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Characterization of a mutation demonstrating a link between the lack of S-adenosylmethionine and cell division in *Escherichia coli*

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ABSTRACT

Characterization of a mutation demonstrating a link between the lack of S-adenosylmethionine and cell division in *Escherichia coli*

Lorne Budman

The *metK* gene of *Escherichia coli* encodes for the enzyme S-adenosylmethionine (SAM) synthetase, which catalyzes the formation of S-adenosylmethionine. Isolated as a γ-glutamylmethyl ester-resistant mutant by Greene *et al.* (1973), a *metK84* mutant grows normally on rich media (Greene *et al.*1973; Lin *et al.*, 1990). This mutation has been transferred via transduction to an *lrp*$^+$ background, where normal growth rates on minimal medium require the presence of 50 μg/ml L-leucine. When the leucine concentration is reduced to 25 μg/ml or less, cells fail to divide, resulting in the formation of long filaments. These filaments can undergo DNA replication, but fail to form crosswalls. SAM synthetase activity assays indicate that *metK84* mutants maintain only 1.7% of the wild type activity when grown on minimal medium without added leucine. A mutation in *lrp*, or the addition of leucine increases *metK* expression in both wild type and mutant strains. When *metK* activity is increased to a level of 0.26 mmol/min/mg of protein (11.3% of wild type activity), the mutant strain undergoes normal growth and cell division. The wild type *metK* gene has been cloned, and expression of the plasmid-borne gene complements for the growth-deficiency phenotypes of *metK84*. However, when the plasmid is over-expressed, the culture becomes auxotrophic for methionine. This work indicates that a SAM deficiency results in a cell division defect in *E. coli*.

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

INTRODUCTION

1. S-adenosylmethionine is a major metabolite in *Escherichia coli* and other cells

S-Adenosylmethionine (SAM) is important in the cellular metabolism of many organisms. SAM is a methionine derivative associated with many metabolic reactions including the formation of the polyamine spermidine, the production of biotin, and the synthesis of the tRNA wobble base queuine (Fukuchi *et al.*, 1994; Guianvarc'h *et al.*, 1997; Slany *et al.*, 1993). SAM also contributes to the regulation of the methionine biosynthetic enzymes as a cofactor of the methionine repressor protein, MetJ (Greene and Radovich, 1975). The major role attributed to SAM, however, is as the primary methyl donor in metabolism, where many cellular processes including the biosynthesis of amino acids and other compounds, chemotaxis, DNA replication, DNA repair, and restriction modification are controlled by methylation (Hughes *et al.*, 1987).

SAM is produced from methionine and ATP in a reaction catalyzed by the SAM synthetase enzyme (Cantoni, 1951). While yeast mutants lacking a functional SAM synthetase require exogenous SAM in the medium to support growth, similar experiments cannot be performed with *E. coli*, since the cell cannot incorporate exogenous SAM to support growth (Charest *et al.*, 1978; Holloway *et al.*, 1970). Thus, while SAM has been proven to be an essential metabolite in yeast by comparing growth of SAM synthetase mutants in the presence and absence of SAM, studies in *E. coli* have relied on mutants with some residual SAM synthetase activity (Holloway, 1970).

In *E. coli*, it is the *metK* gene that encodes the SAM synthetase enzyme (Hunter *et al.*, 1975). Mutants in *metK* have been isolated by screening for resistance to the

methionine analogues ethionine and γ-glutamylmethyl ester (GGME) (Holloway *et al.*, 1970; Kraus *et al.*, 1979). However, none of the *metK* mutants isolated in this way is completely devoid of SAM synthetase activity. As well, attempts to isolate temperature sensitive *metK* mutants in *E. coli* have failed to yield mutants without some residual SAM synthetase activity (Satischandran *et al.*, 1990). Nevertheless, many different leaky *metK* mutants, all with reduced SAM synthetase activity, have been described. These mutants demonstrate phenotypes that include resistance to methionine analogues, elevated levels of the methionine biosynthetic enzymes cystathionine synthetase (MetB) and cystathionase (MetC) (contributing to an overproduction of methionine), and methionine/B12 auxotrophy (Holloway *et al.*, 1970; Kraus *et al.*, 1979; Greene *et al.*, 1973; Satischandran *et al.*, 1990). It has also been reported that *metK* mutants have a higher level of serine hydroxymethyltransferase activity, an enzyme which is essential for the formation of glycine, and other cellular components including the purines, thymine and methionine (Greene and Radovitch, 1975). However, the appearance of these phenotypes is often dependent on the extracellular environment.

The type of growth medium, and the presence, or absence of some factor(s) affects many of the phenotypes described for *metK* mutants. Greene *et al.* (1973) showed that mutants grown on rich medium (LB, or minimal medium containing 1 mg/ml casein hydrolysate) develop colonies after 24 h, while growth on minimal medium requires 5 mM L-leucine to yield colonies after two days. At the biochemical level, the *metK* mutants grown on minimal medium show very low SAM synthetase activity and elevated activity of cystathionine synthetase and cystathionase over that of a wild type strain. SAM synthetase activity increases, and a repression of the cystathionine synthetase and

cystathionase activity is noted for mutants grown on LB, although wild type levels are not restored by any of these factors (Greene *et al.*, 1973).

Another factor contributing to the difficult study of the leaky *metK* mutants, is the quick accumulation of suppressor mutations. To date the only mutations identified as suppressors of *metK* are in the *lrp* gene, but these are not the only type of suppressing mutations (Lin *et al.*, 1990). The *lrp* gene codes for the leucine-responsive regulatory protein (LRP), a DNA binding protein that regulates the expression of 35 to 75 genes in *E. coli* (see Newman *et al.*, 1996 for review). The presence of a secondary mutation in *lrp* increases the growth rate of *metK* mutants, and allows for growth on minimal medium in the absence of exogenous leucine (Lin *et al.*, 1990). Through the study and comparison of *metK* mutants versus *metK lrp* double mutants, Lin discovered another phenotype caused by the SAM synthetase deficiency of *metK*—the formation of long filamentous cells in the unsuppressed strain (Lin *et al.*, 1990).

2. Purpose of this study

This study is an attempt to characterize the effects of a true *metK* mutant. Such a study can answer a number of questions. First, what are the phenotypes of a true *metK* mutation? This requires the transfer of a *metK84* marker into a *lrp*$^+$ background, where growth becomes dependent on the leucine concentration. Second, why is growth of these mutants leucine-dependent, and what concentration of leucine is required to restore normal growth? Third, Lin *et al.* (1990) reported that *metK*$^-$ *lrp*$^+$ strains not only grow poorly on minimal media, but also fail to undergo normal cell division. What role does MetK, or ultimately SAM, play in cell division? What is the importance of Lrp in this

process? Is the formation of filaments truly related to the *metK84* marker, to Lrp, or is there some other factor causing this phenotype? By restoring the SAM synthetase levels of a *metK* mutant strain to normal, one can determine if normal growth and cell division are restored. This could be achieved by inducing expression of a plasmid-borne copy of *metK*$^+$. Such a construct could also be used to answer the question of whether or not *metK* is an essential gene in *E. coli*. Finally, if it is confirmed that SAM is essential for cell division, what product(s) is (are) methylated to allow the cell to divide?

This work was performed to address some of these questions, and by studying the *metK84* mutant, it was possible to characterize further the importance of the *metK* gene in *E. coli*. The *metK84* mutation leads to a reduction in the SAM synthetase activity, and this leads to a number of phenotypes, which I define here. Such a mutation leads to leucine-dependent growth, such that 50 μg/ml of leucine are required to support normal growth rates in minimal medium. When leucine concentration is reduced to 25 μg/ml or less, the culture grows poorly, and the cells develop into long filaments. Finally, I show that the leucine-dependent growth and filamentation phenotypes brought on by *metK84* can be eliminated by complementation from expression of a plasmid-borne copy of the wild type *metK* gene.

3. S-adenosylmethionine plays many roles in *E. coli*

SAM is directly and indirectly involved in many key metabolic processes in *E. coli*. To better understand the importance of SAM in metabolism, a description of some of the key roles played by SAM is presented below.

3.1 S-adenosylmethionine and the synthesis of polyamines

Polyamines are involved in many important biological processes of both eukaryotes and bacteria, including nucleic acid and protein synthesis (Fukuchi *et al.*, 1994). Arginine is converted to ornithine, which is then decarboxylated to yield the polyamine putrescine. In *E. coli*, putrescine is also synthesized by a second pathway, which converts arginine into agmantine, which is then hydrolyzed to form putrescine (Moore and Boyle, 1991). These two reactions are catalyzed respectively by the products of the *speB* and *speA* genes, which are located immediately upstream of the *metK* coding region. *metK* encodes the SAM synthetase gene required for the formation of SAM. SAM is then decarboxylated by the product of the *speD* gene, SAM decarboxylase. This form of SAM then reacts with putrescine to form the triamine spermidine. Spermidine is an essential polyamine, which is involved in DNA packaging, and also acts as regulator for the production of putrescine (Figure 1; see Glansdorff, 1996, for review).

3.2 S-adenosylmethionine and queuine formation

The tRNA molecules specific for the amino acids aspartate, asparagine, histidine, and tyrosine in bacteria and eukaryotes (other than yeast and archaea), are modified such that the wobble position of the anti-codons contains the nucleoside queuosine in place of guanine (Slany *et al*, 1994). The direct precursor to queuosine is epoxy-queuosine, and this is formed from tRNA precursors containing the product of the *queA* gene (Slany *et al.*, 1993). It has recently been demonstrated that the synthesis of epoxy-queuosine from the QueA protein requires a single cofactor—SAM (Slany *et al.*, 1993). It is in fact the

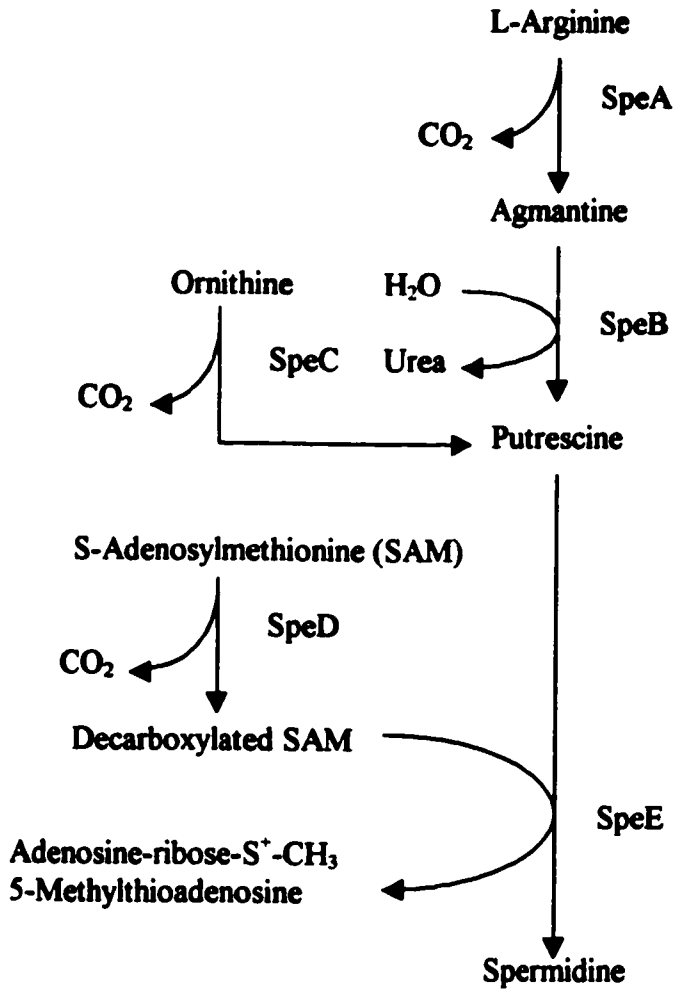

Figure 1. Biosynthesis of spermidine in *E. coli*. SpeA, arginine decarboxylase; SpeB, arginine ureohydrolase; SpeC, ornithine decarboxylase; SpeD, SAM decarboxylase; SpeE, Spermidine synthase.

ribosyl moiety of SAM that is required for the proposed reaction. The ribosyl moiety is transferred and isomerized to an epoxycyclopentane residue of epoxy-queuosine (Slany *et al.*, 1993). This represents the first example for which the ribosyl moiety of SAM has been shown to participate directly in a unique biosynthesis reaction (Slany *et al.*, 1993).

3.3 S-adenosylmethionine and biotin formation

Biotin is a vitamin produced in microorganisms and plants. The final step in the biotin biosynthetic pathway is catalyzed by the enzyme biotin synthase. This enzyme requires many cofactors, one of which is SAM (Shaw *et al.*, 1998). It has been suggested recently that biotin synthase may be a member of a larger family of enzymes, which requires a deoxyadenosyl radical generated from the reductive cleavage of SAM (Guianvarc'h *et al.*, 1997). Other enzymes dependent on the SAM-derived deoxyadenosyl radical include pyruvate formate-lyase, lysine 2,3-aminomutase, and anaerobic ribonucleotide reductase (Guianvarc'h *et al.*, 1997). A mechanism describing the stoichiometry of SAM usage in biotin synthesis has recently been proposed. Evidence suggests that dethiobiotin, the precursor of biotin, requires one SAM-derived radical for the formation of an intermediate, and a second radical is required to convert the intermediate into biotin (Guianvarc'h *et al.*, 1997; Shaw *et al.*, 1998).

3.4 S-adenosylmethionine as methyl donor

Methylation is a key metabolic process for the control of many cellular functions. Reactions dependent on methylation include the biosynthesis of amino acids, chemotaxis, DNA replication, DNA repair, and restriction modification (Hughes *et al*, 1987). For

instance, proteins interacting with DNA will react differently depending on the methylation state of the DNA. An example of this phenomenon is the sequence 5'-GATC-3', which in *Escherichia coli* can be modified at the A residue by the *dam*-encoded methylase. When methylated, the sequence can be cleaved by the restriction enzyme *Dpn*I, but not by the enzyme *Mbo*I, which cuts only unmethylated GATC sites to initiate cleavage (Barras and Marinus, 1989).

Modification of DNA by methylation is also important for the mismatch repair pathway, where the MutS protein recognizes the modified strand as the original template, and the MutH protein targets the newly formed, unmethylated strand for correction (Barras and Marinus, 1989). SAM supplies the methyl groups for *dam*-mediated modification of DNA.

A third example of SAM mediated methylation is the formation of cyclopropane fatty acids (CFAs). CFAs are present in the phospholipids of many bacterial species, and the formation of these molecules is 100% dependent on SAM methylation. Unsaturated fatty acids (UFAs) contain a methylene group which, upon receipt of a methyl group from SAM, is converted to a cyclopropane ring (see Grogan and Cronan, 1997, for review). The timing of CFA formation is typically at the onset of the stationary phase, and after the phospholipid molecules have been incorporated into the membrane bilayers (Grogan and Cronan, 1997). It has been reported that *metK* strains are still capable of producing CFAs, but that the rate of synthesis is slowed from that of the wild type (Greene *et al.*, 1973). While the widespread presence of CFAs throughout the cell suggests that these molecules are important constituents of the cell, there is currently no clear understanding of the physiological need for CFAs (Grogan and Cronan, 1997).

As the examples listed above indicate, methylation is essential to the proper control and function of many cellular processes. While it is SAM that acts as a donor to drive many methyl-directed processes, to fully appreciate the mechanisms of C_1 metabolism, it is important to consider the origin of the methyl group being transferred. The complete picture of C_1 metabolism requires a review of the biosynthesis of methionine.

4. The biosynthesis of methionine

The production of methionine has been extensively studied and many of the biosynthetic enzymes have been well defined (see Old *et al.*, 1991; Greene, 1996, for review). While a number of reactions form a dedicated methionine biosynthetic pathway, the production of methionine is also dependent on other converging metabolic pathways (Figure 2). As a member of the aspartate family of amino acids, which includes lysine and threonine, the carbon skeleton of methionine is derived from aspartate. Later a sulfur atom from cysteine, and a methyl group from the metabolism of serine are added to yield the completed methionine molecule (Figure 2; Old *et al.*, 1991). Once the methionine molecule has been produced, it can react with ATP to form SAM, the activated form of methionine. As will be discussed below, it is SAM, more so than methionine itself, that plays a role in the regulation of the methionine biosynthetic pathway (Greene *et al.*, 1970, 1973; Hobson and Smith, 1973).

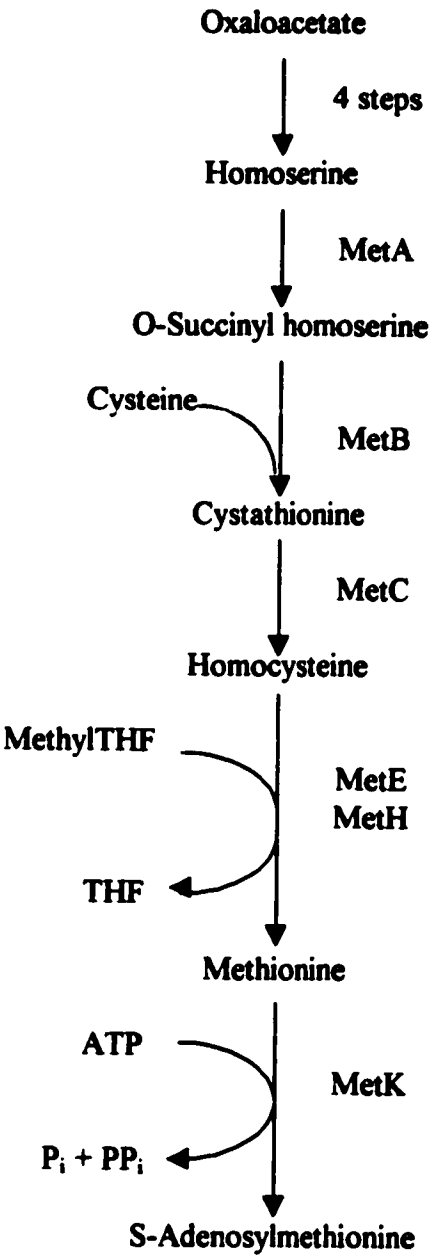

Figure 2. Biosynthesis of methionine and subsequent conversion to S-Adenosylmethionine (SAM). MetA, homoserine transsuccinylase; MetB, cystathionine γ-synthase; MetC, cystathionine β-lyase; MetE, vitamin B_{12}-independent homocysteine methyl-transferase; MetH, vitamin B_{12}-dependent homocysteine methyl-transferase.

4.1 The methionine biosynthetic enzymes

The methionine regulon consists of several genes scattered throughout the *E. coli* genome. Listed in Table 1 is a summary of the *met* genes, their genome map location, as well as the name and size of their protein product. Figure 2 shows a diagram of the methionine biosynthetic pathway, which begins with the conversion of aspartate to homoserine. This conversion takes place in three steps, which are reactions common to the production of threonine, lysine, and valine, in addition to methionine formation.

The next step is the formation of O-succinyl homoserine from homoserine and succinyl-CoA, in a reaction catalyzed by the product of the *metA* gene (Figure 2). The *metA* gene is located at 90.8' on the *E. coli* map, and it encodes a 35.7 kDa enzyme called homoserine trans-succinylase (Duclos *et al.*, 1989). This reaction represents the first step committed to the biosynthesis of methionine. As such, it also represents the first target of inhibition (Greene, 1996). While high concentrations of either methionine or SAM can inhibit MetA, a lesser concentration of the two metabolites together can more efficiently inhibit this enzyme (Lee *et al.*, 1966).

The next reaction in the methionine biosynthetic pathway provides the sulfur atom derived from the metabolism of cysteine. The product of the *metB* gene, cystathionine γ-synthase, catalyzes the conversion of O-succinylhomoserine to cystathionine (Figure 2). This gene has been cloned, and its product is a tetramer with a total molecular weight of 160 kDa (Kaplan and Flavin, 1965). The *metB* gene is located at 88.9 minutes on the *E. coli* map, as part of a cluster consisting of the *metJBLF* genes that map together (Old *et al.*, 1991). Together with the *metL* gene, *metB* comprises half of the only operon component of the methionine biosynthetic pathway (Greene and Smith, 1984).

The next step along this biosynthetic pathway results in the formation of homocysteine, the direct precursor to methionine (Figure 2). Homocysteine, ammonia, and pyruvate are produced from cystathionine in a reaction catalyzed by cystathionine β-lyase (cystathionase), the product of the *metC* gene. This gene, located at 67.9' on the *E. coli* map, encodes a protein with a molecular weight of 43 kDa (Belfaiza *et al.*, 1986). The structure of the MetC enzyme shows over 30% identity to that of MetB, and both of these enzymes show elevated activity in *metK* strains (Belfaiza *et al.*, 1986; Greene *et al.*, 1973). This suggests that MetB and MetC may have originated from a common ancestor.

The final step in the biosynthesis of methionine requires the convergence between the folate and methionine pathways (Old *et al.*, 1991). It is from the metabolism of serine that the methyl group of methionine is obtained, and this is passed along the folate pathway from serine to 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate which reacts with homocysteine to produce methionine (Figure 3).

Serine reacts with tetrahydrofolate to produce glycine and 5,10-methylenetetrahydrofolate, using serine hydroxymethyltransferase, the product of the *glyA* gene, as a catalyst (Greene and Radovich, 1975). Methyl groups are generated by this reaction for use in the synthesis of methionine, purines, and thymine (Old *et al.*, 1991). The regulation of *glyA* is dependent on the combined presence of serine, glycine, methionine, thymine, guanine, and adenine, but the repression from methionine involves a different mechanism from that which represses the other *met* genes (Old *et al.*, 1991).

The next step in the folate pathway is the irreversible transfer of a methyl group from 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate (Figure 3). This

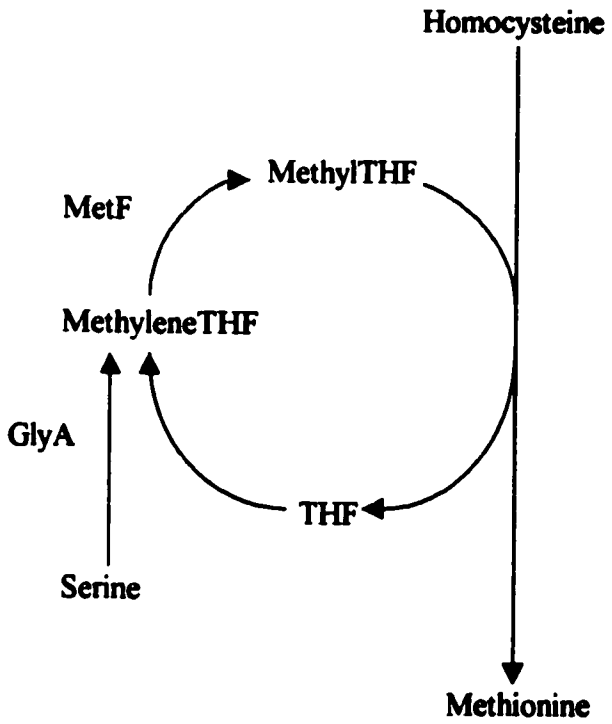

Figure 3. Intersection of the folate and methionine pathways. A methyl group derived from the degradation of serine is transferred to methionine with tetrahydrofolate (THF) as methyl donor. GlyA, serine hydroxymethyltransferase; MetF, 5,10-methyleneTHF reductase.

reduction reaction requires $FADH_2$ as a cofactor, and is catalyzed by the product of the *metF* gene, 5,10-methylenetetrahydrofolate reductase. This gene has been cloned, and the MetF enzyme consists of several peptides, each having a mass of about 33 kDa (Stauffer and Stauffer, 1988).

The final step in the biosynthesis of methionine is the transfer of a methyl group from 5-methyltetrahydrofolate to homocysteine (Figure 2). Unlike the other steps in the pathway described above, the methylation of homocysteine can be performed via two different mechanisms: one uses the *metH* gene product as a catalyst, while the second utilizes the *metE* gene product. The major difference between the two enzymes is that MetH has a requirement for vitamin B_{12} (cobalamine) as a cofactor, while the MetE enzyme works independently of this cofactor (Old *et al.*, 1991). Tetrahydrofolate derivatives can be found in two different forms: as a poly-glutamate, or as a mono-glutamate. While MetH can transfer a methyl group from either form in the conversion of homocysteine to methionine, MetE is only capable of a methyl group transfer from a tetrahydrofolate derivative with no fewer than three glutamate residues (Greene, 1996).

While both *metH* and *metE* have been cloned, the mechanism using the MetH enzyme is better understood. This enzyme is a monomer of molecular weight 136 kDa (Drummond *et al.*, 1993). The MetE protein is slightly smaller, with a molecular weight of about 85 kDa for the native enzyme (Greene, 1996). The MetE enzyme is also less efficient than that encoded by *metH*, showing less than 2% of the activity of MetH (Old *et al.*, 1991). Thus, a larger amount of this enzyme is required for the biosynthesis of methionine by the cobalamin-independent pathway (Greene, 1996).

The description of the methionine biosynthetic pathway is now complete. Once methionine is formed, it can now be used to initiate peptide synthesis in the form of *N*-Formylmethionyl-tRNA^{fMet}, or be added to a growing peptide chain. It can also react with ATP to form SAM, PP_i, and P_i. This reaction is catalyzed by SAM synthetase, product of the *metK* gene. Located at 66.5' on the *E. coli* map, this gene encodes a 180 kDa enzyme, which is a tetramer consisting of identical sub-units (Markham *et al.*, 1980). Together, methionine and SAM each play a role in the regulation of the methionine biosynthesis enzymes described above.

4.2 Regulation of the methionine biosynthetic pathway

The genes encoding the methionine biosynthetic enzymes are not part of a single operon system like that of the *lac* or *trp* genes. Nevertheless, most of these genes are regulated at the level of gene transcription. With the exception of the MetA enzyme, which as described above is inhibited directly by a combination of methionine and SAM, the other *met* genes are regulated by an aporepressor encoded by the *metJ* gene (Su and Greene, 1971). Strains defective in *metJ* expression overproduce methionine, and *metJ metK* double mutants were even more derepressed for methionine biosynthesis (Su and Greene, 1971; Greene *et al.*, 1970, 1973). These results led to the discovery that MetJ is a repressor of the *met* genes, and that SAM is required as a corepressor.

The MetJ DNA binding site is the eight base consensus sequence 5'-AGACGTCT-3', which is called a MET box (Belfaiza *et al.*, 1986; Old *et al.*, 1991). The sequence is often repeated, two or more times in a row, within the upstream region of the *met* biosynthetic genes, and as the number of repeats increases, so does the amount of

repression controlled by MetJ (Greene, 1996). The genes for which MetJ is greatest at repressing are *metF*, *metB*, *metL*, and *metJ*, which were all protected from DNaseI cleavage during DNA footprinting experiments (Shoeman *et al.*, 1985a, 1985b). In addition to controlling these genes, MET boxes have also been located upstream of the *metA*, *metC*, and *metE* coding regions (Greene, 1996). MetJ also represses the transcription of *metR*, a regulatory gene divergently transcribed from the *metE* coding region. The MetR protein is a regulatory protein that improves expression of *metE*, and stimulates the expression of *metH* (Greene, 1996). Although this protein is repressed by the MetJ repression system, the role it plays in the regulation of the MetE and MetH methyltransferases is carried out independent of SAM.

There are two exceptions to the rule with respect to regulation by the MetJ protein. The first is the *metH* gene, where there are no MET boxes in the region upstream of its coding sequence, and expression of *metH* is partly dependent on MetR (Greene, 1996). The second exception is the regulation of *glyA*, which encodes the serine hydroxymethyltransferase. While this gene undergoes some repression when methionine is supplemented in the medium, there is no significant increase in GlyA activity in *metJ* strains (Greene and Radovich, 1975). On the other hand, there is a significant increase to twice the wild type GlyA activity level in *metK* strains (Greene and Radovich). When SAM synthetase levels are increased, by growing the *metK* mutants on minimal medium supplemented with leucine, the *glyA* expression is repressed to near wild type levels (Greene and Radovich, 1975). These results suggest that while methionine offers some control of *glyA* expression, the mechanism of repression requires SAM, without the involvement of the *metJ* repressor protein.

5. Cell division proteins in *E. coli*

Through the study of cell division mutants, a number of proteins essential to the cell division process in *E. coli* have been identified. Together, these proteins make up the class of proteins called Fts, or Filamentous Temperature Sensitive proteins. Mutants lacking functional FtsA, I, K, L, N, Q, W, or Z are unable to undergo cell division, although chromosome replication and nuclear segregation continue normally (Hale and de Boer, 1997). These and other cell division mutants develop into filaments, long multinucleate cells, which are unable to propagate, and eventually die (Hale and de Boer, 1997).

5.1 The FtsZ protein

One of the most widely studied cell division proteins is the *ftsZ* gene product. Within the cell, it has been determined that FtsZ behaves in a manner similar to the eukaryotic protein tubulin. Like tubulin, FtsZ acts as a GTPase, and polymerizes into long tubules in a GTP-dependent manner (RayChaudhuri and Park, 1992; Lutkenhaus, 1993; Ma *et al.*, 1996). The interest in the FtsZ protein also stems from the discovery that FtsZ forms a cytokinetic ring at the site of septum formation in the dividing cell (See Lutkenhaus, 1993, for review). This ring is believed to drive the formation of the septum, and it was recently determined that FtsZ binds to the inner membrane protein ZipA. It has been suggested that ZipA connects FtsZ with the cytoplasmic membrane, and therefore is

essential to the assembly and/or function of the FtsZ ring in septum formation (Hale and de Boer, 1997).

Another aspect of the FtsZ has also been investigated. Using the multicopy plasmid pZAQ, which carries wild type copies of the *ftsZ*, *ftsA*, and *ftsQ* genes, it was determined that overproduction of FtsZ causes minicell formation in wild type cells (Lutkenhaus and Ward, 1985). The same plasmid was used to show that overproduction of FtsZ could suppress the formation of filaments normally resulting from a mutation at the *sulA* locus (Lutkenhaus, 1986).

5.2 FtsA, I, L, and Q

In addition to the FtsZ protein, analysis of other mutants has led to the discovery of other proteins essential to cell division, and the best described include FtsA, FtsI, FtsL, and FtsQ. The FtsA protein plays a role as an ATPase. As the FtsZ ring can be formed in *ftsA* mutants, this protein must act at a later stage in the septum formation process than FtsZ (Addinall *et al.*, 1996; Ma *et al.*, 1996). While it is known to be an essential cell division component localized to the cytoplasmic membrane, the exact role it plays in cell division remains unknown (Ma *et al.*, 1996).

The FtsI protein also acts after the formation of the FtsZ ring (Addinall *et al.*, 1996). This protein, also known as the penicillin binding protein PBP3, plays a role in peptidoglycan biosynthesis (Addinall *et al.*, 1996; Guzman *et al.*, 1997). Cells without functional FtsI, resulting from mutation or antibiotic deactivation, elongate, but fail to form septa (Guzman *et al.*, 1997). Thus the action of FtsI is expected to take place only

at the later stages of cell growth, and to aid in peptidoglycan formation required specifically for the formation of the septation (Guzman *et al.*, 1997).

The *ftsL* and *ftsQ* genes are very similar to the *ftsI* gene, and mutations at these loci result in a similar phenotype to that described for *ftsI* mutants (Guzman *et al.*, 1997). Very little is currently known about the roles played by FtsL or FtsQ. It has been suggested that FtsL may function as a leucine zipper, but additional evidence is required to confirm this hypothesis (Guzman *et al.*, 1997). The function of the FtsQ protein remains unknown.

5.3 The FtsJ protein

Also described recently was the discovery of the FtsJ protein in *E. coli*. This protein is essential for cell division, but not for growth (Ogura *et al.*, 1991). A noteworthy feature of FtsJ is that it has a SAM binding motif, and may play a role as a methyl-transferase enzyme (Ogura *et al.*, 1991).

The many roles played by SAM, as well as its complex regulation, reflect the importance of this molecule to the proper functioning of the cell. While many of these roles are well defined, little has been reported on the link between a lack of SAM and the formation of filaments in *E. coli*. For this reason, I set out to characterize further the importance of SAM for the proper growth and division of the cell. This goal was successfully achieved following the isolation of an unsuppressed *metK*⁻ mutant strain.

MATERIALS AND METHODS

1. Bacterial strains and plasmids

The bacterial strains used and isolated in this study are all derivatives of *E. coli* K-12, and are described in Table 2. Also included in Table 2 is a list of the plasmids used in this study.

2. Conditions for growth of bacteria

The bacteria were grown in either rich medium or minimal medium. The nutrient Luria-Bertani (LB) medium is a rich nutrient source consisting of 0.5% yeast extract, 0.5% NaCl, and 1% tryptone. The minimal medium was composed of 1.5% K_2HPO_4, 0.52% KH_2PO_4, 0.2% $(NH_4)_2SO_4$, 0.02% $MgSO_4$, 0.001% $CaCl_2$, 50 μg/ml L-isoleucine, 50 μg/ml L-valine. The added carbon source was 0.2% glucose or 0.2% glycerol, forming minimal glucose or minimal glycerol medium, respectively. The presence of isoleucine and valine is required due to the *ilvA* deletion of MEW1 (Table 2), the strain of *E. coli* from which all the other strains used in this study are derived (Lin *et al.*, 1990). The minimal medium containing no carbon source is called NIV solution. Antibiotics were added to the medium in the following concentrations: 200 μg/ml ampicillin, 25 μg/ml chloramphenicol, 50 μg/ml kanamycin, and 10 μg/ml tetracycline.

Bacterial growth in liquid medium was carried out in a 50 ml flask containing 5 ml of medium. The flask was agitated in a shaker and the culture incubated at a temperature of 37°C. Solid medium for bacterial growth consisted of 2% agar added to

either LB or NIV medium. Solid media (plates) were incubated in a humid environment at 37°C. Incubation times were approximately 16 hours, unless stated otherwise.

3. DNA manipulation

Enzymatic reactions with restriction endonuclease, and T4 ligase, were carried out according to the suppliers' instructions (MBI Fermentas, Pharmacia Biotech). Kits supplied by Qiagen were used for plasmid isolation, PCR product purification, and for extracting DNA from agarose gels following electrophoresis. Phage transduction and isolation, transformation of plasmid DNA into competent bacterial cells, agarose gel electrophoresis, and all other DNA manipulation was carried out according to a laboratory manual (Sambrook *et al.*, 1989).

4. Strain construction

The *metK84* mutation, first isolated in strain RG62, was transferred by Lin to the CU1008 strain background by cotransduction with *serA*$^+$ forming strain MEW30 (Lin *et al.*, 1990; Miller, 1972). The mutation was then transferred to recipient strains MEW311 *serA::λplac*Mu Δara$_{714}$, and DRN-1 by using phage grown on MEW30 and selecting on minimal glucose supplemented with leucine (100 μg/ml). Recipients were screened for leucine-dependent growth, and resistance to GGME. Strains with the *lrp* mutation were isolated by growing phage on MEW26 lrp::Tn10 and selecting on LB+tet. These transductants were then screened by Chen for the ability to grow using serine as a carbon source (Miller, 1972).

5. Construction of plasmids pKK*metK* and pBAD*metK*

Two unique products were amplified by PCR using chromosomal DNA extracted from strain MEW1 as template. Primer 1, CGAATTCCATCCATACCGATTAACACTC, corresponds to the region from –118 to –95 upstream of the ATG transcriptional start site of *metK*, with five additional nucleotides which add an *Eco*RI site to the sequence. Primer 2, ACGAAGCTTGAACGCAGGTGAAGAAAGATTAC, is identical to 24 bases at the 3' end of the gene, and includes six additional bases, which provide a *Hind*III site. The PCR product was digested with both *Eco*RI and *Hind*III, and then ligated to plasmid pKK223-3 previously digested with the same enzymes, thereby forming plasmid pKK*metK*.

A second PCR product was prepared using the same MEW1 chromosomal DNA as template. For this product, a different 5' primer was used. The primer CATCCCATGGCAAAACACCTTTTTACGTCC corresponds to bases +1 to +24 of the *metK* coding region, with a six base *Nco*I site added to the 5' end. The same 3' primer used for the pKK223-3 insert was utilized for this reaction. Following digestion with *Nco*I and *Hind*III, the DNA fragment containing the *metK*$^{+}$ sequence was ligated to plasmid pBAD22 pre-digested with the same enzymes, creating plasmid pBAD*metK*.

RESULTS

1. Isolation of a leucine-requiring GGME-resistant *metK* mutant

Strain MEW30, a *metK84* mutant constructed by Lin, requires leucine for growth, is resistant to GGME, and grows long filaments when leucine is limiting. The strain grows normally in a rich medium (LB), and the leucine requirement is suppressed by a mutation in the *lrp* gene (Lin *et al.*, 1990). To study the effects of the *metK84* mutation it is necessary to recreate the strain free of suppressors, at regular intervals. I, therefore, began this work by recreating the strain by transducing *metK84* into a fresh isolate with the genetic background of MEW.

To do this, I used phage P1 grown on MEW30 to transduce strain MEW311 (*serA::λPlac*Mu) to serine independence by selecting for growth on glucose minimal medium plates containing isoleucine, valine, and leucine (100 μg/ml). To determine if the transductants were leucine requiring, I streaked the colonies on minimal glucose plates with and without added leucine (100 μg/ml). While most of the colonies grew equally well independent of leucine concentration, some colonies grew better on plates containing leucine. The transductants requiring leucine were further screened on minimal glucose plates supplemented with leucine and GGME (500 μg/ml, a normally toxic amount). One leucine-requiring and GGME-resistant isolate, designated as strain MEW402, was studied further for this work.

2. Quantifying the leucine requirement of strain MEW402

To determine the quantity of leucine required for strain MEW402 to grow, I diluted a single colony of MEW402 in NIV liquid, and inoculated aliquots of the cells into flasks containing glucose minimal medium (5 ml) supplemented with 0, 2.5, 10, 25, and 50 μg/ml L-leucine. After an incubation period of 16 hours at 37°C, it was already evident from the turbidity of the cultures that growth was dependent on the leucine concentration. At this time, the 50 μg/ml leucine culture was very turbid with an OD_{600} reading of 1.518. Of the remaining cultures, only the 25 μg/ml leucine sample showed visible turbidity (OD_{600} = 0.445), but the culture was significantly less dense than the 50 μg/ml sample. The 0, 2.5, and 10 μg/ml cultures were all relatively clear, with measured OD_{600} values of 0.100, 0.097, and 0.083, respectively. Thus, 50 μg/ml leucine was sufficient for a fully grown culture, but 25 μg/ml leucine was not.

3. Filamentation in strain MEW402

The MEW402 cultures assayed for growth as a function of leucine concentration were also examined under the microscope at 400X magnification. After 16 hours of incubation at 37°C, the 50 μg/ml leucine culture consisted of cells of normal size (approximately 2 μm in length). However, in the culture supplemented with 25 μg/ml leucine, most of the cells were clearly not of the normal, wild type length, but had instead grown as long filaments. The majority of the cells growing in the 0, 2.5, and 10 μg/ml leucine- supplemented cultures were also filaments, but the number of cells growing was much lower in these cultures than for the 25 μg/ml leucine sample. While the length of the

filaments in each culture varied from near wild type length to an estimated 100 μm, or 50-fold longer than the normal cell length, the majority of cells were closer in length to the longer extreme. Samples of cells grown for 16 hours in minimal glucose with 25 or 50 μg/ml leucine were brought to Dr. E. Chan of McGill University, who produced the photos presented as Figures 4 and 5 using dark field photography. Note that for limiting leucine concentrations, longer filaments predominated when examined at 16-24 hours following inoculation, but by 36 to 48 hours of incubation, the cultures contained many normal sized cells.

To determine if these filamentous cells were still carrying the *metK84* marker, I grew MEW1 and MEW402 cells in glucose minimal medium supplemented with 500 μg/ml GGME and limiting leucine (25 μg/ml). The MEW1 culture failed to grow, while the MEW402 culture contained many long filaments. This indicates that the GGME-resistant filaments are derivatives of the *metK84*-carrying MEW402 strain.

All of the experiments described above were repeated by inoculating MEW402 into minimal medium cultures with glycerol as the carbon source in place of glucose. Again, the cells required 50 μg/ml leucine for normal growth, grew filaments at 25 μg/ml leucine, and were resistant to GGME.

4. The link between *metK84* and filamentation

One way to link a phenotype to a particular gene is to restore the function of the mutated gene, and measure the effect on the (potentially) associated phenotype. This can be achieved by ligating a copy of the wild type gene to an inducible promoter on an expression vector. The vector can be transformed into the mutant strain, and wild type

Figure 4: Strain MEW402 grown in minimal glucose medium with limiting amounts of l-leucine. The photomicrographs were taken under dark field illumination by Dr. E. Chan. Bar, 10 μm.

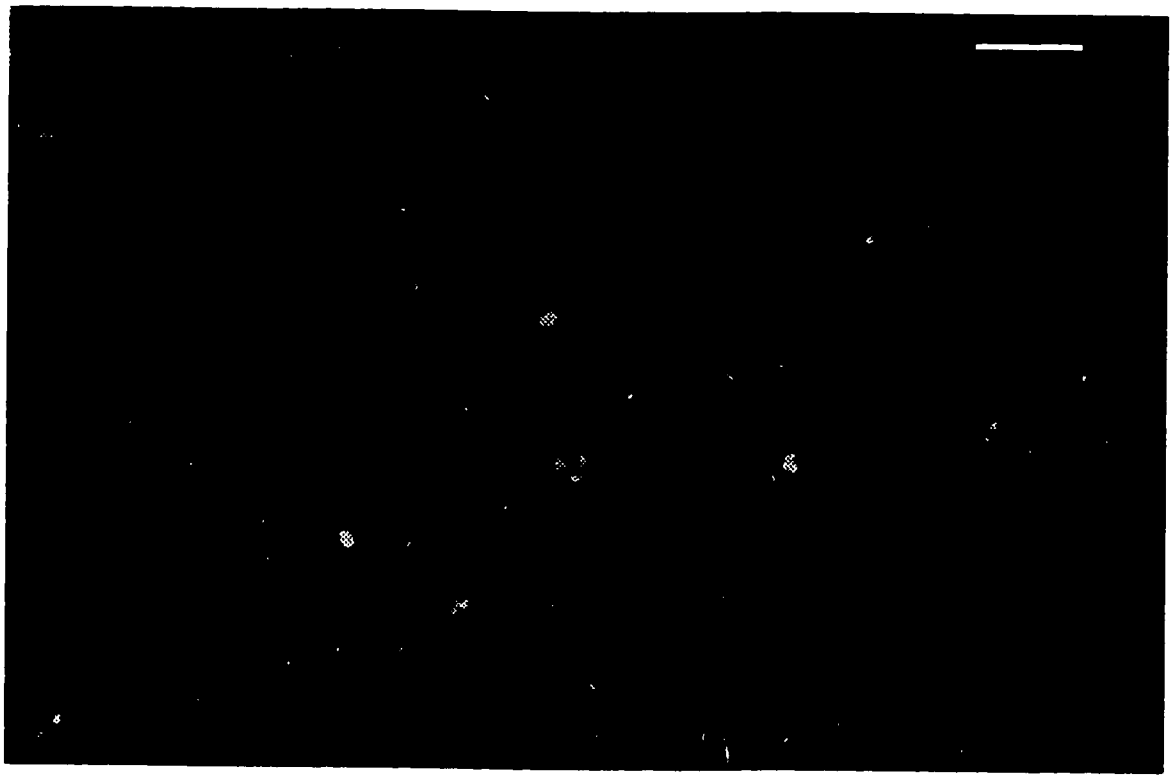

Figure 5: MEW402 growing in minimal glucose supplemented with 50 μg/ml leucine. Photomicrograph taken under dark field illumination by Dr. E. Chan. Bar 40 μm.

gene expression can be turned on by addition of the appropriate inducer. If the gene is able to complement the mutant, i.e., restore the cell to wild type with respect to that phenotype, then a link between the phenotype and the mutation has been confirmed.

To confirm that the *metK84* mutation is the origin of the limiting leucine-dependent filamentation phenotype, I amplified the *metK* gene from strain MEW1 via PCR, and ligated the products to the multiple cloning sites of plasmids pKK223-3 and pBAD22, forming plasmids pKK*metK* and pBAD*metK*, respectively.

4.1 Plasmid pKK*metK*

A PCR-amplified *metK*$^+$ gene was ligated to plasmid pKK223-3. By restriction enzyme analysis, it was determined that the inserted fragment was the size expected for the amplified *metK* gene (~1.3 Kb). The pKK223-3 plasmid carries an ampicillin resistance gene, so the pKK*metK* plasmid was transformed into MEW1 and MEW402 cells with selection on LB+amp plates.

The pKK223-3 vector places the expression of the *metK* gene under the control of the P_{tac} promoter (Pharmacia Biotech). This is an IPTG-inducible promoter, such that in the presence of IPTG, expression of *metK* is turned on. To determine if the plasmid-borne copy of *metK* could complement the *metK84* mutation, I sub-cultured MEW402/pKK*metK* transformants grown in liquid LB+amp into minimal glucose cultures (with ampicillin) in the presence and absence of IPTG (0.2 μM final concentration). Neither culture grew after overnight incubation at 37°C. To determine if leucine was required for growth, I repeated the experiment by inoculating these transformants into minimal glucose cultures supplemented with leucine (25, 50, and 100 μg/ml). None of

these liquid cultures grew. Attempts to grow these transformants on minimal glucose plates with or without added leucine (100 μg/ml) failed to yield any colonies. I also tested MEW1/pKK*metK* transformants for growth in minimal glucose liquid and solid media, and found that no cells could grow in either case.

It is evident from the fact that the pKK*metK* transformants could grow on rich media (LB), but not on minimal media, that there is some factor absent in the media required to support the growth of these transformants. To determine if the plasmid causes the host to become auxotrophic for an amino acid, I inoculated the MEW402/pKK*metK* and MEW1/pKK*metK* transformants in minimal glucose cultures supplemented with 0.1% casamino acids. This time the cultures were fully grown after 16 hours at 37°C, and all cells were of normal size. Thus, I concluded that the pKK*metK* plasmid results in a requirement for amino acid(s)—even in the absence of IPTG inducer.

To determine which amino acid(s) needed to be added to support the growth of the pKK*metK* transformants, I inoculated these transformants in cultures each containing the natural amino acids except one. All amino acids were added at 100 μg/ml, except for serine (500 μg/ml), and cysteine, which was not added at all. Each culture grew with the exception of that which was lacking methionine. Those cultures lacking alanine, arginine, glycine, or leucine, grew some normal sized cells, but had less growth than each of the other cultures, which were fully grown after overnight incubation at 37°C. These results suggested that methionine was essential for growth of the pKK*metK* transformants, and that alanine, arginine, glycine, and leucine may also help to support growth of these cells.

I tested the importance of these amino acids by inoculating MEW402 and MEW1 transformants with pKK*metK* into minimal glucose cultures with 100 μg/ml of alanine,

arginine, glycine, leucine, or methionine. Only the culture containing methionine was able to grow, and all cells were of normal size. Cultures of minimal glucose supplemented with alanine, arginine, glycine, and leucine failed to grow when inoculated with the pKK*metK* transformants, but grew fully when methionine was also added.

While it is true that induction of the P_{tac} promoter requires IPTG, this promoter is known to be leaky, such that the gene being carried by this plasmid is not completely repressed in the absence of inducer (Guzman *et al.* 1995). The expression of the plasmid-borne *metK*$^+$ gene, even without IPTG, is sufficiently high as to result in a requirement for methionine. When the pKK223-3 vacant vector was transformed into MEW1 or MEW402, the transformants showed no methionine requirement for growth. This confirms that the methionine requirement is the result of the expression of the *metK*$^+$ insert carried by the pKK*metK* plasmid.

Due to the fact that this plasmid leads to an entirely new phenotype for the host cell, it was not possible to confirm the link between filamentation and *metK84* using this plasmid. Thus, a second attempt at answering this question was made by ligating the wild type *metK* gene from MEW1 to a second inducible-expression plasmid, pBAD22. This is an ampicillin resistant plasmid, which uses the arabinose-induced P_{araBAD} promoter to regulate gene expression. The repression of the *araBAD* promoter is significantly greater than that of the P_{tac} promoter of pKK223-3 (Guzman *et al.*, 1995).

4.2 Plasmid pBAD*metK*

The pBAD*metK* plasmid was prepared by ligating a PCR-amplified copy of *metK*$^+$ from MEW1 into plasmid pBAD22. MEW402 cells were transformed with either plasmid

pBAD*metK*, or the vacant vector pBAD22, with selection on LB+amp plates. The *araBAD* promoter of pBAD22 is induced by arabinose, a sugar that cannot be used in the presence of glucose. For this reason, the transformants were tested for growth in non-catabolite-repressing glycerol minimal medium, supplemented with 25 μg/ml leucine. Cultures inoculated with MEW402/pBAD*metK* transformants grew fully, and no filaments were present. When inoculated in the same medium, MEW402 transformants with the vacant vector grew poorly, and developed many filaments. It is clear from these results that even in the absence of arabinose, there is enough expression from this promoter to prevent filamentation (Guzman *et al.*, 1995).

To test if the pBAD*metK* plasmid could complement the leucine requirement for MEW402 cells, transformants with this plasmid, or with the vacant vector, were inoculated in glycerol minimal medium without added leucine. After 16 hours of incubation at 37°C, the culture containing pBAD*metK* transformants grew fully. There were no filaments noted. Again, the culture of transformants with the vacant vector grew poorly, and most of the cells grew as filaments. This indicates that the limited expression of the pBAD*metK* plasmid, in the absence of inducer, is sufficient to overcome the leucine requirement for the growth of MEW402 cells.

I repeated the experiment a third time, this time by adding arabinose to induce further the expression of the plasmid-borne *metK*. When arabinose was added to the medium at a concentration of 1 μg/ml or greater, a culture of MEW402/pBAD*metK* transformants failed to grow. Under the same conditions, the vacant vector transformants grew filaments, independently of the presence or absence of arabinose. The normal growth of the culture was restored by the addition of methionine to the culture containing

the pBAD*metK* transformants. I determined that 100 μg/ml of methionine was required to restore normal growth of the culture when 1.0 μg/ml of arabinose was added. The same induction level of the pBAD*metK* plasmid was also toxic when it was transformed into MEW1 cells. These results show that over-expression of the pBAD*metK* plasmid is toxic to the cell, and results in auxotrophies for methionine.

In an attempt to minimize the leakiness of the *araBAD* promoter, the pBAD*metK* and vacant vector transformants were inoculated into cultures containing glucose minimal medium. Both the vacant vector and the pBAD*metK* transformants required leucine for growth, and produced long filaments when leucine was limiting. However, the pBAD*metK* transformants grew fewer long filaments and more normal-sized cells at 25 μg/ml of added leucine, than did the pBAD22 transformants under the same conditions. This is surprising in that, despite the expected catabolite repression of the plasmid's *araBAD* promoter by glucose, the plasmid-borne *metK* is expressed enough to have an effect on the phenotype of the cells (Guzman *et al.*, 1995). To further induce *metK* expression from pBAD*metK*, I added a large amount of arabinose (500 μg/ml) to the minimal glucose culture inoculated with the pBAD22 and pBAD*metK* transformants of MEW402. The pBAD*metK* transformants now grew like a wild type culture, i.e. normal sized cells able to grow independently of leucine concentration. The wild type growth must be due to the expression of the plasmid-borne *metK* and not to the presence of excess arabinose, as the transformants with the vacant vector continued to show the leucine dependent growth and filamentous phenotypes even when arabinose concentration was 500 μg/ml.

To determine whether the normal sized cells are truly MEW402, and not some other ampicillin-resistant contaminant, I attempted to cure the cell of the plasmid in order to verify that the host cell would then show MEW402 phenotypes. I sample of the culture containing normal sized cells was diluted, and then spread on a LB plate. After overnight incubation at 37°C, several hundred single colonies were present on the plate. The colonies were then replica plated onto LB and LB+amp plates, which were incubated overnight at 37°C. By comparing the growth on the two plates, I was able to isolate some ampicillin-sensitive colonies, which presumably were MEW402 transformants that had lost the plasmid. I inoculated five of these single colonies in minimal glucose medium with 25 μg/ml leucine, and after 16 hours at 37°C each culture had grown long filaments. This confirms that the normal growing cells in the original minimal glucose culture supplemented with arabinose were in fact MEW402 transformants with pBAD*metK*.

4.2.1 The inserted sequence of pBAD*metK*

The pBAD*metK* plasmid can be used to complement the *metK84* phenotypes by restoring wild type growth and cell division when partially induced by arabinose in glucose minimal medium. However, this does not prove that the DNA fragment carried by the pBAD22 vector is truly a copy of the wild type *metK* gene. To verify if the insertion consists of the desired sequence, samples of the pBAD*metK* plasmid, and the primers used to amplify the inserted PCR product, were forwarded to Dr. Ian Krantz to be sequenced.

The insert of the pBAD*metK* plasmid was sequenced in both the 5' to 3' and 3' to 5' directions using an automatic sequencer. In the 5' to 3' direction, the first 9 codons (27 nucleotides) at the 5' end were illegible. In the 3' to 5' direction, however, the full

sequence, including the 5' ATG start codon was determined with the exception of the first four nucleotides at the 3' end, which were listed as four unknowns (NNNN). Using a computer program called PCGene, both determined sequences were compared to each other, and the sequence was 100% consistent in overlapping regions. The inserted sequence was then compared to the *metK* sequence published at GenBank determined as part of the *E. coli* Genome Project. The sequences were found to be 100% identical, although the genome sequence, posted in September 1997, is significantly different from the sequence originally published for *metK* (Markham *et al.*, 1984). I conclude that the inserted region in the pBAD*metK* plasmid is a copy of the wild type *metK* gene.

4.2.2 Plasmid pBAD*metK* and GGME resistance

The pBAD*metK* plasmid is able to eliminate the leucine dependence and filamentation phenotypes of strain MEW402. To test if this is true of the third phenotype attributed to *metK84*, i.e. resistance to GGME, I grew MEW402 transformants with pBAD*metK* in glucose minimal medium liquid cultures supplemented with 500 μg/ml GGME with and without added leucine (50 μg/ml). The growth of each culture resembled that which was observed under these conditions in the absence of GGME, i.e., wild type-like growth with leucine, and a lack of significant growth in the absence of leucine. When these transformants were grown in glucose with GGME under induced conditions (500 μg/ml arabinose added) wild type growth resulted, as was observed for these cells in the absence of GGME.

5. The link between *metK84* and Lrp

To express the leucine requirement and filament formation phenotypes, Lin showed that the *metK84* mutation must be in a host with a functional *lrp* gene (Lin *et al.*, 1990). *lrp* encodes the leucine-responsive regulatory protein, a DNA binding protein which regulates the expression of 35 to 75 genes in *E. coli* (Newman, *et al.*, 1996). Lrp can be inactivated either by binding leucine, which prevents the molecule from reaching DNA binding sites, or through a mutation at the *lrp* locus. Since the *metK84* mutation is suppressed either by the addition of leucine or a mutation in *lrp*, it is likely that there is some direct interaction between Lrp and *metK*, and that the suppression of *metK84* by leucine is through the inactivation of Lrp. Thus, Lrp may be repressing the *metK* gene, and the absence of Lrp may allow more S-adenosylmethionine to be produced leading to an increase in the cell's SAM pools. To test this possibility, I sent strains MEW1, MEW45 (*lrp::kan*), MEW402 (*metK84*), and MEW403 (*lrp::kan metK84*) to Dr. R. Greene, who assayed SAM synthetase activity in extracts of each strain following growth in the presence or absence of leucine, with the results presented in Table 3. The results indicate that either the addition of leucine or a mutation at *lrp* will increase SAM synthetase activity. This is true for both the wild type strain, where the increase in *metK* induction was smaller, and for the mutant strain, where SAM synthetase activity was increased 7-fold by either method of Lrp inactivation. The assay results also indicate that normal cell division can occur even when SAM synthetase activity is 0.26

nmoles/min/mg protein, equivalent to a 9-fold decrease from wild type levels. Conversely, The conditions at which the cells formed filaments, i.e. a SAM synthetase activity of 0.04 nmoles/min/mg protein is insufficient to support normal cell growth and division.

6. Nucleoid segregation in MEW402 filaments

I grew a culture of MEW402 cells for 16 hours in minimal medium supplemented with 25 and 50 μg/ml leucine. I examined samples from each culture using a microscope (40X objective lens), and confirmed the presence of filamentous and wild type cells for the 25 and 50 μg/ml cultures, respectively. I took samples of each culture, mixed them with formaldehyde at 1.25% final concentration, and sent them to Dr. Conrad Woldringh of the University of Amsterdam, who prepared the photomicrographs shown in figure 6 using a fluorescence microscope. The photos show that there is even segregation of DNA throughout the length of the filament, indicating that *metK84* mutants can continue DNA synthesis during filamentation.

7. Effects of *metK84* on DNA methylation

The *metK84* mutation causes a reduction in the activity of SAM synthetase, presumably leading to a reduction in the cell's SAM pools as compared to wild type cell levels. One of the products methylated by SAM is DNA, and a reduction in the SAM pools by expression of a SAM hydrolase has been shown to cause the DNA to be undermethylated (Hughes *et al.*, 1987). To test if the *metK84* mutation causes a similar effect on the state of DNA methylation, I transformed ampicillin-resistant plasmid

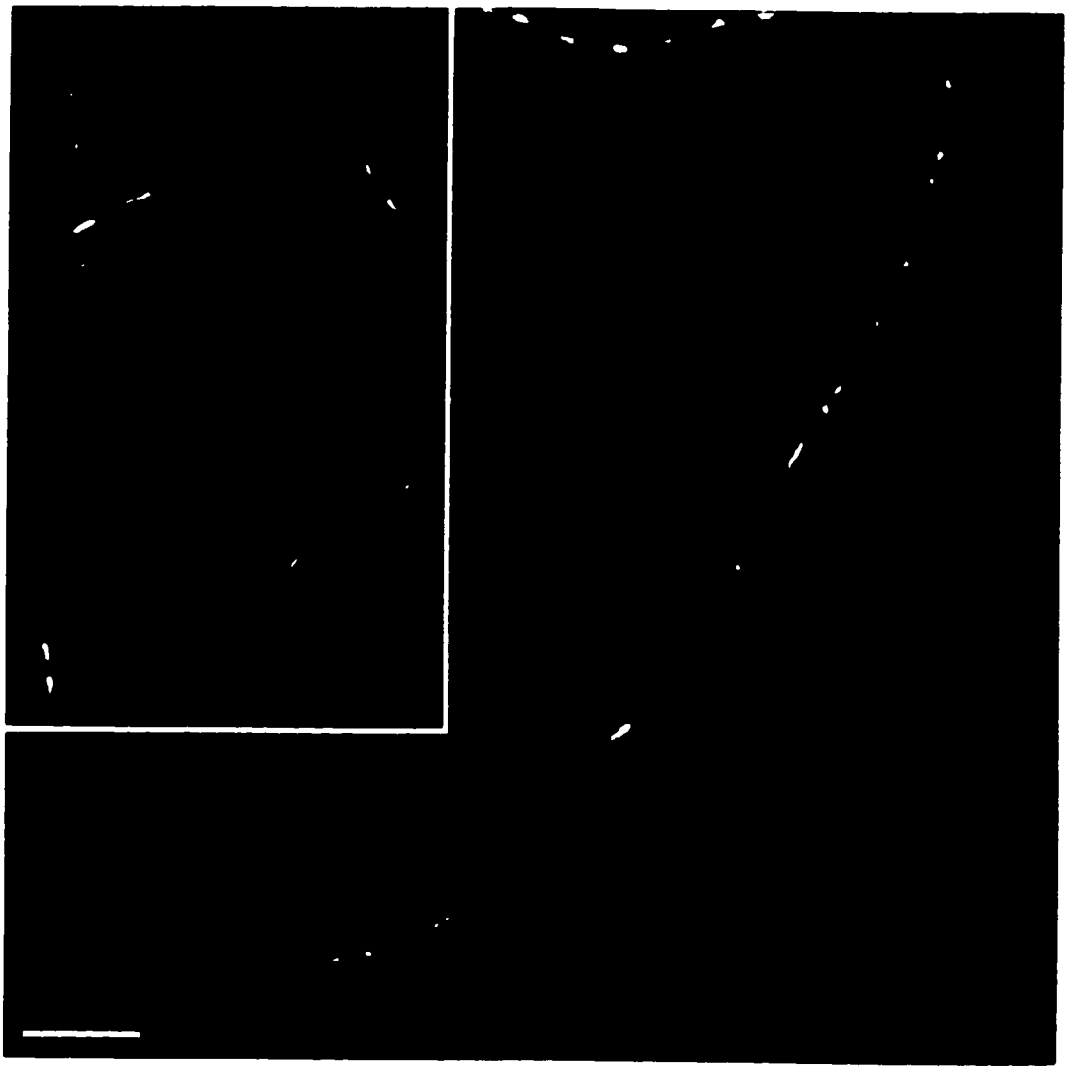

Figure 6. Nuclear segregation in MEW402 filaments and control cells. MEW402 cells were grown for 19 hours in minimal glucose medium with 25 μg/ml (filaments) or 50 μg/ml (control, inset) of leucine added. Samples were forwarded to Conrad Woldringh of the University of Amsterdam who prepared the samples for fluorescent microscopy. Uniform nucleoid segregation is noted along the length of the filament. The nucleoid regions appear mostly in the shape of a dumbbell, which presumably represents two chromosomes that have not fully separated. Integrated density measurement of the relative amount of DNA per individual nucleoid region indicated the presence of 16 nucleoid equivalents for this filament. Bar, 5 μm.

pBR322 into MEW402 competent cells. Purified transformants were grown overnight in liquid cultures of LB and glucose minimal medium with limiting leucine (25 μg/ml) and ampicillin, with the latter culture growing filaments. I then extracted plasmid DNA from both samples and digested the DNA with each of three restriction enzymes. *Dpn*I, *Mbo*I, and *Sau*3a each recognize the DNA sequence 5'-GATC-3', and pBR322 has 22 of these sites. However, *Dpn*I only recognizes methylated sites, *Mbo*I exclusively targets unmethylated sites, and *Sau*3a cuts these sites independently of the methylated state of the site (Barras and Marinus, 1989). I analyzed the digested products by agarose gel electrophoresis, and determined that in both the filamentous and normal sized cells, *Dpn*I and *Sau*3a cut the plasmid, while *Mbo*I digested samples are indistinguishable from undigested DNA used as a control (Figure 7). I conclude that pBR322 is normally methylated in the *metK84* mutant.

8. Plasmid pZAQ and filamentation in MEW402

The plasmid pZAQ contains copies of the *ftsZ*, *ftsA*, and *ftsQ* genes, each of which codes for a protein essential for cell division (Ward and Lutkenhaus, 1985). This plasmid was transformed into MEW1 and MEW402 strains, and streaking for single colonies on LBtet plates purified the transformants. Both types of cells were inoculated into glucose minimal cultures supplemented with 0, 2.5, 10, 25, 50, 100, and 200 μg/ml leucine, and incubated at 37°C. After 24 hours, samples examined under the microscope at 400X magnification revealed that the MEW1 transformants grew independently of leucine concentration, and cell size in each culture was very short, even less than the normal size for the host cells. However, the MEW402 transformants grew differently. There was

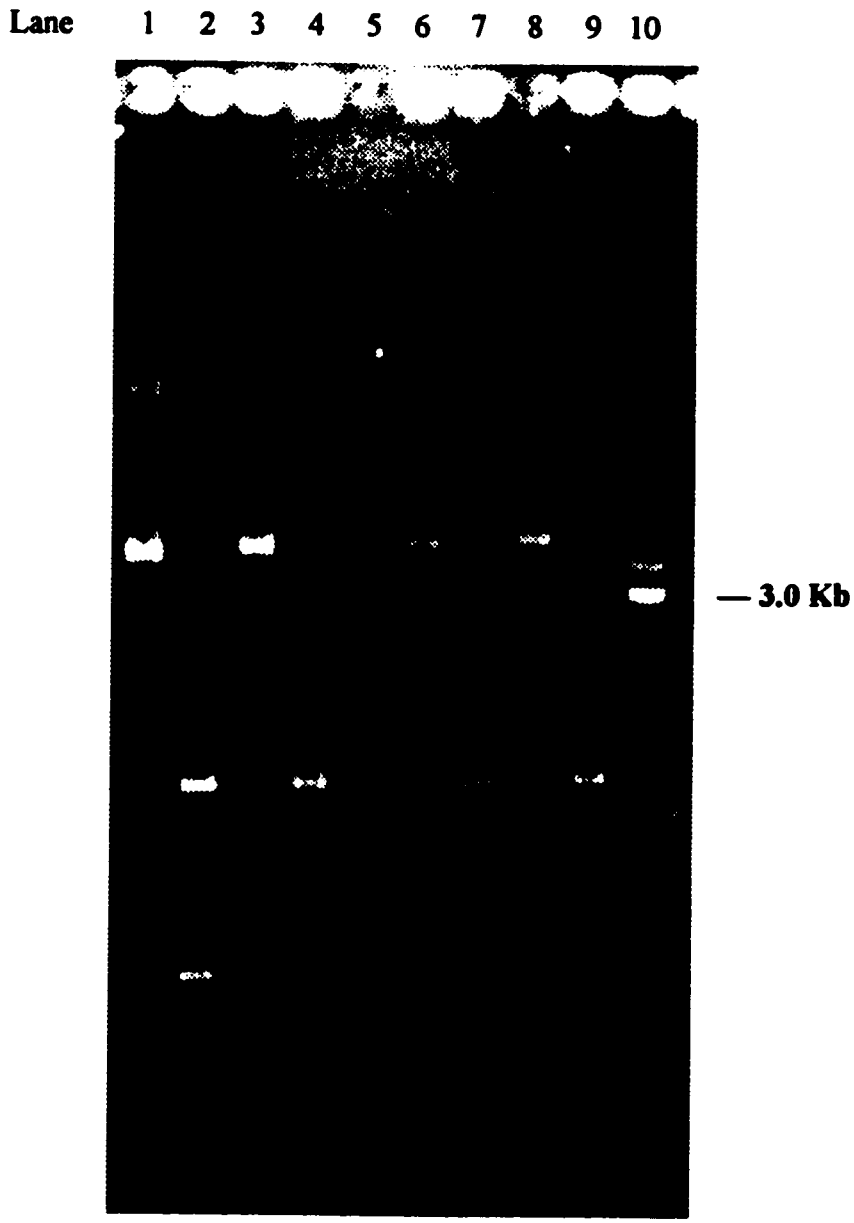

Figure 7. Methylation of DNA at GATC sites in a *metK84* mutant of *E. coli*. A 1% agarose gel containing pBR322 DNA isolated from MEW402 grown overnight in A. LBamp (lanes 1-4), and B. in minimal glucose amp supplemented with 25 μg/ml L-leucine (lanes 6-9). Lanes 1 and 6—undigested DNA; lanes 2 and 7—digested with *Dpn*I; lanes 3 and 8—digested with *Mbo*I; lanes 4 and 9—digested with *Sau*3a; lane 10—1 Kb DNA ladder. *Dpn*I targets methylated GATC sites, *Mbo*I unmethylated sites, and *Sau*3a both methylated and unmethylated sites (Barras and Marrinus, 1989). Plasmid pBR322 contains 22 GATC sites.

little growth with low leucine concentrations (0 – 10 μg/ml), but a majority of cells grew as very long filaments. These were confirmed to be pZAQ transformants by plating cells on LB+tet plates. When cultures grown with greater leucine concentrations (25 – 200 μg/ml), were examined after 24 hours of shaking at 37°C, cultures were a mix of short cells with some very long filaments. The 50, 100, and 200 μg/ml cultures were each densely grown with cells, the majority of which were short in length. However, when 200 μg/ml leucine was added, a concentration at which all MEW402 cells are of normal cell length, each field contained several long filaments. Thus, the combination of a mutated *metK* gene and the over-expression of *ftsZ*, *ftsA*, and/or *ftsQ* leads to the formation of very long filaments, some of which are not suppressed by large amounts of leucine.

DISCUSSION

A mutation at the *metK* locus of *Escherichia coli* leads to a decrease in S-adenosylmethionine synthetase activity, and a subsequent reduction in the cell's SAM pools (Greene *et al.*, 1973). A total deletion of *metK* is lethal in other species, such as in certain strains of yeast, but in *E. coli* no *metK* deletion has been produced, and the well-studied mutant *metK84* is not lethal despite a reduction in SAM synthetase activity to 3% of the wild type level. Still, this mutant demonstrates some phenotypic reaction to the reduced enzyme activity, such that *metK84* confers resistance to ethionine and GGME, and the host strain carrying this marker grows poorly in minimal medium unless leucine is added to the medium (Greene *et al.*, 1973). Lin reported that the formation of filaments is a third phenotype associated with this mutation, i.e. that cells with a defective *metK* gene are unable to undergo normal cell division (Lin *et al*, 1990). This work was performed to characterize further the role of *metK* in *E. coli*, while confirming the link between *metK* and cell division.

1. *lrp* is a suppressor of *metK84*

The *metK84* strain MEW402 grows normally when the exogenous leucine concentration is 50 μg/ml or greater, but the medium provides insufficient leucine at 25 μg/ml. This is surprising in that other leucine auxotrophs, i.e. strains which are unable to synthesize sufficient amounts of leucine *in vivo*, require only 10 μg/ml to yield a fully grown culture (Burns *et al.*, 1966). The leucine requirement here does not necessarily

relate to a breakdown in the cell's leucine production machinery, but more likely reflects the need to block or intensify the action of Lrp.

Lrp is a DNA binding protein that induces or represses the expression of 35 to 75 genes in *E. coli* (Newman *et al.*, 1996). Several different loci could be the target of Lrp binding in the regulation of SAM synthetase activity. It is evident from Greene's assays that the inactivation of Lrp, through the addition of leucine or by a mutation at *lrp*, induces the activity of SAM synthetase. However, the assays do not indicate whether Lrp acts directly on *metK*, or indirectly through interaction at another locus. Lrp may induce a gene whose expression inhibits the induction of *metK*, or there may be a gene other than *metK*, which encodes an active SAM synthetase. If this is the case, then Lrp may be repressing the expression of this second gene, which would then be induced by the inactivation of Lrp to provide sufficient SAM synthetase to overcome the mutation at *metK*.

Other species, including *S. cerevisiae* are known to have two SAM synthetases, and the existence of a second SAM synthetase enzyme for *E. coli* was once proposed (Cherest *et al.*, 1978; Satishchandran *et al.*, 1990). However, a BLAST search failed to locate a gene homologous to *metK* in *E. coli* (Altschul *et al.*, 1990; Newman *et al.*, 1998). The search result is significant considering the fact that SAM synthetase is highly conserved among different species. From this finding, it is clear that there is only one enzyme in *E. coli* that has SAM synthetase activity, and that is the product of the *metK* gene.

Since *metK* is the only gene whose product acts as a SAM synthetase, it is likely that Lrp interacts directly at the *metK* locus, although the possibility remains that Lrp

could act on a gene whose product then induces *metK* expression. If Lrp acts directly as a repressor of *metK*, then blocking the action of Lrp will alleviate the block on *metK* expression. This could be achieved by supplying leucine to bind Lrp, leaving the protein unable to attach to the DNA region controlling the expression of *metK*. Similarly, a mutation in *lrp* will also prevent a block of *metK* expression by Lrp. This model is analogous to the model for the regulation of *sdaA* by Lrp (Lin *et al.*, 1990). This model also accounts for the increased SAM synthetase activity over wild type levels brought on by a mutation in *lrp*. To show further support for this model, I examined the region upstream of the *metK* transcriptional start site for possible Lrp binding sites.

Different sources have proposed various consensus sequences for Lrp binding. According to a review by Newman and Lin (1995), the sequences AGAATTTTATTCT and (g/a)(g/c)nnnTTTATtCTgG, proposed by two separate groups, each contain the core sequence TTTATtCT with different flanking regions. It was also noted in the review (Newman and Lin, 1995), that a third group suggested that it is AT-richness, rather than a particular sequence which is recognized by Lrp as a potential binding site. I examined the 500 nucleotides immediately upstream of the ATG start of *metK* to determine if there are any probable Lrp binding sites. The complete sequence TTTATtCT did not appear, but TTTAT appears twice, once within a run of 11 straight A and T bases (-314 to -304). This particular sequence lies within the proposed *speA-metK* intergenic region (GenBank). Other AT-rich sites also appear closer to the *metK* coding region including -289 to -270 (17 of 20 bases A or T), -209 to -195 (12 of 15), -96 to -85 (9 of 12), and -31 to -15 (13 of 17). In addition to these AT-rich regions, the proposed -35 (TGGAAA; -175 to -170) and -10 (TAAAAT; -151 to -146) boxes of *metK* are located within large AT-rich

regions. Finally, the region immediately upstream of *metK* (-9 to +2) consists entirely of A and T bases. Any of these regions could be potential Lrp binding sites. However, the possibility remains that Lrp does not interact with *metK* at all, but that there is an intermediary gene whose protein then helps to regulate the expression of the *metK* gene.

2. Plasmid expression of *metK*

An attempt was made to compare the growth of MEW402/pBAD*metK* transformants in medium where *metK* is expressed, to medium in which *metK* is not expressed. This proved to be difficult, as only a small increase in SAM synthetase activity is required to restore normal growth and cell division. Normally, it would be possible to compare growth of these transformants in glycerol medium with and without arabinose, but it turns out that the plasmid expression is high enough without arabinose to complement the growth and division deficiencies of MEW402. Since arabinose is catabolite repressed, the plasmid expression should be reduced when grown on glucose. As expected, these transformants grow filaments on glucose, but the addition of arabinose at 500 μg/ml leads to sufficient plasmid induction to show complementation of *metK84*.

The level of pBAD*metK* induction by 500 μg/ml arabinose in glucose must be less than, or equivalent to, the plasmid's induction in glycerol minimal medium in the absence of arabinose. This is evident from the observation that the addition of arabinose, at any concentration, reduces the ability of the culture to grow fully in glycerol, while full growth is observed on glycerol without arabinose.

When cultured on glycerol, MEW402/pBAD*metK* transformants fail to grow when arabinose is added at a concentration of 1.0 μg/ml or greater. The over-production of

SAM synthetase under these conditions results in a requirement for an exogenous supply of methionine. That pBAD*metK* expression results in a methionine requirement is consistent with the experiments performed with pKK*metK*, where growth in minimal medium requires methionine. A similar result was reported by Satishchandran *et al.* (1990), who described that even expression of a single chromosomal equivalent from a plasmid-borne wild type *metK* results in a methionine requirement for *metG*⁻ *metK*⁻ transformants. The methionine requirement may be explained by considering the role of *metK* in the biosynthesis of methionine.

The methionine biosynthetic pathway begins with three reactions shared by other biosynthetic processes, including those for the formation of lysine, threonine, and the branched chain amino acids (Greene, 1996; Figure 2). The fourth reaction in the *met*-synthesis pathway, the *O*-succinylation of homoserine, is the first reaction in the pathway committed to the formation of homocystine, the direct precursor to methionine. The succinylation reaction is catalyzed by the enzyme homoserine transsuccinylase, product of the *metA* gene (see Greene, 1996 for review). Through a process of feedback inhibition, SAM or methionine inhibit homoserine trans-succinylase (MetA), and a mixture of both products leads to an even greater inhibition of the enzyme (Greene, 1996). The methionine requirement described here could be explained if, as *metK* is over-expressed, SAM synthetase activity is increased, and most of the available methionine molecules are converted to SAM. Then, the increase in the SAM pools might inhibit further production of methionine by inhibiting *metA* expression. SAM also contributes to the repression of other genes in the methionine biosynthetic pathway by acting as the corepressor to the MetJ aporepressor (Su *et al.*, 1971; Greene *et al.*, 1970, 1973). Thus, over-production of

SAM may block the production of methionine by feedback inhibition of the methionine biosynthetic pathway.

3. GGME resistance of *metK84*

Despite inhibition of the use of arabinose by glucose, I showed that there is sufficient induction of plasmid pBAD*metK* when arabinose is present in glucose minimal medium to overcome two of the three phenotypes associated with the *metK84* mutation. Resistance to other amino acid analogues has been attributed to alterations in aminoacyl-tRNA synthetases, or tRNA modifying enzymes (Kraus *et al.*, 1979). It is conceivable that GGME resistance could arise from the undermethylation of some factor(s) in the translation pathway of the *metK* mutants. However, this is unlikely to be the case, as unlike the growth and cell division phenotypes of the *metK*$^-$ strain, GGME sensitivity is not restored by the expression of the plasmid-borne *metK*$^+$ gene. That the wild type *metK* expression corrects for both growth and cell division deficiencies, but not for GGME resistance indicates that the resistance develops from a factor other than a mutation in *metK*, or the absence of SAM synthetase activity. It has been suggested previously that GGME resistance, like resistance to other amino acid analogues, may be the result of an altered aminoacyl-tRNA synthetase, or tRNA modifying enzyme (Kraus *et al.*, 1979). The study of other GGME-resistant mutants suggested that the resistance could be linked to an altered glutamyl-tRNA synthetase (Kraus *et al.*, 1979), but no test has been performed to determine the source of the GGME-resistant strains described here.

4. Filamentation in *E. coli*

The formation of filaments is not unique to the *metK84* mutation. Mutations at genes encoding the cell division proteins FtsA, I, K, L, N, Q, W, and Z lead to filaments which fail to propagate and eventually die (Hale and de Boer, 1997). In these filaments, cell growth, DNA replication and nucleoid segregation take place as in a normal cell, except for the absence of cell division. The *metK84* filaments, which are considerably longer than those caused by *fts* mutations, also show even nucleoid segregation and DNA replication suggesting that the cell is unable to form crosswalls. This may indicate that a SAM-dependent methylation step is required for septation to occur.

One possibility relates to the relationship between the dam methylase and the *sfiA* gene. It has been reported that *sulA* expression increases in a *dam*$^-$ strain (Barras and Marinus, 1989). The SulA protein is induced in response to DNA damage as part of the cells SOS response system. This protein acts to inhibit the GTPase and polymerization activity of FtsZ, an action that results in the formation of filaments (Trusca *et al.*, 1998). If a lack of SAM reduces the methylation by the dam methylase, this could conceivably result in the increased induction of the SulA protein, and the subsequent formation of filaments.

5. Methylation and cell division

Currently the target of SAM methylation required for cell division to occur is unknown. A number of possible targets exist, including a number of the Fts proteins. These proteins have been isolated for playing essential roles in the cell division of *E. coli*. A leading candidate is the product of the *ftsJ* gene. The FtsJ protein has a SAM-binding

motif, and it has been suggested that this protein may function as a methyl transferase (Ogura *et al.*, 1991). It is therefore possible that in the absence of SAM, the FtsJ protein is unable to function, resulting in the failure of the cell to divide.

Other cell division proteins such as FtsZ, FtsA, FtsQ, ZipA, and others could easily be the target of SAM methylation. Each of these proteins is required to form the complexes required for the cell to constrict and ultimately divide into two daughter cells (Hale and de Boer, 1997). To date there has been no study of the methylation state of these and other proteins essential for cell division, and any or all of these may require SAM methylation to function properly with respect to cell division.

6. Future considerations

Among the unanswered questions about *metK* and SAM in *E. coli* is whether or not the cell can survive without the *metK* gene. Is this gene truly essential to the *E. coli* cell, as it is in yeast? Or could there be some alternate pathway, which could compensate for the resultant lack of SAM. One way to answer this question is through the use of the pBAD*metK* plasmid. This plasmid can be transformed into a wild type strain and then grown in the presence of arabinose to induce expression of the plasmid-borne *metK* gene. The chromosomal copy of *metK* could now be disrupted or deleted without a loss of SAM synthetase activity. Once the strain lacking chromosomal *metK* function has been isolated, it can be shifted to a medium lacking arabinose. If the cell continues to grow in the absence of both the chromosomal and plasmid-borne *metK* genes, then the *metK* is not essential to the survival of the *E. coli* cell. Conversely, the absence of growth would

indicate that *metK* is an essential gene. Thus, the pBAD*metK* plasmid is a useful construct for the further analysis of the *metK* gene, and ultimately, of SAM.

Another topic for future consideration is the role of SAM or SAM-dependent methylation in the SulA/FtsZ relationship. It is believed that a factor, found in the inner membrane, is required to nucleate or stabilize FtsZ polymers in the cell (Trusca *et al*, 1998). Perhaps SAM methylation is required for the proper functioning or regulation of this proposed stabilizing factor.

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