of the L-SD protein, i.e. the assembly of Fe-S cluster in the protein. However, I noticed that even after activation by iron and DTT, the L-SD activity of MEW128 was only about 60% of

the parental strain *in vitro*. I wondered if the transcriptional regulation of the L-SD coding genes in MEW128 is also changed as a result of this point mutation? To answer this question, I decided to assay the expression of the *sdaA* gene in MEW128. This is relatively straightforward because *sdaA* is the only L-SD encoding gene which can be expressed in the presence of glucose in minimal medium. Furthermore, the regulatory mechanisms controlling expression of this gene are already very clear.

As shown in Table 10, in glucose minimal medium with glycine and leucine, MEW128 lost almost all of its L-SD activity *in vivo*. The triple mutant MEW999 showed no detectable L-SD activity in cells grown in the same medium. There was very little difference observed in the β-galactosidase activity made by these three strains. These data suggested that the *sdaA* gene still could be expressed and probably also could be translated normally by MEW128. That is, its loss of L-SD activity is not due to a failure of expression of the L-SD encoding genes.

By adding iron and DTT to the *in vivo* assay directly, a certain amount of L-SD activity was obtained by MEW128 cell culture. It is about 40% of the L-SD activity obtained by MEW1 with iron and DTT. This suggested that even *in vivo*, the inactive L-SD protein could be activated by inorganic iron and the reducing agent. Even the triple mutant showed some activity *in vivo*, though a very low amount (1.6 units L-SD activity as compared to 14.2 units for the parental strain) when provided with iron and DTT, which previously was ascribed to the Fenton-type nonenzymatic deamination of L-serine by iron and DTT (Newman *et al.*, 1990). The nonenzymatic deamination is slow and does not interfere with the interpretation of the results. In fact, the triple mutant results give the best measure of the nonenzymatic deamination as seen in the presence of many other

proteins.

The parental strain showed 29% more activity when iron and DTT were added *in vivo* than when they were not. Even allowing for the nonenzymatic deamination, this probably suggests that not only is L-SD made by the cell in an inactive form requiring further post-translational activation, but also some of the L-SD protein synthesized remains in an inactive form within the cytoplasm, even in the parental strain.

8.2 Location of the MEW128 mutation in the *ygfZ* gene

I constructed genomic libraries to screen for genes which allowed strain MEW128 to produce colonies on SGL medium. Very few colonies were found on the SGL plates.

Two complementing plasmids were isolated. As shown in Figure 48, with either of these two plasmids, MEW128 could grow on SGL plates, whether or not thiamine was added. Thus, SGL^- and $thiamine^-$ phenotypes are likely to be caused by the same gene mutation as originally suggested. Both plasmids also restored the L-SD activity of MEW128 grown in glucose minimal medium with glycine and L-leucine, the medium in which only *sdaA* is usually expressed (Table 11). With these two plasmids, MEW128 showed more L-SD activity, perhaps because the complementing gene was on a high copy plasmid pBR322. Therefore, the gene product was overexpressed in the cell and hence activated higher proportion of the expressed inactive L-SD protein than the parental strain.

Sequencing showed that the DNA fragments inserted in these two plasmids were the same. The inserted fragment is 5.24 kbp long and includes seven open reading frames: *fldB*, *ygfX*, *ygfY*, *ygfZ*, *yqfA*, *yqfB* and *bglA* (Figure 49). These seven genes are located at 65.5 min on the chromosomal DNA. Further restriction enzyme analysis of the plasmid

showed that *ygfZ* was the gene which could restore the L-SD deficiency of MEW128. I amplified the *ygfZ* gene from strain MEW128 and sent it for sequencing. A nonsense mutation which changed a glutamine-coding codon CAA into a stop codon TAA was identified at 559 bp inside the *ygfZ* gene.

The 981 bp *ygfZ* gene codes for a 36 kDa protein composed of 326 amino acid residues. The point mutation in MEW128 causes the premature termination of *ygfZ* transcription. The truncated message would be 561 bp long and would generate a 21 kDa protein. This shorter peptide presumably cannot function as the whole length protein, whatever that function might be, and indeed might not be stable enough to persist in the cell. This is the malfunction, presumably, which causes the phenotype of MEW128 (i.e. no L-SD activity, thiamine dependent).

8.3 pLtet*ygfZ* expression in MEW128

The plasmid pLtet*ygfZ* is induced in MEW128. The results shown in Table 12 and 13 suggested that *ygfZ* expression could restore L-SD activity in MEW128 as judged by the effects of cloning the plasmid pLtet*ygfZ* into it. Moreover, strain MEW128 with pLtet*ygfZ* grew well without thiamine. Thus, I concluded that the *ygfZ* mutation in MEW128 caused both the loss of L-SD activity and the requirement for thiamine.

I found that a change in the concentration of the inducer of *ygfZ* i.e. of aTc from 0 to 100 ng/mL did not increase the L-SD activity produced by MEW128 much (Table 13). This is interesting because without the inducer, the promoter PLtet01 of the plasmid pLtet should be highly repressed. The promoter strength in the presence of aTc (100 ng/mL) is about 3600 fold higher than without aTc as indicated by the increasing of luciferase activity under the regulation of PLtet01 (Lutz and Bujard, 1997). It is hard to imagine

that such a big difference in protein concentration would not alter L-SD activity if YgfZ acts as an enzyme. Furthermore, it seems unlikely that the small amount of YgfZ made in the absence of inducer by the plasmid pLtet, would be enough to allow the post-translational modification of L-SD enzyme, as by providing for the synthesis of the Fe-S cluster in L-SD. However, if YgfZ acts as a regulator of a promoter, it would be possible that the protein acts at a low concentration and that a small change in its concentration might cause the promoter to switch off or on. Therefore, rather than an enzyme which catalyzes specific reaction, YgfZ protein seems more likely involved indirectly in the activating reaction of L-SD. For instance, YgfZ might be a regulator of the promoter for the enzymes which are for Fe-S cluster synthesis of L-SD.

8.4 The regulation of *ygfZ* expression

I tried to investigate the regulation of *ygfZ* and deduce its possible metabolic role. Compared to the β-galactosidase activity expressed from the promoter of *sdaA* (524, Table 10), the activity generated from the promoter of *ygfZ* is lower (13.8 units), this indicates that the promoter of *ygfZ* is weak, suggesting that YgfZ functions at a low concentration in *E. coli.*

Since a mutation in the *ygfZ* gene caused a deficiency of L-SD activity in *E. coli.* I wondered if the regulators of the expression of L-SD encoding genes affected the expression of *ygfZ.* L-leucine and glycine are two inducers of *sdaA* gene and thus were tested. Furthermore, the other four amino acids L-serine, L-methionine, L-lysine and L-threonine and CAA, which were known to have a great impact on the growth of cells with L-SD activity deficiency, were also tested for effects on the promoter of *ygfZ* gene.

However, as shown in Table 14, with strain MEW1, adding amino acids to the

medium did not show an obvious impact on the expression of *ygfZ*. However, CAA caused a considerable increase (1.7 fold). This is also true for MEW999, which had a β-galactosidase activity of about 17.8 in glucose minimal medium and 36.6 with CAA added to the medium. This might be a sign that when *E. coli* need L-SD activity to maintain normal growth, it will try to activate more L-SD than usual and this will consequently affect the expression of *ygfZ*.

8.5 Study on YgfZ

YgfZ has been relatively little studied, and what studies there are focused on the protein's structure, crystal structure and ligand binding characteristics.

The homologs of YgfZ are found in many organisms such as bacteria, fungi, plants and mammals, but not in archaea. Three amino acid residues Arg68, Arg237 and Lys245 are conserved throughout the different species (Teplyakov *et al.*, 2004). However, there is no further report about the possible role of these three amino acid residues in YgfZ structure or function.

The crystal structure of YgfZ was determined by Teplyakov *et al.* (2004). As shown in Figure 53, its three-dimensional structure is very similar to GcvT (aminomethyltransferase) of the GCV system from *Thermotoga maritime* and *Pyrococcus horikoshii* and to the C-terminal THF-binding region of enzyme DMGO (dimethylglycine oxidase) from the bacterium *Arthrobacter globiformis* (Lokanath *et al.*, 2004; Scrutton and Leys, 2005; Teplyakov *et al.*, 2004). All of these three proteins compose a folate-binding structure composed of three domains which form a cloverleaf-like or ring-like structure with a central hole accommodating folate.

The enzymatic functions of two earlier known proteins are well studied. GcvT

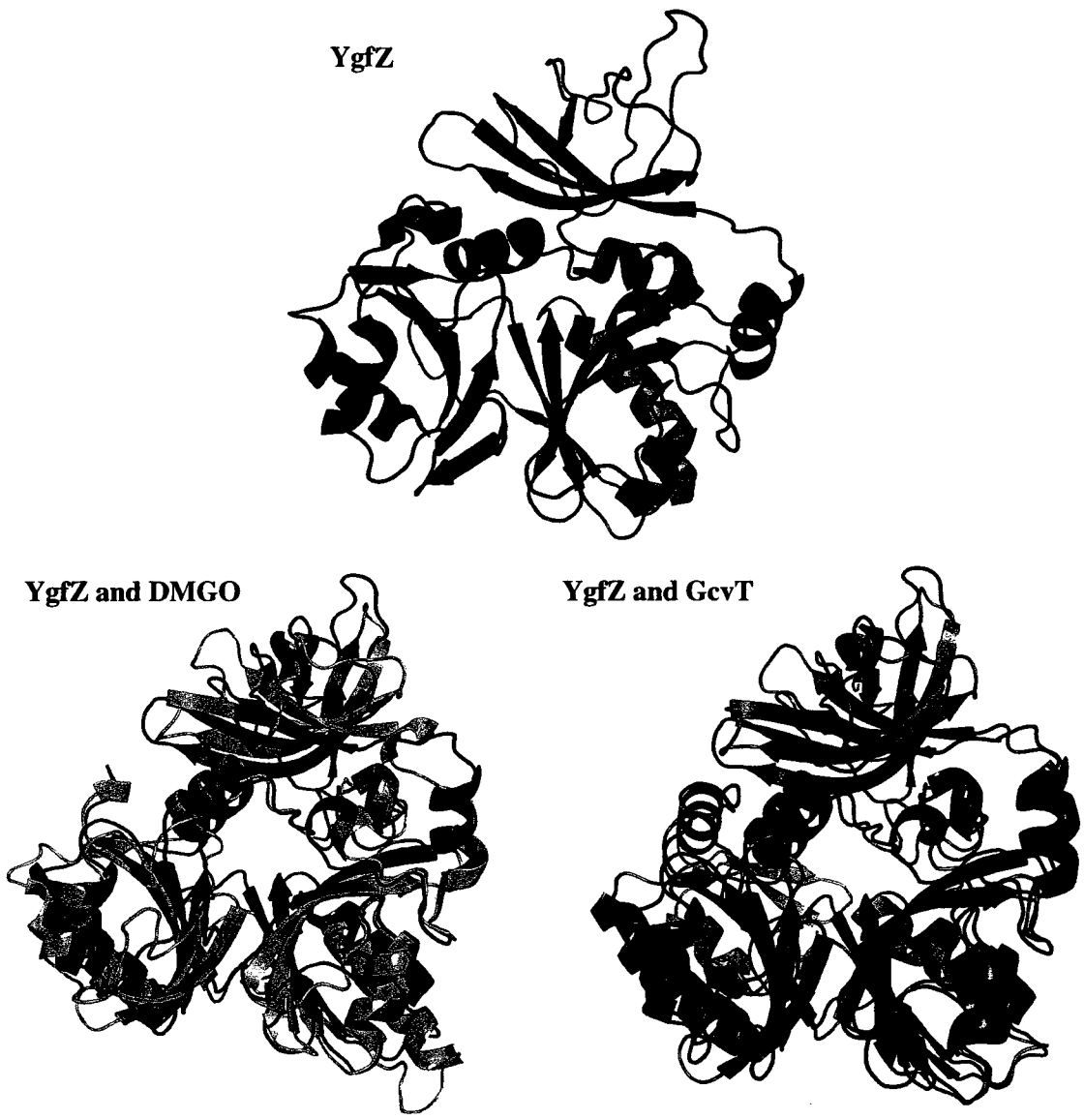


Figure 53 Structural superimposition of the YgfZ with GcvT and the THF-binding region of DMGO

YgfZ is depicted in the top in red. The bottom-left view depicts a superimposition of YgfZ with the corresponding C-terminal THF-binding region of DMGO (lime green). YgfZ is coloured red. The bottom-right view depicts a superimposition of GcvT (skyblue) with the YgfZ (red) structure.

catalyzes the reaction forming methylTHF from glycine, releases ammonia from the intermediate attached to the H-protein, and transfers the methyl group to THF (tetrahydrofolate). Dimethylglycine oxidase (DMGO) is a bifunctional enzyme that catalyzes the oxidation of N, N-dimethylglycine and the formation of N_5, N_{10} methyleneTHF. Although the crystal structure of YgfZ is similar to that of the other two proteins, YgfZ just has marginal sequence identity to GcvT (15%) and DMGO (26%). The abilities of YgfZ to bind folic acid and tetrahydrofolate were confirmed by measuring the fluorescence of tryptophan residues. It was suggested that two tryptophan residues, Trp27 and Trp189, which are located in the central cavity interact with the ligand (Teplyakov *et al.*, 2004). Although YgfZ has the structure for binding a folic acid derivative, an acidic residue Asp552 which is conserved in all GcvT enzymes is missing in YgfZ. This residue is very important for the catalytic ability of the enzyme. Thus, it is very likely that YgfZ does not have the same enzyme function as the GcvT, i.e. transferring the methyl group to the folic acid (Teplyakov *et al.*, 2004).

Most of the YgfZ family has a structure of a concave surface covered by basic amino acid residues. This suggests its ability to bind nucleic acids and act as a transcriptional regulator. These investigators suggested that the YgfZ family functions in regulation of transcription presumably of genes involved in C1 metabolism (Teplyakov *et al.*, 2004).

The possible function of YgfZ protein in C1 metabolism regulation is very interesting in view of my study about the metabolic function of L-SD in the first part of this thesis, which suggests that L-SD plays a very important role in C1 metabolism. Deleting all three L-SD encoding genes in *E. coli* will result in unbalanced C1 unit metabolism, thus causing a serious growth deficiency of the cells incubated in minimal medium with CAA

or in rich medium LB. Thus YgfZ seems to act in an area of metabolism in which L-SD is also involved, even if neither mechanism is known.

A possible role of YgfZ in relieving oxidative stress was suggested by Chen *et. al.* in 2006. The induction of YgfZ was observed in the presence of plumbagin, a redox-cycling chemical, and this induction was mediated by SoxS, a transcriptional regulator participating in controlling several genes involved in the response to oxidative stress (Chen *et al.*, 2006; Keseler *et al.*, 2005). Inaddition, Ote *et al.* (2006) found that the *ygfZ* mutant had a lower level of methylated tRNA. That the *ygfZ* mutant showed less methylated tRNA might be due to C1 metabolsim deficiency and scarcity of SAM in the cell because of the loss of L-SD activity.

8.6 YgfZ and Fe-S cluster synthesis

Why a *ygfZ* mutation should cause a loss of post-translational activation of L-SD and a deficiency in thiamine synthesis is unknown.

The thiamine biosynthetic pathway in *E. coli* involves 12 genes (Begley *et al.*, 1999). Among these, ThiH seems a likely candidate for a relation with L-serine metabolism because it is a radical SAM enzyme and it uses a [4Fe-4S] cluster. ThiH is a subunit of the ThiGH complex, which catalyzes the synthesis of 4-methyl-5-(β-hydroxyethyl) thiazole phosphate, the rate-limiting step in thiamine synthesis (Leonardi *et al.*, 2003). The enzymes of the radical SAM protein super family to which ThiH belongs display a wider variety of biochemical reactions. They contain a Cys-X-X-X-Cys-X-X-Cys signature motif (Frey *et al.*, 2008), used for catalysis a [4Fe-4S] cluster in which three sulfurs are contributed by cysteine, and the last by SAM (Sofia *et al.*, 2001). Since this enzyme requires SAM, it is clear that starvation for SAM and/or C1 units could prevent

its function. This is clearly shown by the fact that the *in vitro* activity of ThiH was doubled by adding SAM and reducing agent (NADPH) (Leonardi and Roach, 2004).

Problems in thiamine synthesis might arise from the deficiency in L-SD, or from a deficiency in the assembly of Fe-S clusters. I have shown in part one of this thesis that deletion of three L-SD coding genes in *E. coli* causes a deficiency in C1 metabolism. Since MEW128 is also deficient in L-SD activity, it too might be short of SAM. If the Km for inserting SAM into the Fe-S cluster of ThiH were high, the strain might be deficient in thiamine biosynthesis. However, one would then expect that the triple mutant would also require thiamine.

The use of radical SAM enzymes for the assembly of metallocofactors has been a subject of considerable recent study, particularly with respect to hydrogenase and nitrogenase (Curatti *et al.*, 2006; Leach and Zamble, 2007; Peters *et al.*, 2006). Two enzymes, HydE and HydG, involved in the synthesis of cluster H in hydrogenase were identified as radical SAM enzymes. The radical SAM motifs of HydE and HydG, involved in the synthesis of cluster H of the hydrogenase, are essential for production of active hydrogenase in *E. coli* (King *et al.*, 2006).

A SAM radical enzyme is also involved in the assembly of the iron-molybdenum cofactor FeMoCo of nitrogenase. Thus NifB, which is involved in the synthesis of the iron-molybdenum cofactor FeMoCo uses this method of catalysis (Curatti *et al.*, 2006). There is no further report indicating the involvement of SAM radical enzyme in the maturation of Fe-S enzymes. However, it is possible that such an enzyme carries out the assembly of Fe-S cluster in L-SD so that the cell can regulate the activity of L-SD in response to the C1 unit level inside the cell. ThiH might share the same Fe-S assembly

enzyme system as L-SD.

Reference

Adam, M., Fraipont, C., Rhazi, N., Nguyen-Disteche, M., Lakaye, B., Frere, J.M., Devreese, B., Van Beeumen, J., van Heijenoort, Y., van Heijenoort, J., and Ghuysen, J.M. (1997) The bimodular G57-V577 polypeptide chain of the class B penicillin-binding protein 3 of *Escherichia coli* catalyzes peptide bond formation from thiolesters and does not catalyze glycan chain polymerization from the lipid II intermediate. *J Bacteriol* **179**: 6005-6009.

Agar, J.N., Krebs, C., Frazzon, J., Huynh, B.H., Dean, D.R., and Johnson, M.K. (2000) IscU as a scaffold for iron-sulfur cluster biosynthesis: sequential assembly of [2Fe-2S] and [4Fe-4S] clusters in IscU. *Biochemistry* **39**: 7856-7862.

Ayala-Castro, C., Saini, A., and Outten, F.W. (2008) Fe-S cluster assembly pathways in bacteria. *Microbiol Mol Biol Rev* **72**: 110-125, table of contents.

Ayala, J.A.G., T.;de Pedro, M A.; Vicente,M. (1994) *Molecular biology of bacterial separation.* . Amsterdam: The Netherlands: Elsevier Science.

Baba, T., Ara, T., Hasegawa, M., Takai, Y., Okumura, Y., Baba, M., Datsenko, K.A., Tomita, M., Wanner, B.L., and Mori, H. (2006) Construction of *Escherichia coli* K-12 in-frame, single-gene knockout mutants: the Keio collection. *Mol Syst Biol* **2**: 2006 0008.

Bach, T., and Skarstad, K. (2004) Re-replication from non-sequesterable origins generates three-nucleoid cells which divide asymmetrically. *Mol Microbiol* **51**: 1589-1600.

Bairoch, A., and Boeckmann, B. (1993) The SWISS-PROT protein sequence data bank, recent developments. *Nucleic Acids Res* **21**: 3093-3096.

Balasubramanian, R., Shen, G., Bryant, D.A., and Golbeck, J.H. (2006) Regulatory roles for IscA and SufA in iron homeostasis and redox stress responses in the cyanobacterium *Synechococcus sp.* strain PCC 7002. *J Bacteriol* **188**: 3182-3191.

Barreteau, H., Kovac, A., Boniface, A., Sova, M., Gobec, S., and Blanot, D. (2008) Cytoplasmic steps of peptidoglycan biosynthesis. *FEMS Microbiol Rev* **32**: 168-207.

Begley, T.P., Downs, D.M., Ealick, S.E., McLafferty, F.W., Van Loon, A.P., Taylor, S., Campobasso, N., Chiu, H.J., Kinsland, C., Reddick, J.J., and Xi, J. (1999) Thiamin biosynthesis in prokaryotes. *Arch Microbiol* **171**: 293-300.

Beinert, H., and Kennedy, M.C. (1993) Aconitase, a two-faced protein: enzyme and iron regulatory factor. *Faseb J* **7**: 1442-1449.

Beinert, H., Holm, R.H., and Munck, E. (1997) Iron-sulfur clusters: nature's modular, multipurpose structures. *Science* **277**: 653-659.

Beinert, H. (2000) Iron-sulfur proteins: ancient structures, still full of surprises. *J Biol Inorg Chem* **5**: 2-15.

Bowler, L.D., and Spratt, B.G. (1989) Membrane topology of penicillin-binding protein 3 of *Escherichia coli*. *Mol Microbiol* **3**: 1277-1286.

Brendler T, A.A., Austin S. (1995) A protein that binds to the P1 origin core and the *oriC* 13mer region in a methylation-specific fashion is the product of the host *seqA* gene. *EMBO J.* **14**: 4083-4089.

Britten, R.J., Roberts, R.B., and French, E.F. (1955) Amino acid adsorption and protein synthesis in *Escherichia Coli*. *Proc Natl Acad Sci U S A* **41**: 863-870.

Britton, R.A., Lin, D.C., and Grossman, A.D. (1998) Characterization of a prokaryotic SMC protein involved in chromosome partitioning. *Genes Dev* **12**: 1254-1259.

Brown, E.A., D'Ari, R., and Newman, E.B. (1990) A relationship between L-serine degradation and methionine biosynthesis in *Escherichia coli* K12. *J Gen Microbiol* **136**: 1017-1023.

Buddelmeijer, N., and Beckwith, J. (2002) Assembly of cell division proteins at the *E. coli* cell center. *Curr Opin Microbiol* **5**: 553-557.

Burman, J.D., Harris, R.L., Hauton, K.A., Lawson, D.M., and Sawers, R.G. (2004) The iron-sulfur cluster in the L-serine dehydratase TdcG from *Escherichia coli* is required for enzyme activity. *FEBS Lett* **576**: 442-444.

Carballido-Lopez, R., and Errington, J. (2003) The bacterial cytoskeleton: *in vivo* dynamics of the actin-like protein Mbl of *Bacillus subtilis*. *Dev Cell* **4**: 19-28.

Carter, J.E., and Sagers, R.D. (1972) Ferrous ion-dependent L-serine dehydratase from *Clostridium acidiurici*. *J Bacteriol* **109**: 757-763.

Chen, J.W., Sun, C.M., Sheng, W.L., Wang, Y.C., and Syu, W.J. (2006) Expression Analysis of Up-Regulated Genes Responding to Plumbagin in *Escherichia coli*. *J Bacteriol* **188**: 456-463.

Cherepanov, P.P., and Wackernagel, W. (1995) Gene disruption in *Escherichia coli*: TcR and KmR cassettes with the option of Flp-catalyzed excision of the antibiotic-resistance determinant. *Gene* **158**: 9-14.

Cicchillo, R.M., Baker, M.A., Schnitzer, E.J., Newman, E.B., Krebs, C., and Booker, S.J. (2004) *Escherichia coli* L-serine deaminase requires a [4Fe-4S] cluster in catalysis. *J Biol Chem* **279**: 32418-32425.

Clarke, S. (1993) Protein methylation. *Curr Opin Cell Biol* **5**: 977-983.

Cohen, G.N. (1969) *The aspartokinases and homoserine dehydrogenases of Escherichia coli.* New York: Academic Press Inc.

Cosloy, S.D., and McFall, E. (1970) L-Serine-sensitive mutants of *Escherichia coli* K-12. *J Bacteriol* **103**: 840-841.

Cronan, J.E., Jr. (2002) Phospholipid modifications in bacteria. *Curr Opin Microbiol* **5**: 202-205.

Curatti, L., Ludden, P.W., and Rubio, L.M. (2006) NifB-dependent in vitro synthesis of the iron-molybdenum cofactor of nitrogenase. *Proc Natl Acad Sci U S A* **103**: 5297-5301.

Dai, K., and Lutkenhaus, J. (1992) The proper ratio of FtsZ to FtsA is required for cell division to occur in *Escherichia coli*. *J Bacteriol* **174**: 6145-6151.

Danilova, O., Reyes-Lamothe, R., Pinskaya, M., Sherratt, D., and Possoz, C. (2007) MukB colocalizes with the *oriC* region and is required for organization of the two

Escherichia coli chromosome arms into separate cell halves. *Mol Microbiol* **65**: 1485-1492.

Dasgupta, S., Maisnier-Patin, S., and Nordstrom, K. (2000) New genes with old modus operandi. The connection between supercoiling and partitioning of DNA in *Escherichia coli*. *EMBO Rep* **1**: 323-327.

Davis, L., and Metzler, D.E. (1972) *The Enzymes.* New York: Academic Press.

de Boer, P.A., Crossley, R.E., and Rothfield, L.I. (1989) A division inhibitor and a topological specificity factor coded for by the minicell locus determine proper placement of the division septum in *E. coli*. *Cell* **56**: 641-649.

Den Blaauwen, T., Buddelmeijer, N., Aarsman, M.E., Hameete, C.M., and Nanninga, N. (1999) Timing of FtsZ assembly in *Escherichia coli*. *J Bacteriol* **181**: 5167-5175.

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

Dey, S., Hu, Z., Xu, X.L., Sacchettini, J.C., and Grant, G.A. (2007) The effect of hinge mutations on effector binding and domain rotation in *Escherichia coli* D-3-phosphoglycerate dehydrogenase. *J Biol Chem* **282**: 18418-18426.

Eberhardt, C., Kuerschner, L., and Weiss, D.S. (2003) Probing the catalytic activity of a cell division-specific transpeptidase *in vivo* with beta-lactams. *J Bacteriol* **185**: 3726-3734.

Eccleston, J.F., Petrovic, A., Davis, C.T., Rangachari, K., and Wilson, R.J. (2006) The kinetic mechanism of the SufC ATPase: the cleavage step is accelerated by SufB. *J Biol Chem* **281**: 8371-8378.

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

Flardh, K., Garrido, T., and Vicente, M. (1997) Contribution of individual promoters in the *ddlB-ftsZ* region to the transcription of the essential cell-division gene ftsZ in *Escherichia coli*. *Mol Microbiol* **24**: 927-936.

Fontecave, M., and Ollagnier-de-Choudens, S. (2008) Iron-sulfur cluster biosynthesis in bacteria: Mechanisms of cluster assembly and transfer. *Arch Biochem Biophys* **474**: 226-237.

Fraser, J., and Newman, E.B. (1975) Derivation of glycine from threonine in *Escherichia coli* K-12 mutants. *J Bacteriol* **122**: 810-817.

Frey, P.A., Hegeman, A.D., and Ruzicka, F.J. (2008) The Radical SAM Superfamily. *Crit Rev Biochem Mol Biol* **43**: 63-88.

Fuller, R.S., Funnell, B.E., and Kornberg, A. (1984) The dnaA protein complex with the *E. coli* chromosomal replication origin (oriC) and other DNA sites. *Cell* **38**: 889-900.

Gayda, R.C., Henk, M.C., and Leong, D. (1992) C-shaped cells caused by expression of an *ftsA* mutation in *Escherichia coli*. *J Bacteriol* **174**: 5362-5370.

Giel, J.L., Rodionov, D., Liu, M., Blattner, F.R., and Kiley, P.J. (2006) IscR-dependent gene expression links iron-sulphur cluster assembly to the control of O2-regulated genes in *Escherichia coli*. *Mol Microbiol* **60**: 1058-1075.

Grabowski, R., and Buckel, W. (1991) Purification and properties of an iron-sulfur-containing and pyridoxal-phosphate-independent L-serine dehydratase from *Peptostreptococcus asaccharolyticus*. *Eur J Biochem* **199**: 89-94.

Grant, G.A., Xu, X.L., and Hu, Z. (2004) Quantitative relationships of site to site interaction in *Escherichia coli* D-3-phosphoglycerate dehydrogenase revealed by asymmetric hybrid tetramers. *J Biol Chem* **279**: 13452-13460.

Graumann, P.L. (2007) Cytoskeletal elements in bacteria. *Annu Rev Microbiol* **61**: 589-618.

Greene, R.C., and Radovich, C. (1975) Role of methionine in the regulation of serine hydroxymethyltransferase in *Eschericia coli*. *J Bacteriol* **124**: 269-278.

Guo, F., and Adhya, S. (2007) Spiral structure of *Escherichia coli* HUalphabeta provides foundation for DNA supercoiling. *Proc Natl Acad Sci U S A* **104**: 4309-4314.

Guzman, L.M., Barondess, J.J., and Beckwith, J. (1992) FtsL, an essential cytoplasmic membrane protein involved in cell division in *Escherichia coli*. *J Bacteriol* **174**: 7716-7728.

Hale, C.A., and de Boer, P.A. (1999) Recruitment of ZipA to the septal ring of *Escherichia coli* is dependent on FtsZ and independent of FtsA. *J Bacteriol* **181**: 167-176.

Hale, C.A., Meinhardt, H., and de Boer, P.A. (2001) Dynamic localization cycle of the cell division regulator MinE in *Escherichia coli*. *Embo J* **20**: 1563-1572.

Hama, H., Sumita, Y., Kakutani, Y., Tsuda, M., and Tsuchiya, T. (1990) Target of serine inhibition in *Escherichia coli*. *Biochem Biophys Res Commun* **168**: 1211-1216.

Hama, H., Kayahara, T., Tsuda, M., and Tsuchiya, T. (1991) Inhibition of homoserine dehydrogenase I by L-serine in Escherichia coli. *J Biochem* **109**: 604-608.

Hara, H., Yasuda, S., Horiuchi, K., and Park, J.T. (1997) A promoter for the first nine genes of the *Escherichia coli* mra cluster of cell division and cell envelope biosynthesis genes, including ftsI and ftsW. *J Bacteriol* **179**: 5802-5811.

Hesslinger, C., Fairhurst, S.A., and Sawers, G. (1998) Novel keto acid formate-lyase and propionate kinase enzymes are components of an anaerobic pathway in *Escherichia coli* that degrades L-threonine to propionate. *Mol Microbiol* **27**: 477-492.

Hiraga, S., Niki, H., Ogura, T., Ichinose, C., Mori, H., Ezaki, B., and Jaffe, A. (1989) Chromosome partitioning in *Escherichia coli*: novel mutants producing anucleate cells. *J Bacteriol* **171**: 1496-1505.

Hofmeister, A.E., Grabowski, R., Linder, D., and Buckel, W. (1993) L-serine and L-threonine dehydratase from *Clostridium propionicum*. Two enzymes with different prosthetic groups. *Eur J Biochem* **215**: 341-349.

Hofmeister, A.E., Albracht, S.P., and Buckel, W. (1994) Iron-sulfur cluster-containing L-serine dehydratase from *Peptostreptococcus asaccharolyticus*: correlation of the cluster type with enzymatic activity. *FEBS Lett* **351**: 416-418.

Hofmeister, A.E., Textor, S., and Buckel, W. (1997) Cloning and expression of the two genes coding for L-serine dehydratase from *Peptostreptococcus asaccharolyticus*: relationship of the iron-sulfur protein to both L-serine dehydratases from *Escherichia coli*. *J Bacteriol* **179**: 4937-4941.

Holmes, V.F., and Cozzarelli, N.R. (2000) Closing the ring: links between SMC proteins and chromosome partitioning, condensation, and supercoiling. *Proc Natl Acad Sci U S A* **97**: 1322-1324.

Holtje, J.V. (1996) Molecular interplay of murein synthases and murein hydrolases in *Escherichia coli*. *Microb Drug Resist* **2**: 99-103.

Holtje, J.V. (1998) Growth of the stress-bearing and shape-maintaining murein sacculus of *Escherichia coli*. *Microbiol Mol Biol Rev* **62**: 181-203.

Hu, Z., Mukherjee, A., Pichoff, S., and Lutkenhaus, J. (1999) The MinC component of the division site selection system in *Escherichia coli* interacts with FtsZ to prevent polymerization. *Proc Natl Acad Sci U S A* **96**: 14819-14824.

Hu, Z., and Lutkenhaus, J. (2001) Topological regulation of cell division in *E. coli*. spatiotemporal oscillation of MinD requires stimulation of its ATPase by MinE and phospholipid. *Mol Cell* **7**: 1337-1343.

Huang, K.C., Meir, Y., and Wingreen, N.S. (2003) Dynamic structures in *Escherichia coli*: spontaneous formation of MinE rings and MinD polar zones. *Proc Natl Acad Sci U S A* **100**: 12724-12728.

Hughes, J.A., Brown, L.R., and Ferro, A.J. (1987) Expression of the cloned coliphage T3 S-adenosylmethionine hydrolase gene inhibits DNA methylation and polyamine biosynthesis in *Escherichia coli*. *J Bacteriol* **169**: 3625-3632.

Huisman, O., D'Ari, R., and George, J. (1980) Further characterization of *sfiA* and *sfiB* mutations in *Escherichia coli*. *J Bacteriol* **144**: 185-191.

Huisman, O., D'Ari, R., and Gottesman, S. (1984) Cell-division control in *Escherichia coli*: specific induction of the SOS function SfiA protein is sufficient to block septation. *Proc Natl Acad Sci U S A* **81**: 4490-4494.

Isenberg, S., and Newman, E.B. (1974) Studies on L-serine deaminase in *Escherichia coli* K-12. *J Bacteriol* **118**: 53-58.

Jacob, F., and Brenner, S. (1963) [On the regulation of DNA synthesis in bacteria: the hypothesis of the replicon.]. *C R Hebd Seances Acad Sci* **256**: 298-300.

Johnson, D.C., Dean, D.R., Smith, A.D., and Johnson, M.K. (2005) Structure, function, and formation of biological iron-sulfur clusters. *Annu Rev Biochem* **74**: 247-281.

Joseleau-Petit, D., Liebart, J.C., Ayala, J.A., and D'Ari, R. (2007) Unstable *Escherichia coli* L forms revisited: growth requires peptidoglycan synthesis. *J Bacteriol* **189**: 6512-6520.

Kar, S., Edgar, R., and Adhya, S. (2005) Nucleoid remodeling by an altered HU protein: reorganization of the transcription program. *Proc Natl Acad Sci U S A* **102**: 16397-16402.

Keseler, I.M., Collado-Vides, J., Gama-Castro, S., Ingraham, J., Paley, S., Paulsen, I.T., Peralta-Gil, M., and Karp, P.D. (2005) EcoCyc: a comprehensive database resource for *Escherichia coli*. *Nucleic Acids Res* **33**: D334-337.

King, P.W., Posewitz, M.C., Ghirardi, M.L., and Seibert, M. (2006) Functional studies of [FeFe] hydrogenase maturation in an *Escherichia coli* biosynthetic system. *J Bacteriol* **188**: 2163-2172.

Koch, A.L. (2000) The bacterium's way for safe enlargement and division. *Appl Environ Microbiol* **66**: 3657-3663.

Kroos, L., and Maddock, J.R. (2003) Prokaryotic development: emerging insights. *J Bacteriol* **185**: 1128-1146.

Lang, T., and Kessler, D. (1999) Evidence for cysteine persulfide as reaction product of L-Cyst(e)ine C-S-lyase (C-DES) from *Synechocystis*. Analyses using cystine analogues and recombinant C-DES. *J Biol Chem* **274**: 189-195.

Leach, M.R., and Zamble, D.B. (2007) Metallocenter assembly of the hydrogenase enzymes. *Curr Opin Chem Biol* **11**: 159-165.

Lederberg, J. (1956) Bacterial Protoplasts Induced by Penicillin. *Proc Natl Acad Sci U S A* **42**: 574-577.

Lemon, K.P., and Grossman, A.D. (1998) Localization of bacterial DNA polymerase: evidence for a factory model of replication. *Science* **282**: 1516-1519.

Lemon, K.P., and Grossman, A.D. (2000) Movement of replicating DNA through a stationary replisome. *Mol Cell* **6**: 1321-1330.

Leonard, A.C., and Grimwade, J.E. (2005) Building a bacterial orisome: emergence of new regulatory features for replication origin unwinding. *Mol Microbiol* **55**: 978-985.

Leonardi, R., Fairhurst, S.A., Kriek, M., Lowe, D.J., and Roach, P.L. (2003) Thiamine biosynthesis in *Escherichia coli*: isolation and initial characterisation of the ThiGH complex. *FEBS Lett* **539**: 95-99.

Leonardi, R., and Roach, P.L. (2004) Thiamine biosynthesis in *Escherichia coli*: in vitro reconstitution of the thiazole synthase activity. *J Biol Chem* **279**: 17054-17062.

Lill, R., Diekert, K., Kaut, A., Lange, H., Pelzer, W., Prohl, C., and Kispal, G. (1999) The essential role of mitochondria in the biogenesis of cellular iron-sulfur proteins. *Biol Chem* **380**: 1157-1166.

Lin, R., D'Ari, R., and Newman, E.B. (1992) Lambda placMu insertions in genes of the leucine regulon: extension of the regulon to genes not regulated by leucine. *J Bacteriol* **174**: 1948-1955.

Lin, R.T., D'Ari, R., and Newman, E.B. (1990) The leucine regulon of *Escherichia coli* K-12: a mutation in *rblA* alters expression of L-leucine-dependent metabolic operons. *J Bacteriol* **172**: 4529-4535.

Lin, R.T. 1992, Characterization of the leucine/Lrp regulon in *Escherichia coli* K-12. Ph. D. thesis at Concordia University.

Loiseau, L., Ollagnier-de-Choudens, S., Nachin, L., Fontecave, M., and Barras, F. (2003) Biogenesis of Fe-S cluster by the bacterial Suf system: SufS and SufE form a new type of cysteine desulfurase. *J Biol Chem* **278**: 38352-38359.

Lokanath, N.K., Kuroishi, C., Okazaki, N., and Kunishima, N. (2004) Purification, crystallization and preliminary crystallographic analysis of the glycine-cleavage system component T-protein from Pyrococcus horikoshii OT3. *Acta Crystallogr D Biol Crystallogr* **60**: 1450-1452.

Lu, M., Campbell, J.L., Boye, E., and Kleckner, N. (1994) SeqA: a negative modulator of replication initiation in *E. coli*. *Cell* **77**: 413-426.

Lutkenhaus, J. (2007) Assembly dynamics of the bacterial MinCDE system and spatial regulation of the Z ring. *Annu Rev Biochem* **76**: 539-562.

Lutz, R., and Bujard, H. (1997) Independent and tight regulation of transcriptional units in *Escherichia coli* via the LacR/O, the TetR/O and AraC/I1-I2 regulatory elements. *Nucleic Acids Res* **25**: 1203-1210.

Margolin, W. (2001) Spatial regulation of cytokinesis in bacteria. *Curr Opin Microbiol* **4**: 647-652.

Margulies, C., and Kaguni, J.M. (1996) Ordered and sequential binding of DnaA protein to *oriC*, the chromosomal origin of *Escherichia coli*. *J Biol Chem* **271**: 17035-17040.

Matthews, R.G. (1996) *One-Carbon Metabolism.* Washington D. C.: American Society for Microbiology.

McKitrick, J.C., and Pizer, L.I. (1980) Regulation of phosphoglycerate dehydrogenase levels and effect on serine synthesis in *Escherichia coli* K-12. *J Bacteriol* **141**: 235-245.

Meisel, U., Holtje, J.V., and Vollmer, W. (2003) Overproduction of inactive variants of the murein synthase PBP1B causes lysis in *Escherichia coli*. *J Bacteriol* **185**: 5342-5348.

Mengin-Lecreulx, D., Ayala, J., Bouhss, A., van Heijenoort, J., Parquet, C., and Hara, H. (1998) Contribution of the Pmra promoter to expression of genes in the *Escherichia coli mra* cluster of cell envelope biosynthesis and cell division genes. *J Bacteriol* **180**: 4406-4412.

Migocki, M.D., Freeman, M.K., Wake, R.G., and Harry, E.J. (2002) The Min system is not required for precise placement of the midcell Z ring in *Bacillus subtilis*. *EMBO Rep* **3**: 1163-1167.

Mihara, H., and Esaki, N. (2002) Bacterial cysteine desulfurases: their function and mechanisms. *Appl Microbiol Biotechnol* **60**: 12-23.

Miller, B.A., and Newman, E.B. (1974) Control of serine transhydroxymethylase synthesis in *Escherichia coli* K12. *Can J Microbiol* **20**: 41-47.

Miller, J.H. (1972) *Experiments in molecular genetics.* Cold Spring Harbor, N.Y.: Cold Spring Harbor Laboratory Press.

Mingorance, J., Tamames, J., and Vicente, M. (2004) Genomic channeling in bacterial cell division. *J Mol Recognit* **17**: 481-487.

Monette, A. 2006. The Effects of *cpx* Genes on the Regulation of L-serine metabolism in *Escherichia coli* K-12. M. Sc. thesis at Concordia University.

Moriya, S., Kato, K., Yoshikawa, H., and Ogasawara, N. (1990) Isolation of a *dnaA* mutant of *Bacillus subtilis* defective in initiation of replication: amount of DnaA protein determines cells' initiation potential. *Embo J* **9**: 2905-2910.

Morris, J.F., and Newman, E.B. (1980) Map location of the *ssd* mutation in *Escherichia coli* K-12. *J Bacteriol* **143**: 1504-1505.

Mudd, S.H., and Cantoni, G.L. (1964) *Biological transmethylation, methyl-group neogenesis and other "one-carbon"metabolic reactions dependent upon tetrahydrofolic acid.* Elsevier, Amsterdam.

Mudd, S.H., Finkelstein, J.D., Irreverre, F., and Laster, L. (1965) Threonine dehydratase activity in humans lacking cystathionine synthase. *Biochem Biophys Res Commun* **19**: 665-670.

Mulder, E., and Woldringh, C.L. (1989) Actively replicating nucleoids influence positioning of division sites in *Escherichia coli* filaments forming cells lacking DNA. *J Bacteriol* **171**: 4303-4314.

Nachin, L., Loiseau, L., Expert, D., and Barras, F. (2003) SufC: an unorthodox cytoplasmic ABC/ATPase required for [Fe-S] biogenesis under oxidative stress. *Embo J* **22**: 427-437.

Nakamura, M., Maruyama, I.N., Soma, M., Kato, J., Suzuki, H., and Horota, Y. (1983) On the process of cellular division in *Escherichia coli*: nucleotide sequence of the gene for penicillin-binding protein 3. *Mol Gen Genet* **191**: 1-9.

Nanninga, N. (1998) Morphogenesis of *Escherichia coli*. *Microbiol Mol Biol Rev* **62**: 110-129.

Nash, H.A. (1996) *Regulation of gene expression in Escherichia coli,*. Austin, Texas Landes Company.

Newman, E.B., and Magasanik, B. (1963) The Relation of Serine--Glycine Metabolism to the Formation of Single-Carbon Units. *Biochim Biophys Acta* **78**: 437-448.

Newman, E.B., B. Miller, and Kapoor, a.V. (1974a) Biosynthesis of single-carbon units in *Escherichia coli* K12. *Biochim Biophys Acta* **338**: 529-539.

Newman, E.B., Fida, A., and Kapoor, V. (1974b) Causes of the inhibition of growth of *Escherichia coli* K12 by chlorate. *Can J Microbiol* **20**: 65-74.

Newman, E.B., and Kapoor, V. (1980) In vitro studies on L-serine deaminase activity of *Escherichia coli* K12. *Can J Biochem* **58**: 1292-1297.

Newman, E.B., Morris, J.F., Walker, C., and Kapoor, V. (1981) A mutation affecting L-serine and energy metabolism in *E. coli* K12. *Mol Gen Genet* **182**: 143-147.

Newman, E.B., Malik, N., and Walker, C. (1982) L-serine degradation in *Escherichia coli* K-12: directly isolated ssd mutants and their intragenic revertants. *J Bacteriol* **150**: 710-715.

Newman, E.B., and Walker, C. (1982) L-serine degradation in *Escherichia coli* K-12: a combination of L-serine, glycine, and leucine used as a source of carbon. *J Bacteriol* **151**: 777-782.

Newman, E.B., Dumont, D., and Walker, C. (1985a) *In vitro* and *in vivo* activation of L-serine deaminase in *Escherichia coli* K-12. *J Bacteriol* **162**: 1270-1275.

Newman, E.B., Miller, B., Colebrook, L.D., and Walker, C. (1985b) A mutation in *Escherichia coli* K-12 results in a requirement for thiamine and a decrease in L-serine deaminase activity. *J Bacteriol* **161**: 272-276.

Newman, E.B., Walker, C., and Ziegler-Skylakakis, K. (1990) A possible mechanism for the *in vitro* activation of L-serine deaminase activity in *Escherichia coli* K12. *Biochem Cell Biol* **68**: 723-728.

Newman, E.B., Budman, L.I., Chan, E.C., Greene, R.C., Lin, R.T., Woldringh, C.L., and D'Ari, R. (1998) Lack of S-adenosylmethionine results in a cell division defect in *Escherichia coli. J Bacteriol* **180**: 3614-3619.

Nievera, C., Torgue, J.J., Grimwade, J.E., and Leonard, A.C. (2006) SeqA blocking of DnaA-*oriC* interactions ensures staged assembly of the *E. coli* pre-RC. *Mol Cell* **24**: 581-592.

Niki, H., Jaffe, A., Imamura, R., Ogura, T., and Hiraga, S. (1991) The new gene *mukB* codes for a 177 kd protein with coiled-coil domains involved in chromosome partitioning of *E. coli. Embo J* **10**: 183-193.

Ogawa, H., Gomi, T., Konishi, K., Date, T., Nakashima, H., Nose, K., Matsuda, Y., Peraino, C., Pitot, H.C., and Fujioka, M. (1989) Human liver serine dehydratase. cDNA cloning and sequence homology with hydroxyamino acid dehydratases from other sources. *J Biol Chem* **264**: 15818-15823.

Ollagnier-de-Choudens, S., Mattioli, T., Takahashi, Y., and Fontecave, M. (2001) Iron-sulfur cluster assembly: characterization of IscA and evidence for a specific and functional complex with ferredoxin. *J Biol Chem* **276**: 22604-22607.

Ollagnier-de-Choudens, S., Sanakis, Y., and Fontecave, M. (2004) SufA/IscA: reactivity studies of a class of scaffold proteins involved in [Fe-S] cluster assembly. *J Biol Inorg Chem* **9**: 828-838.

Ollagnier-de Choudens, S., Nachin, L., Sanakis, Y., Loiseau, L., Barras, F., and Fontecave, M. (2003) SufA from Erwinia chrysanthemi. Characterization of a scaffold protein required for iron-sulfur cluster assembly. *J Biol Chem* **278**: 17993-18001.

Osborn, M.J., and Rothfield, L. (2007) Cell shape determination in *Escherichia coli*. *Curr Opin Microbiol* **10**: 606-610.

Ote, T., Hashimoto, M., Ikeuchi, Y., Su'etsugu, M., Suzuki, T., Katayama, T., and Kato, J. (2006) Involvement of the *Escherichia coli* folate-binding protein YgfZ in RNA modification and regulation of chromosomal replication initiation. *Mol Microbiol* **59**: 265-275.

Outten, F.W., Wood, M.J., Munoz, F.M., and Storz, G. (2003) The SufE protein and the SufBCD complex enhance SufS cysteine desulfurase activity as part of a sulfur transfer pathway for Fe-S cluster assembly in *Escherichia coli*. *J Biol Chem* **278**: 45713-45719.

Outten, F.W., Djaman, O., and Storz, G. (2004) A suf operon requirement for Fe-S cluster assembly during iron starvation in *Escherichia coli*. *Mol Microbiol* **52**: 861-872.

Paik, W.K., and Kim, S. (1990) *Protein Methylation.* Boca Baton: FL: CRC Press.

Pardee, A.B., and Prestidge, L.S. (1955) Induced formation of serine and threonine deaminases by *Escherichia coli*. *J Bacteriol* **70**: 667-674.

Pardee, A.B., and Yates, R.A. (1956a) Control of pyrimidine biosynthesis in *Escherichia coli* by a feed-back mechanism. *J Biol Chem* **221**: 757-770.

Pardee, A.B., and Yates, R.A. (1956b) Pyrimidine biosynthesis in *Escherichia coli*. *J Biol Chem* **221**: 743-756.

Peters, J.W., Szilagyi, R.K., Naumov, A., and Douglas, T. (2006) A radical solution for the biosynthesis of the H-cluster of hydrogenase. *FEBS Lett* **580**: 363-367.

Pichoff, S., and Lutkenhaus, J. (2007) Overview of cell shape: cytoskeletons shape bacterial cells. *Curr Opin Microbiol* **10**: 601-605.

Pizer, L.I. (1963) The Pathway and Control of Serine Biosynthesis in *Escherichia Coli*. *J Biol Chem* **238**: 3934-3944.

Pizer, L.I., and Potochny, M.L. (1964) Nutritional and Regulatory Aspects of Serine Metabolism in *Escherichia Coli. J Bacteriol* **88**: 611-619.

Pizer, L.I. (1965) Glycine Synthesis and Metabolism in *Escherichia Coli. J Bacteriol* **89**: 1145-1150.

Plamann, M.D., Rapp, W.D., and Stauffer, G.V. (1983) *Escherichia coli* K12 mutants defective in the glycine cleavage enzyme system. *Mol Gen Genet* **192**: 15-20.

Posnick, L.M., and Samson, L.D. (1999) Influence of S-adenosylmethionine pool size on spontaneous mutation, dam methylation, and cell growth of *Escherichia coli. J Bacteriol* **181**: 6756-6762.

Prozorov, A.A. (2005) [The bacterial cell cycle: DNA replication, nucleoid segregation, and cell division]. *Mikrobiologiia* **74**: 437-451.

Pruss, B.M., Nelms, J.M., Park, C., and Wolfe, A.J. (1994) Mutations in NADH:ubiquinone oxidoreductase of *Escherichia coli* affect growth on mixed amino acids. *J Bacteriol* **176**: 2143-2150.

Raskin, D.M., and de Boer, P.A. (1999a) MinDE-dependent pole-to-pole oscillation of division inhibitor MinC in *Escherichia coli. J Bacteriol* **181**: 6419-6424.

Raskin, D.M., and de Boer, P.A. (1999b) Rapid pole-to-pole oscillation of a protein required for directing division to the middle of *Escherichia coli. Proc Natl Acad Sci U S A* **96**: 4971-4976.

Reyes, R. (2005) Studies on a strain of *Escherichia coli* K-12 dependent on an exogenous supply of S-adenosylmethionine. In *Biology* Vol. M.S. Montreal: Concordia.

Rhee, S.G., Chock, P.B., and Stadtman, E.R. (1989) Regulation of *Escherichia coli* glutamine synthetase. *Adv Enzymol Relat Areas Mol Biol* **62**: 37-92.

Roberts, R.B., Abelson, P.H., Cowie, D.B., Bolton, E.T., and Britten, R.J. (1955) *Studies of biosynthesis in Escherichia coli.* Washington, D. C. : Carnegie Inst. Wash. Publ.

Rothfield, L.I., and Justice, S.S. (1997) Bacterial cell division: the cycle of the ring. *Cell* **88**: 581-584.

Russell, D.W., and Zinder, N.D. (1987) Hemimethylation prevents DNA replication in *E. coli*. *Cell* **50**: 1071-1079.

Sawers, G. (2001) A novel mechanism controls anaerobic and catabolite regulation of the *Escherichia coli tdc* operon. *Mol Microbiol* **39**: 1285-1298.

Sawitzke, J.A., and Austin, S. (2000) Suppression of chromosome segregation defects of *Escherichia coli muk* mutants by mutations in topoisomerase I. *Proc Natl Acad Sci USA* **97**: 1671-1676.

Schirch, V., and Szebenyi, D.M. (2005) Serine hydroxymethyltransferase revisited. *Curr Opin Chem Biol* **9**: 482-487.

Scrutton, N.S., and Leys, D. (2005) Crystal structure of DMGO provides a prototype for a new tetrahydrofolate-binding fold. *Biochem Soc Trans* **33**: 776-779.

Sendra, M., Ollagnier de Choudens, S., Lascoux, D., Sanakis, Y., and Fontecave, M. (2007) The SUF iron-sulfur cluster biosynthetic machinery: sulfur transfer from the SUFS-SUFE complex to SUFA. *FEBS Lett* **581**: 1362-1368.

Shao, Z. 1993. Molecular characterization of the *sdaCB* operon in *Escherichia coli* K-12. Ph. D. thesis at Concordia University.

Shao, Z., and Newman, E.B. (1993) Sequencing and characterization of the *sdaB* gene from *Escherichia coli* K-12. *Eur J Biochem* **212**: 777-784.

Slater S, W.S., Lu M, Boye E, Skarstad K, Kleckner N. (1995) *E. coli* SeqA protein binds oriC in two different methyl-modulated reactions appropriate to its roles in DNA replication initiation and origin sequestration. *Cell.* **82**: 927-936.

Sofia, H.J., Chen, G., Hetzler, B.G., Reyes-Spindola, J.F., and Miller, N.E. (2001) Radical SAM, a novel protein superfamily linking unresolved steps in familiar biosynthetic pathways with radical mechanisms: functional characterization using new analysis and information visualization methods. *Nucleic Acids Res* **29**: 1097-1106.

Spratt, B.G. (1977) Temperature-sensitive cell division mutants of *Escherichia coli* with thermolabile penicillin-binding proteins. *J Bacteriol* **131**: 293-305.

Stauffer, G.V., Baker, C.A., and Brenchley, J.E. (1974) Regulation of serine transhydroxymethylase activity in *Salmonella typhimurium*. *J Bacteriol* **120**: 1017-1025.

Stauffer, G.V., and Brenchley, J.E. (1974) Evidence for the involvement of serine transhydroxymethylase in serine and glycine interconversions in *Salmonella typhimurium*. *Genetics* **77**: 185-198.

Stauffer, G.V. (1996) *Biosynthesis of Serine, Glycine, and One-Carbon Units.* Washington, D. C.: American Society for Microbiology.

Stauffer, L.T., Plamann, M.D., and Stauffer, G.V. (1986) Cloning and characterization of the glycine-cleavage enzyme system of *Escherichia coli*. *Gene* **44**: 219-226.

Stauffer, L.T., and Stauffer, G.V. (1994) Characterization of the gcv control region from *Escherichia coli*. *J Bacteriol* **176**: 6159-6164.

Stover, P., and Schirch, V. (1991) 5-Formyltetrahydrofolate polyglutamates are slow tight binding inhibitors of serine hydroxymethyltransferase. *J Biol Chem* **266**: 1543-1550.

Su, H. 1991. Molecular studies of the *sdaA* and *sdaB* genes and their products in *Escherichia coli* K-12. Ph.D. thesis at Concordia University.

Su, H., and Newman, E.B. (1991) A novel L-serine deaminase activity in *Escherichia coli* K-12. *J Bacteriol* **173**: 2473-2480.

Su, H.S., Lang, B.F., and Newman, E.B. (1989) L-serine degradation in *Escherichia coli* K-12: cloning and sequencing of the *sdaA* gene. *J Bacteriol* **171**: 5095-5102.

Sumner, J., Jencks, D.A., Khani, S., and Matthews, R.G. (1986) Photoaffinity labeling of methylenetetrahydrofolate reductase with 8-azido-S-adenosylmethionine. *J Biol Chem* **261**: 7697-7700.

Sun, Q., Yu, X.C., and Margolin, W. (1998) Assembly of the FtsZ ring at the central division site in the absence of the chromosome. *Mol Microbiol* **29**: 491-503.

Sun, Q., and Margolin, W. (2001) Influence of the nucleoid on placement of FtsZ and MinE rings in *Escherichia coli*. *J Bacteriol* **183**: 1413-1422.

Sun, Q., and Margolin, W. (2004) Effects of perturbing nucleoid structure on nucleoid occlusion-mediated toporegulation of FtsZ ring assembly. *J Bacteriol* **186**: 3951-3959.

Szebenyi, D.M., Musayev, F.N., di Salvo, M.L., Safo, M.K., and Schirch, V. (2004) Serine hydroxymethyltransferase: role of *glu75* and evidence that serine is cleaved by a retroaldol mechanism. *Biochemistry* **43**: 6865-6876.

Tamames, J., Gonzalez-Moreno, M., Mingorance, J., Valencia, A., and Vicente, M. (2001) Bringing gene order into bacterial shape. *Trends Genet* **17**: 124-126.

Tang, Y.M. 2004. Role of nine cysteine residues in L-serine deaminase 1 from *Escherichia coli* K-12. M. Sc thesis at Concordia University.

Tazuya-Murayama, K., Aramaki, H., Mishima, M., Saito, K., Ishida, S., and Yamada, K. (2006) Effect of L-serine on the biosynthesis of aromatic amino acids in *Escherichia coli*. *J Nutr Sci Vitaminol (Tokyo)* **52**: 256-260.

Templin, M.F., Ursinus, A., and Holtje, J.V. (1999) A defect in cell wall recycling triggers autolysis during the stationary growth phase of *Escherichia coli*. *Embo J* **18**: 4108-4117.

Teplyakov, A., Obmolova, G., Sarikaya, E., Pullalarevu, S., Krajewski, W., Galkin, A., Howard, A.J., Herzberg, O., and Gilliland, G.L. (2004) Crystal structure of the YgfZ protein from *Escherichia coli* suggests a folate-dependent regulatory role in one-carbon metabolism. *J Bacteriol* **186**: 7134-7140.

Tucker, A.M., Winkler, H.H., Driskell, L.O., and Wood, D.O. (2003) S-adenosylmethionine transport in Rickettsia prowazekii. *J Bacteriol* **185**: 3031-3035.

Uzan, M., and Danchin, A. (1978) Correlation between the serine sensitivity and the derepressibility of the ilv genes in *Escherichia coli relA-* mutants. *Mol Gen Genet* **165**: 21-30.

Van Helvoort, J.M., Huls, P.G., Vischer, N.O., and Woldringh, C.L. (1998) Fused nucleoids resegregate faster than cell elongation in *Escherichia coli pbpB*(Ts) filaments after release from chloramphenicol inhibition. *Microbiology* **144 (Pt 5)**: 1309-1317.

Vicente, M., and Errington, J. (1996) Structure, function and controls in microbial division. *Mol Microbiol* **20**: 1-7.

Vicente, M., Gomez, M.J., and Ayala, J.A. (1998) Regulation of transcription of cell division genes in the *Escherichia coli dcw* cluster. *Cell Mol Life Sci* **54**: 317-324.

Vicente, M., and Lowe, J. (2003) Ring, helix, sphere and cylinder: the basic geometry of prokaryotic cell division. *EMBO Rep* **4**: 655-660.

Vicente, M., Rico, A.I., Martinez-Arteaga, R., and Mingorance, J. (2006) Septum enlightenment: assembly of bacterial division proteins. *J Bacteriol* **188**: 19-27.

Vollmer, W., and Bertsche, U. (2008) Murein (peptidoglycan) structure, architecture and biosynthesis in *Escherichia coli. Biochim Biophys Acta* **1778**: 1714-1734.

Vollmer, W., Joris, B., Charlier, P., and Foster, S. (2008) Bacterial peptidoglycan (murein) hydrolases. *FEMS Microbiol Rev* **32**: 259-286.

Wachi, M., Doi, M., Tamaki, S., Park, W., Nakajima-Iijima, S., and Matsuhashi, M. (1987) Mutant isolation and molecular cloning of *mre* genes, which determine cell shape, sensitivity to mecillinam, and amount of penicillin-binding proteins in *Escherichia coli. J Bacteriol* **169**: 4935-4940.

Wachtershauser, G. (1992) Groundworks for an evolutionary biochemistry: the iron-sulphur world. *Prog Biophys Mol Biol* **58**: 85-201.

Wang, S., Arends, S.J., Weiss, D.S., and Newman, E.B. (2005) A deficiency in S-adenosylmethionine synthetase interrupts assembly of the septal ring in *Escherichia coli* K-12. *Mol Microbiol* **58**: 791-799.

Wei, Y., and Newman, E.B. (2002) Studies on the role of the *metK* gene product of *Escherichia coli* K-12. *Mol Microbiol* **43**: 1651-1656.

Weigel, C., Schmidt, A., Ruckert, B., Lurz, R., and Messer, W. (1997) DnaA protein binding to individual DnaA boxes in the *Escherichia coli* replication origin, oriC. *Embo J* **16**: 6574-6583.

Weiss, D.S., Chen, J.C., Ghigo, J.M., Boyd, D., and Beckwith, J. (1999) Localization of FtsI (PBP3) to the septal ring requires its membrane anchor, the Z ring, FtsA, FtsQ, and FtsL. *J Bacteriol* **181**: 508-520.

Weiss, D.S. (2004) Bacterial cell division and the septal ring. *Mol Microbiol* **54**: 588-597.

Wilson, R.L., Stauffer, L.T., and Stauffer, G.V. (1993) Roles of the GcvA and PurR proteins in negative regulation of the *Escherichia coli* glycine cleavage enzyme system. *J Bacteriol* **175**: 5129-5134.

Woldringh, C.L., Mulder, E., Valkenburg, J.A., Wientjes, F.B., Zaritsky, A., and Nanninga, N. (1990) Role of the nucleoid in the toporegulation of division. *Res Microbiol* **141**: 39-49.

Woldringh, C.L., Mulder, E., Huls, P.G., and Vischer, N. (1991) Toporegulation of bacterial division according to the nucleoid occlusion model. *Res Microbiol* **142**: 309-320.

Woldringh, C.L., and Nanninga, N. (2006) Structural and physical aspects of bacterial chromosome segregation. *J Struct Biol* **156**: 273-283.

Young, K.D. (2003) Bacterial shape. *Mol Microbiol* **49**: 571-580.

Young, K.D. (2006) The selective value of bacterial shape. *Microbiol Mol Biol Rev* **70**: 660-703.

Yu, X.C., and Margolin, W. (1999) FtsZ ring clusters in min and partition mutants: role of both the Min system and the nucleoid in regulating FtsZ ring localization. *Mol Microbiol* **32**: 315-326.

Yuvaniyama, P., Agar, J.N., Cash, V.L., Johnson, M.K., and Dean, D.R. (2000) NifS-directed assembly of a transient [2Fe-2S] cluster within the NifU protein. *Proc Natl Acad Sci U S A* **97**: 599-604.

Zheng, L., Cash, V.L., Flint, D.H., and Dean, D.R. (1998) Assembly of iron-sulfur clusters. Identification of an iscSUA-hscBA-fdx gene cluster from *Azotobacter vinelandii*. *J Biol Chem* **273**: 13264-13272.

Zheng, M., Wang, X., Templeton, L.J., Smulski, D.R., LaRossa, R.A., and Storz, G. (2001) DNA microarray-mediated transcriptional profiling of the *Escherichia coli* response to hydrogen peroxide. *J Bacteriol* **183**: 4562-4570.

Zimmerman, S.B., and Murphy, L.D. (1996) Macromolecular crowding and the mandatory condensation of DNA in bacteria. *FEBS Lett* **390**: 245-248.

Zimmerman, S.B. (2006) Shape and compaction of *Escherichia coli* nucleoids. *J Struct Biol* **156**: 255-261.

Zinecker, H., Andreesen, J.R., and Pich, A. (1998) Partial purification of an iron-dependent L-serine dehydratase from *Clostridium sticklandii*. *J Basic Microbiol* **38**: 147-155.

Zyskind, J.W., and Smith, D.W. (1992) DNA replication, the bacterial cell cycle, and cell growth. *Cell* **69**: 5-8.